Comparative Genomic Analysis of a New *Vibrio* Strain (J383), and the Evaluation of Vaccines Against *Moritella viscosa* in Farmed Atlantic Salmon (*Salmo salar*)

by

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Abstract

Winter Ulcer Disease (WUD) is a prominent health concern for marine fish, particularly affecting farmed salmonids. It has hemorrhagic effects and leads to dermal lesions and ulcers. This disease often reduces the fish's market value and increases mortality rates. *Moritella viscosa*, a gram-negative bacterium, is mainly, but not exclusively, linked to the appearance of WUD, especially during the colder months. Notably, however, there is significant variation in the prevalence and characteristics of WUD in the Eastern North Atlantic (Europe) as compared to in Eastern Canada. The disease in Europe often presents in the winter, and the severity and frequency of outbreaks is affected by water temperature and salinity. In contrast, in Eastern Canada, ulcerative disease outbreaks generally occur in the summer and mid-autumn, and mortality persists until September.

Although various polyvalent vaccines are used against *M. viscosa*, several outbreaks of this disease have been reported in Eastern Canada in recent years. It is unknown whether undescribed bacterial pathogens are causing ulcerative disease in vaccinated farmed Atlantic salmon in this region, or whether current vaccines against *M. viscosa* are ineffective.

This study analyzed the phenotypical and genomic characteristics of a new *Vibrio* spp. (*Vibrio* sp. J383) isolated from internal organs of vaccinated farmed Atlantic salmon displaying clinical signs of ulcer disease (**Chapter 2**). Additionally, the susceptibility of vaccinated farmed Atlantic salmon to *M. viscosa* challenge was determined, and the haemato-immune response of immunized farmed Atlantic salmon to *M. viscosa* vaccine preparations was investigated (**Chapter 3**).

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List of Abbreviations

°C	Degrees centigrade
µL	Microliter
ANOVA	Analysis of variance
CDRF	Cold-Ocean and Deep-Sea Research Facility
CFU	Colony forming unit
DAPI	4′, 6-diamidino-2-phenylindole
DiOC6	3,3 dihexyloxacarbocyanine
dpc	Days post-challenge
dpi	Days post-infection
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GG	Genomes gaps
GIs	Genomic islands
h	Hour
HPR	Horseradish peroxidase
Ig	Immunoglobulin
ip	Intraperitoneal
IROMP	Iron regulated outer membrane proteins
JBARB	Dr. Joe Brown Aquatic Research Building
L	Liter
LCBs	Locally collinear blocks
mg	Milligram
min	Minute
mL	Milliliter
MUN	Memorial University of Newfoundland
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding RNAs
O.D.	Optical density
OMVs	Outer membrane vesicles
PBS	Phosphate buffered saline
SEM	Standard error of the mean
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
wpi	Weeks post-infection
wpv	Weeks post-vaccination
WUD	Winter ulcer disease

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Co-Authorship Statement

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1. Chapter 1: General Introduction

1.1. Canadian Aquaculture Industry

The Canadian aquaculture industry has experienced dramatic growth over the past 50 years. The industry's production was less than 10,000 tonnes in 1980. It then saw a substantial increase, reaching 49,500 tonnes by 1991, with a value of approximately CAN$ 233.6 million (Noakes, 2018). By 2009, Canadian aquaculture production ranked 20th globally, with Atlantic salmon farming accounting for nearly 70% of the national output and Canada being the 4th largest producer of farmed salmon worldwide (Nguyen & Williams, 2013).

Canadian aquaculture has continued to increase. In 2011, the industry accounted for 162,000 tonnes, about 0.25% of global aquaculture production. A significant portion of this production, 60%, was attributed to British Columbia, however, New Brunswick and Newfoundland and Labrador also emerged as important provinces in salmon production (Nguyen & Williams, 2013). Farmed salmon production, a key economic driver in rural and coastal areas, was Canada's third-largest seafood export. This industry has played a pivotal role in provinces like Newfoundland and Labrador, New Brunswick, Nova Scotia, Prince Edward Island, and British Columbia (DFO, 2017). Globally, Canada is the fourth-largest producer of farmed Atlantic salmon (*Salmo salar*), emphasizing its significant contribution to sustainable seafood production (FAO, 2020). In 2019, farmed salmon production accounted for 118,630 tonnes, with a value exceeding $900 million (CAD) (Statistics Canada, 2021). The Canadian aquaculture industry, especially Atlantic salmon farming, is experiencing rapid growth, and its contribution to enhancing economic development and employment opportunities has been highlighted (Osmond et al., 2023).

1.2. Diseases in Atlantic Salmon

Infectious Salmon Anemia virus (ISAv) and several bacterial pathogens, including: *Renibacterium salmoninarum*, which causes bacterial kidney disease; *Vibrio anguillarum*, which

also causes vibriosis; *Aeromonas salmonicida*, the etiological agent of furunculosis; and *Moritella viscosa*, the main causative agent of winter ulcer disease (WUD), are common infectious diseases in the aquaculture industry (Brooker et al., 2018; Nilsen et al., 2017; Powell et al., 2018; Stentiford et al., 2017).

Winter ulcer disease typically affects Atlantic salmon at cold temperatures, usually in the winter (Sørum et al., 2000; Toranzo et al., 2005). According to studies by Björnsdóttir et al. (2011), Björnsdóttir et al. (2012), Grove et al. (2008), Heidarsdottir, et al. (2008), Olsen et al. (2011) and Tunsjø et al. (2009), it can happen when ocean temperatures fall below 7°C. The disease initially appears as superficial skin lesions on scaled areas but can turn into skin ulcers (Benediktsdóttir et al., 1998; Tunsjø et al., 2009; Tunsjø et al., 2011).

Winter ulcer disease poses a serious threat to Norwegian salmonid farming. It is the most prevalent bacterial infection that cannot be prevented by immunization and antibiotics (Løvoll et al., 2009). Winter ulcer disease has a negative economic impact on Norway and Iceland (Jansson & Vennerström, 2014). Downgrading of fillet quality and mortalities cause severe financial losses (Grove et al., 2008; Jansson & Vennerström, 2014). Winter ulcer disease typically leads to a 10% mortality rate during an outbreak (Lunder et al., 1995; Olsen et al., 2011). However, one study suggested that mortality rates could exceed 40% (Hoffman et al., 2012). Low temperature contributes to the development of disease (Kent & Poppe, 1999). When temperatures rise over 8°C (Lunder et al., 1995) or salinity drops below 12–15 ppt (Løvoll et al., 2009), fish can recover. Although the disease can affect juvenile and adult fish, it usually affects fish in their first year at sea in Norway (Coyne et al., 2006; Lillehaug et al., 2003). Winter ulcer disease in 2-3 kg salmon was reported by Bruno et al. (1998) in Scotland. However, infections in fish over 1 kg were less

frequent (Lillehaug et al., 2003). Similarly, farm-raised Atlantic salmon on Canada's east coast frequently develop ulcers when they weigh less than 1 kg (MacKinnon et al., 2019).

Several investigations have been carried out to identify the bacterial species and strains leading to infection and ulcer formation. Karlsen et al. (2012) showed that bacteria in the seawater was the primary cause of skin ulcers in Atlantic salmon and Lunder et al. (1995) reported that skin ulcers formed after bath infection in Norwegian Atlantic salmon with *M. viscosa*. Also, they mainly occurred at the injection site following intraperitoneal or intramuscular challenges (Björnsdóttir et al., 2004; Gudmundsdóttir et al., 2006).

1.3. *Moritella viscosa* and Winter Ulcer Disease

Winter ulcer disease, caused mainly by the bacterium *Moritella viscosa*, is a severe problem in the salmon farming sector. The bacterium is found in farmed Atlantic salmon in the Northern Atlantic Ocean and has been isolated from the marine environment. *Moritella*, is in the family *Alteromonadaceae*, and was initially recognized and distinguished from the genus *Vibrio* by Urakawa et al. (1998). It is a halophilic, psychotropic, motile bacterium which utilizes both fermentative and oxidative metabolism and produces oxidase. The colonies are yellow and round on Trypticase Soy Agar (TSA) supplemented with 2% NaCl (Lunder et al., 1995).

The genome of *M. viscosa* has one chromosome (5.1 Mb) and two small cryptic plasmids called pMVIS41 (4.1 kb) and pMVIS39 (3.9 kb) (Hjerde et al., 2015). This bacterium affects different organs of fish; hemorrhagic signs can be found in the gills, on the head, and different regions of the skin. There are also reports of internal organ necrosis or muscle deterioration. Fish with chronic infections may experience a severe inflammatory response that changes their muscle, and endothelial cells in the hypodermis (Løvoll et al., 2009; Lunder et al., 1995).

However, there is not currently enough conclusive information about the causative agent of WUD. It has been speculated that either multiple opportunistic pathogens or the primary pathogen *M. viscosa* cause this disease. Also, *M. viscosa* virulence factors are not well known. However, it appears that extracellular cytotoxic products (ECPs) play a significant role in causing skin ulcers in Atlantic salmon, and identification of these extracellular products may clarify the pathology related to *M. viscosa* (Björnsdóttir et al., 2011; MacKinnon et al., 2019). The extracellular metallopeptidase MvP1 has been isolated from ECPs and characterized, but its role in virulence has not been established (Björnsdóttir et al., 2011). Also, it has been reported that *M. viscosa* oligosaccharides and unknown non-protein antigens (17–19 kDa) in the outer membrane increase the antigenicity of the bacterium and might be essential virulence factors (Bjornsdottir et al., 2009). Whether these virulence factors can be used as vaccines or immune stimulants has not been determined (Karlsen et al., 2014b).

There are not many documented descriptions of Atlantic salmon winter ulcer epidemics. Most papers cite outbreak-related facts without going into detail about the actual occurrence. For instance, Coyne et al. (2006) claim that post-smolts in their first year at sea are particularly susceptible to outbreaks, but they give no further details about the occurrence of these events. The first outbreak of winter ulcer in farmed Scottish Atlantic salmon is described by Bruno et al. (1998). Market-sized (2–3 kg) Atlantic salmon experienced mortality at low temperatures. More recently, a comprehensive descriptive examination of ulcer diagnosis in Atlantic salmon farms in New Brunswick was provided by MacKinnon et al. (2019). However, they are not directly comparable to ulcerative disease observed in farmed Atlantic salmon in BC or Europe since the water temperatures were between 10 and 13°C when the outbreak occurred.

1.4. Co-Infection

Although *M. viscosa* is thought to be the cause of winter ulcers, other bacteria may also contribute to outbreaks of the disease in Norway (Jansson & Vennerström, 2014; Karlsen et al., 2014b). The most frequently identified bacteria are *Aliivibrio wodanis* and *Tenacibaculum* sp., which have been isolated from cases of ulcer disease (Karlsen et al., 2017; Småge et al., 2016). Småge et al. (2016) also reported that the pathogen *Tenacibaculum finnmarkense* is a novel species within the genus *Tenacibaculum*. It may attack scarified skin and co-infect wounds caused by *M. viscosa* (Olsen et al., 2011).

Although Hjerde et al. (2015) showed that *A. wodanis* may restrict the growth of *M. viscosa*, Toranzo et al. (2005) suggested that *A. wodanis* may inhibit the healing process of skin ulcers resulting from the first infection with *M. viscosa*.

1.5. Regional Differences

Winter ulcer has been known to exist in Norway since 1980 (Lunder et al., 1995). In contrast, ulcerative disease was not noted in Canada (New Brunswick, NB) until 1990 (Whitman et al., 2001), and the first case was not reported in British Columbia (BC) until 2011 (Wade & Weber, 2020). Ulcerative disease caused by *M. viscosa* infection in BC resembles that seen in farmed Atlantic salmon in Norway, Scotland, and Iceland. It is characterized by shallow wounds, the isolation of *M. viscosa*, an onset at temperatures below 8°C, and recovery or no incidence of infection at temperatures above 10°C (Benediktsdóttir et al., 1998; Bruno et al., 1998; Lunder et al., 1995; Sørum et al., 2000).

However, ulcerative disease described on Canada's east coast is more complicated. Whitman et al. (2001) report that ulcerative disease was primarily responsible for the mortality that persisted until the end of September and even with antibiotic treatment, mortality was 31% during the

outbreak (Whitman et al., 2001). According to Brewer-Dalton et al. (2014), the mean monthly temperature for September in the upper 12 m of sea cages in New Brunswick is ~ 13°C (range 9-18°C. Finally, a *Vibrio* species was also identified during the investigation in New Brunswick; and its biochemical and SDS-Page characteristics matched *Allivibrio wodanis* (Whitman et al., 2001).

In Atlantic Canada, MacKinnon et al. (2019) reviewed the risk factors for development of skin ulcers in farmed Atlantic salmon. Although they did not identify the pathogen(s) involved, they demonstrated that the prevalence of ulcerative disease increases in the summer and autumn and water temperature during the epidemics ranged from 10.06°C to 13.36°C (MacKinnon et al., 2019).

This study also assessed the occurrence of skin ulcers based on clinical signs which varied in severity from swollen scales to skin ulceration on the lateral side. Data from 29 farms (2014–2016), with a total of 312 cages were analyzed descriptively. According to MacKinnon et al. (2019), farms near one another occasionally experienced epidemics at the same time. However, in other instances geographically separated farms reported skin ulcers a week apart. Mortality during epidemics lasted approximately eight weeks. However, in certain cages, it was higher for up to 26 weeks.

Thus, there are several differences between Canadian and European outbreaks of ulcerative disease. In European countries, WUD occurs below 7°C (Tunsjø et al., 2007). However, in Canada, ulcerative disease frequently occurs in summer and mid-autumn when the water temperatures are variable and between 10 and 13°C (MacKinnon et al., 2019). Furthermore, the pathogen-associated diseases in the two regions are different. Two main phenotypic and genotypic clades ('typical' and 'variant') have been described in *M. viscosa* (Grove et al., 2010). 'Typical' *M. viscosa* has been isolated from Atlantic salmon farmed in Norway, Scotland, and the Faroe Islands. In contrast,

‘Variant’ *M. viscosa* has been isolated from Atlantic salmon reared in Iceland and Canada and antigenic heterogeneity suggests that *M. viscosa* is serologically diverse (Heidarsdottir et al., 2008).

It is proposed that genetic elements within *M. viscosa* have advanced compatibility factors that adjust ‘typical’ *M. viscosa* to host-specific virulence (Karlsen et al., 2014a). The mortality rate caused by WUD is less than 10% in Norwegian farms during an outbreak in winter, and fish that survive recover when the water temperature increase above 8°C in spring. However, skin ulcers in saltwater net-pen raised Atlantic salmon can lead to an increase in mortality rates as high as 23.32% and economic losses in Atlantic Canada (MacKinnon et al., 2019). The presence of skin ulcers in summer is relatively new, and there is limited published data related to ulcerative disease in Atlantic salmon on the east coast of Canada. Salmon farmers report that skin ulcers in saltwater can progress very fast. Fish may initially exhibit only subtle signs of laterally raised scales, and after a few days, they die with a single large circular ulcerative lesion several centimeters in diameter (MacKinnon et al., 2019; MacKinnon et al., 2020). In contrast, in Norway, Lunder et al. (1995) reported the presence of detectable *M. viscosa* only at the edge of skin lesions and within the underlying muscle.

1.6. Vaccines Against WUD

Vaccination is an effective management strategy for fish health that decreases disease outbreaks and minimizes the use of antibiotics in the aquaculture industry (Shoemaker et al., 2009). The first vaccination trials against *M. viscosa* started in 1993 for Atlantic salmon. After the introduction of vaccines, antibiotic use decreased in the salmonid farming industry (Gudmundsdóttir & Björnsdóttir, 2007). The vaccine structure was based on formalin-killed bacteria and were injected with an oil adjuvant into salmon. This method of vaccination has been

accepted in Norway and Iceland and provides salmon with some protection against *M. viscosa* (Greger & Goodrich, 1999; Mutoloki et al., 2004).

However, it has been suggested that a polyvalent oil-based vaccine against *M. viscosa* is more effective. Polyvalent vaccines contain at least two or more strains of the antigen (Björnsdóttir et al., 2004; Gudmundsdóttir & Björnsdóttir, 2007).

The assessment of vaccine efficacy in test challenges is complex. The occurrence of lesions and infections in fish was not measured in the above studies, and the efficacy of the vaccine was evaluated based on the relative percentage survival (RPS) and the mean day to death (MDD) (Gudmundsdóttir & Björnsdóttir, 2007).

However, on salmon farms, skin ulcers and wound infections cause mortality and low-quality flesh, and therefore establishing a new strategy for vaccine evaluation is required. Even though international surveys suggested that immunization against WUD is effective, several studies reported the occurrence of winter mortality and WUD in vaccinated fish, and thus it appears that current vaccines need to be modified and/or new vaccines must be developed. Over the past decade, vaccine development has been focused on identifying a novel technology that enhances vaccine effectiveness (Tafalla et al., 2013). Modern vaccine studies focus on specific pathogen components and producing vaccines that contain new antigens (Kim et al., 2016). Attenuated vaccines are more effective than killed bacteria due to their ability to induce the cellular immune response in the host. These vaccines are designed using attenuated bacteria or natural strains with low virulence factors (Adams, 2019; Liu et al., 2018). Plasmids in DNA vaccines also play a significant role. They carry specific genes that code antigenic proteins that are produced in high quantities within bacterial cells. Multivalent DNA vaccines are super-efficient because they stimulate the adaptive immune responses in the host (Adams, 2019; Levine & Sztein, 2004).

Finally, one of the most important innovations for enhancing the efficacy of vaccines has been comparative bacterial genomics, which utilizes genome-sequenced information to understand the pathogenesis of bacteria (Fraser et al., 2000; Prentice, 2004).

1.7. Treatment

Nearly half of all antibiotic prescriptions in Norwegian Atlantic salmon farming are for treating winter ulcers. However, they do not substantially reduce disease-related mortality (Coyne et al., 2004; Coyne et al., 2006; Løvoll et al., 2009). This may be partially attributable to the diseased fish's tendency to stop feeding, which prevents them from ingesting the antibiotic (Jansson & Vennerström, 2014). It is crucial that the antibiotic also targets the skin because the disease may not develop into a systemic infection. Antibiotics have previously been recommended for BC farmed Atlantic salmon to treat gram-negative bacteria that cause furunculosis, vibriosis, enteric red mouth (ERM), and stomatitis (Morrison & Saksida, 2013). While immunization against ERM, vibriosis, and furunculosis in fish has significantly decreased antibiotic usage, prescriptions for antibiotics are still required (Morrison & Saksida, 2013). In Canada, only oxytetracycline hydrochloride (Terramycin-Aqua), trimethoprim and sulphadiazine powder (Tribressen 40% powder), sulfadimethoxine and ormetoprim (Romet 30), and florfenicol (Aquaflor) are approved for use in aquaculture (Health Canada, 2010). Research has examined the benefits of urea added to feed to minimize osmotic stress (Rørvik et al., 2000; Rørvik et al., 2001) and trimethylamine oxide has been used to improve fat digestibility to prevent winter ulcers. These mitigating strategies have had some positive effects (Rørvik et al., 2000).

1.8. Comparative Bacterial Genomics as a Beneficial Technology for Vaccine Advancements

According to Pastoret (1999), vaccination has been recognized as the most economical method of managing infectious diseases in the aquaculture industry. Fish immunization began in 1940 to prevent bacterial and viral diseases (Snieszko & Friddle, 1949). Over 26 certified commercial fish vaccines are currently available worldwide for various cultured fish species (Gudding & Van Muiswinkel, 2013; Ma et al., 2019). Vaccines currently consist of inactivated bacterial pathogens and are the most appropriate way to prevent infectious disease in fishes. Vaccines are produced with or without adjuvants, such as mineral oils, which are added mainly to enhance the immune response of the immunized fish (Gudding & Van Muiswinkel, 2013; Sudheesh & Cain, 2017; Tafalla et al., 2013).

Novel vaccine research utilizes live-attenuated, subunit, or recombinant DNA and RNA particle vaccines with new antigenic components (Kim et al., 2016). Live attenuated vaccines are more immunogenic than inactivated bacteria due to their ability to proliferate, enter the host, and trigger innate and adaptive immune responses (Adams, 2019; Liu et al., 2018). DNA vaccines contain an expression plasmid that transfers a specific gene that codes a desired antigenic protein within bacterial cells. The gene is encompassed by promoter and termination components that promote the expression. Multivalent DNA vaccines can strongly stimulate the adaptive immune system and protect the fish (Adams, 2019; Levine & Sztein, 2004).

Comparative bacterial genomics is also a beneficial tool for vaccine development that uses genome-sequenced data to describe different biological aspects (bacterial evolution, physiology, and pathogenesis) of microorganisms (Fraser et al., 2000; Prentice, 2004). Genomics research (e.g., classic microbiology, molecular microbiology, and host-pathogen interactions) can provide

comprehensive information about pathogenesis and immune responses that the host employs to control infectious diseases (García-Angulo et al., 2014; Seib et al., 2009; Weinstock, 2000).

1.9. Research Rationale

Research on Atlantic salmon from Norway, Iceland, and to a lesser extent, Scotland has contributed significantly to our understanding of winter ulcer primarily caused by *M. viscosa*. However, few of these studies have included isolates of *M. viscosa* from Canada, and the differences in the temperature that ulcerative outbreaks occur (< 7°C vs. 10-13°C, respectively) suggest that different pathogens might be involved. In Atlantic Canada, this disease has a significant economic impact, but there is limited published data on the appearance or causes of skin ulcers in Atlantic salmon reared in this region. Consequently, Chapter 2 of this thesis focused on the phenotypic and genomic characterization of a new pathogen isolated from an ulcerative outbreak in Atlantic salmon held at 10°C at Memorial University. Comparative genomic studies of bacteria may provide novel insights into bacterial evolution and virulence and contribute to effective vaccine or drug design programs. Also, they can provide us with useful information on pathogenesis, the host immune response, and different aspects of pathogens-host interactions that are important in preventing infectious diseases.

Ulcerative disease has been frequently reported in vaccinated farmed Atlantic salmon and the occurrence of ulcerative disease in these fish suggests that the current vaccine might not be sufficiently efficacious. Thus, in Chapter 3 of this thesis, farmed Atlantic salmon previously vaccinated with ALPHA JECT micro IV (Pharmaq, Novahalla, Norway) were challenged with *Moritella viscosa,* to examine the effectiveness of this formulation. Mortality rates were recorded and susceptibility of farmed Atlantic salmon were studied following bath and ip challenges. Furthermore, Atlantic salmon were boosted by in-house vaccines to determine the antigenicity of

M. viscosa vaccine components in Atlantic salmon. Hemato immune response was analyzed in vaccinated and boosted animals after the challenge and, antibody titers of vaccinated and challenged Atlantic salmon were measured by performing an indirect Enzyme-Linked Immunosorbent Assay (ELISA).

1.10. General Objective

To identify the causative agent of ulcer outbreak isolated from Atlantic salmon (*Salmo salar*) in Newfoundland and Labrador, and to evaluate the vaccine efficacy and immune response of vaccinated farmed Atlantic salmon to *M. viscosa* challenge.

1.11. Specific Objectives

1. To determine the potential causative agent of an ulcerative disease event in Atlantic salmon in Newfoundland and Labrador, by characterizing its phenotypic traits and performing genomic analyses of the virulent isolate (Chapter 2).
2. To determine the susceptibility of vaccinated Atlantic salmon to *M. viscosa* challenge, and to evaluate the immune response of farmed Atlantic salmon, to *M. viscosa* vaccine preparations (Chapter 3).

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2. Chapter 2: Comparative Genomic Analysis of a Novel *Vibrio* sp. Isolated from an Ulcer Disease Event in Atlantic Salmon (*Salmo salar*)

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2.1. Abstract

Ulcer diseases are a recalcitrant issue at Atlantic salmon (*Salmo salar*) aquaculture cage-sites across the North Atlantic region. Classical ulcerative outbreaks (also called winter ulcer disease) refer to a skin infection caused by *Moritella viscosa*. However, several bacterial species are frequently isolated from ulcer disease events, and it is unclear if other undescribed pathogens are implicated in ulcer disease in Atlantic salmon. Although different polyvalent vaccines are used against *M. viscosa*, ulcerative outbreaks are continuously reported in Atlantic salmon in Canada. This study analyzed the phenotypical and genomic characteristics of *Vibrio* sp. J383 isolated from the internal organs of vaccinated farmed Atlantic salmon displaying clinical signs of ulcer disease. Infection assays were conducted on vaccinated farmed Atlantic salmon and revealed that *Vibrio* sp. J383 causes a low level of mortalities when administered intracelomic at doses ranging from 10^7–10^8 CFU/dose. *Vibrio* sp. J383 persisted in the blood of infected fish for at least 8 weeks at 10 and 12°C. Clinical signs of this disease were greatest at 12°C, but no mortality and bacteremia were observed at 16°C. The *Vibrio* sp. J383 genome (5,902,734 bp) has two chromosomes of 3,633,265 bp and 2,068,312 bp, respectively, and one large plasmid of 201,166 bp. Phylogenetic and comparative analyses indicated that *Vibrio* sp. J383 is related to *V. splendidus*, with 93% identity. Furthermore, the phenotypic analysis showed that there were significant differences between *Vibrio* sp. J383 and other *Vibrio* spp, suggesting that J383 is a novel *Vibrio* species adapted to cold temperatures.

Keywords: Atlantic salmon, *Vibrio* sp. J383, genomics, phylogenetics, phenotype, ulcer disease

2.2. Introduction

Ulcerative diseases in Atlantic salmon (*Salmo salar*) aquaculture were first reported in Norway in 1980, and still are a significant health/economic issue for the North Atlantic region

(Lunder, 1990). The Gram-negative marine pathogen *Moritella viscosa* is typically described as the etiological agent of the classic ulcer disease (also called winter ulcer disease) in European farmed fish (Benediktsdóttir et al., 2000; Løvoll et al., 2009). Although *M. viscosa* is the primary pathogen associated with ulcer disease, it is not the only bacterium isolated from ulcers and lesions in Atlantic salmon. The isolation and identification of various bacterial species from ulcer-disease cases have been reported, including *Aliivibrio wodanis* (formerly *Vibrio wodanis*) and *Tenacibaculum* sp. (Benediktsdottir et al., 1998; Olsen et al., 2011). In fact, *Tenacibaculum* might target scarified skin, and co-infect wounds with *M. viscosa* (Olsen et al., 2011). Also, *M. viscosa* might co-infect with *A. wodanis,* which may limit the growth of *M. viscosa* (Hjerde et al., 2015). In addition, *A. wodanis* has the ability to solo infect Atlantic salmon (Karlsen et al., 2014).

Since the first documented outbreak of ulcerative disease in Eastern Canada (summer 1999) caused by *A. wodanis* in Atlantic salmon (Whitman et al., 2001), several pathogens including *M. viscosa* and *Tenacibaculum* spp. have been isolated (Lunder et al., 1995; Olsen et al., 2011). Despite broad immunization with polyvalent vaccines containing *M. viscosa* antigens, ulcerative disease events continue to be reported, causing poor fillet quality and financial losses (Furevik et al., 2023; Karlsen et al., 2017). This suggests that undescribed bacterial species may be involved in ulcer disease pathology (Karlsen et al., 2017), and that they are not covered by current vaccines.

The occurrence of ulcerative disease in Norway and other European countries is significantly different from in Canada. In European countries, ulcerative disease occurs below 7°C (Tunsjø et al., 2007). However, in Canada, ulcerative disease frequently occurs in summer and mid-autumn when water temperatures are over 10°C (MacKinnon et al., 2019a). The mortality rate caused by ulcerative disease is less than 10% in Norwegian farms during winter outbreaks, and fish that survive recover when the water temperature increases above 8°C in spring (Lunder et al., 1995).

In Atlantic Canada, the highest cumulative cage-level mortality recorded was 31.2% in the first described outbreak (Whitman et al., 2001); however, it seems that mortality has decreased over time, although its frequency has increased (MacKinnon et al., 2019a).

Skin-ulcer disease in Atlantic salmon is relatively unexplored, and there is limited published data on this disease in Atlantic salmon on the east coast of Canada (MacKinnon et al., 2019a, b). Salmon farmers report that skin ulcers in sea-cages can progress very fast. At first, fish may only have laterally raised scales, and after a few days, they die with a single large circular ulcerative lesion of several centimeters in diameter (MacKinnon et al., 2019b). Actually, in Atlantic Canada, "summer" skin ulcers in Atlantic salmon lead to significant mortality rates and economic losses (MacKinnon et al., 2019a).

In this study, we isolated a novel *Vibrio* sp. J383 strain from vaccinated farmed Atlantic salmon exhibiting clinical signs of skin ulcer disease and characterized its phenotype and genome. Infection assays revealed that *Vibrio* sp. J383 does not cause an acute infection, but instead causes a chronic infection in Atlantic salmon. Comparative genomics analyses suggest that *Vibrio* sp. J383 is a new species that might contribute to skin ulcer disease in Atlantic salmon.

2.3. Materials and Methods

2.3.1. Phenotypic Characterization

2.3.1.1. Isolation

Farmed Atlantic salmon vaccinated with ALPHA JECT exhibiting clinical signs of ulcer disease at 12°C (Figure 2-1) were netted and immediately euthanized with an overdose of MS-222 (400 mg/L; Syndel Laboratories, BC, Canada). Tissue samples (spleen, head kidney, and liver) were collected and placed into sterile homogenizer bags (Nasco whirlpak®, Fort Atkinson, WI, USA, then weighed and homogenized in phosphate-buffered saline [PBS; 136 mM NaCl, 2.7 mM

KCl, 10.1 mM Na2HPO4, 1.5 mM KH2PO4 (pH 7.2) up to a final volume of 1 mL] (Vasquez et al., 2020a). From the homogenized tissue suspension, 100 µL was plated onto Trypticase Soy Agar (TSA; Difco, Franklin Lakes, NJ, USA) supplemented with up to 2% NaCl and incubated at 15°C for 48–72 h. Colonies were streak purified on agar for further analysis (Leboffe & Pierce, 2015). Bacterial stocks were preserved at −80°C in 10% glycerol and 1% peptone solution. A single colony of each isolated bacteria was grown in 3 mL Trypticase Soy Broth (TSB) supplemented with 2% NaCl in a 16 mm diameter glass tube and placed in a drum roller (TC7, New Brunswick Scientific, MA, USA) for 24 h at 15°C with aeration (180 rpm). When required, TSB was supplemented with 1.5% bacto-agar (Difco) and 0.02% Congo-red (Sigma-Aldrich, Burlington, MA, USA) (Connors et al., 2019; Soto-Dávila et al., 2019). Luria Bertani (LB; yeast extract 5 g; tryptone 10 g; NaCl 10 g; dextrose 1 g) with different concentrations of NaCl (0, 0.5, 2%) was used to evaluate halophilic growth (Myhr et al., 1991).

2.3.1.2. Biochemical, Enzymatic and Physiological Characterization

The biochemical profile of *Vibrio* sp. J383 was characterized using API 20E, API 20NE, and API ZYM according to the manufacturer's (BioMerieux, Marcy-l'Etoile, France) instructions. The strips were incubated at 15°C for 48 h, and the results were analyzed using API web (BioMerieux). The primary characterization of *Vibrio* sp. J383 was performed based on the Gram stain, capsule stain, and morphological and cultural characteristics (Leboffe & Pierce, 2015). *Vibrio* sp. J383 growth rate was determined in TSB with 2% NaCl at 4, 15, 28, and 37°C. Halophilic growth was evaluated in LB supplemented with 0.5 and 2% of NaCl. Also, catalase and oxidase activity were measured according to standard protocols (Leboffe & Pierce, 2015; Myhr et al., 1991).

Hemolytic activity was assessed in TSA with 5% salmon blood and sheep blood agar at 15 °C (Myhr et al., 1991; Wood, 1983). The bacteria's liposaccharide (LPS) profile was examined

based on previous protocols (Hitchcock & Brown, 1983; Santander et al., 2013). *Vibrio* sp. J383 growth curves were determined in triplicate at 15°C according to established protocols (Connors et al., 2019).

Figure 2-1. *Vibrio* sp. J383 strain was isolated from the spleen of Atlantic salmon exhibiting clinical signs of skin ulcers.

2.3.1.3. Antibiogram

The susceptibility to antimicrobials was determined using sensi-disc diffusion tests (Ramasam et al., 2018). Briefly, *Vibrio* sp. J383 susceptibility was determined for tetracycline (10 µg), oxytetracycline (30 µg), ampicillin (10 µg), sulfamethoxazole (STX) (25 µg), chloramphenicol (30 µg), colistin sulphate (10 µg), and oxalinic acid (2 µg) using standard methods (Myhr et al., 1991; Ramasam et al., 2018).

2.3.1.4. Siderophore Synthesis

A siderophores secretion assay was performed using CAS plates according to standard procedure (Louden et al., 2011). Briefly, previously mentioned conditions were used to cultivate *Vibrio* sp. J383, and mid-log phase bacteria with an optical density (O.D.) at 600 nm of 0.7 were

harvested, washed three times with PBS at 6000 rpm for 10 min, and then resuspended in 1 mL of PBS. This bacterial culture was used to inoculate TSB with 2% NaCl, and TSB with 2% NaCl supplemented with 100 µM of FeCl3 or 100 µM of 2,2 dipyridyl in a ratio 1:10 (bacteria: culture media). *Vibrio* sp. J383 was cultured under aeration for 24 h at 15°C. The cells were collected at the mid-log phase after the incubation time, washed twice with PBS, and resuspended in 100 µL of PBS. After that, CAS agar plates were inoculated with 5 µL of the concentrated bacterial pellet and incubated at 15°C for 48 h (Louden et al., 2011).

2.3.2. Infection Trials

2.3.2.1. Fish Origin and Holding Conditions

Farmed Atlantic salmon (~200–250 g) of New Brunswick (Saint John River) origin that had been vaccinated with ALPHA JECT micro IV (Pharmaq, Overhalla, Norway) were held at the Joe Brown Aquatic Research Building (Ocean Sciences Center, Memorial University; MUN) in 3800 L tanks supplied with 95–100% air saturated, and UV-treated, flow through seawater at 10–12°C, and an ambient photoperiod (spring–summer). The fish were fed three days per week at 1% body weight with a commercial dry pellet (Skretting, BC, Canada; 50% protein, 18% fat, 1.5% carbohydrate, 3% calcium, 1.4% phosphorus). All experiments were conducted under approved institutional animal ethics protocols (#18-1-JS and #18-03-JS).

2.3.2.2. Bacterial Inoculum Preparation

Isolated strains were grown, harvested, and used to infect the Atlantic salmon. Briefly, bacterial cells were harvested at the mid-log phase, at an O.D. at 600 nm of ~0.7, and washed three times with PBS at 6000 rpm for 10 min. Bacterial O.D. was monitored using a Genesys 10 UV

spectrophotometer (Thermo Spectronic, Thermo Fischer Scientific, MA, USA) and by plating to determine the colony forming units (CFU/mL) (Umasuthan et al., 2021; Vasquez et al., 2020b).

2.3.2.3. Koch's Postulates

The infection procedures were conducted in the AQ2 biocontainment unit at the Cold Ocean Deep-Sea Research Facility (CDRF), MUN. Fish were transferred to the AQ2-CDRF unit and acclimated for one week at 10°C before infection. An initial infection screening assay was conducted in Atlantic salmon (200 g) intraperitoneally (ip) injected with a high dose (1×10^8 CFU/dose) of each isolated strain. Each infected group consisted of 6 fish in individual tanks under optimal conditions. Fish ip injected with PBS were used as a negative control, and fish ip injected with *M. viscosa* J311 (ATCC BAA-105) were used as a positive control.

A total of 135 Atlantic salmon (~250 g) were used to evaluate Koch's postulates for *Vibrio* sp. J383 (Koch, 1893; Umasuthan et al. 2021; Vasquez et al., 2020a). The infection procedures were conducted according to established protocols (Chakraborty et al., 2019; Vasquez et al., 2020b). Briefly, fish were anesthetized with 0.05 g/L MS-222 and individually injected with 100 µL of the respective bacterial inoculum. Fish were divided into three 500 L tanks containing 45 Atlantic salmon each, and intracelomic (ic) infected with 10^6, 10^7, and 10^8 CFU/dose, respectively. Mortality was monitored until 12 weeks post-infection (wpi). The water temperature was increased during the experiment, starting at 10°C for 4 wpi, then raised to 12°C at 5 wpi, and finally increased to 16°C at 9 wpi until 12 wpi. Tissue samples (e.g., head kidney, liver, spleen, and blood) from 6 fish of each dose were aseptically taken at 2 wpi. Bacterial loads were determined by established protocols (Chakraborty et al., 2019; Vasquez et al., 2020b). Briefly, liver, spleen and head kidney were aseptically removed, and sections of the collected tissues were placed into sterile homogenizer bags (Nasco whirl-pak®, Fort Atkinson, WI, USA), weighed, and PBS was added to

a final volume of 1 mL. Then, the tissues were homogenized, the suspensions were serially diluted (1:10), and then plated onto TSA supplemented with 2% NaCl. To determine the number of bacteria CFU per g of tissue, the plates were incubated at 15°C for at least 5 days. The total bacterial count was normalized to 1 g of tissue according to the initial weight of the tissue as previously described (Vasquez et al., 2020b). Additionally, blood samples were taken from 6 fish per dose every two weeks until the end of the experiment (i.e., at 2, 4, 6, 8, 10, and 12 wpi). Heparin (100 mg/mL) was added to the blood samples, serially diluted in filtered sterilized seawater (1:10) and plated in TSA with 2% NaCl.

Since mortality was evident at 12°C, a second infection trial was conducted only at 12°C. A total of 160 Atlantic salmon (~250 g) were equally distributed in four 500 L tanks containing 40 fish each and acclimated at 12°C for one week before infection. One group of fish was not infected and used as a negative control. Fish in the other group were ic injected with 10^8 CFU/dose of *Vibrio* sp. J383. Blood samples were collected randomly from 9 fish every two weeks to determine the bacterial load until 14 wpi. Also, tissue samples (e.g., head kidney, liver, and spleen) were collected at 12 and 14 wpi from 9 fish. This experiment was conducted according to established protocols (Chakraborty et al., 2019; Vasquez et al., 2020b), and mortality was monitored daily until 12 wpi.

2.3.3. *Vibrio* sp. J383 Genomics

2.3.3.1. Vibrio sp. J383 DNA Extraction and Sequencing

Vibrio sp. J383 was grown, harvested, and washed as previously described. According to the manufacturer's instructions (Promega, Madison, WI, USA), the Wizard Genomic DNA Purification Kit was used to extract the genomic DNA (gDNA) of *Vibrio* sp. J383. The gDNA was quantified by spectrophotometer using a Genova Nano MicroSpectrophotometer (Jenway, UK)

and evaluated for purity and integrity by electrophoresis (0.8% agarose gel) (Sambrook & Russel, 2001). Libraries and sequencing were conducted at Genome Quebec (Canada) using PacBio and Miseq Illumina sequencers.

2.3.3.2. Genome Assembly, Annotation and Data Submission

Celera Assembler (August 2013 version) was used to assemble the PacBio readings. The assembled contigs were analyzed using a CLC genomic workbench (Qiagen, v22.0). Genome annotations were conducted using the Rapid Annotation Subsystem Technology pipeline (RAST 2.0.) (http://rast.nmpdr.org/; accessed on 2 February 2023) (Aziz et al., 2008; Overbeek et al., 2014), and PATRIC (https://www.patricbrc.org; accessed on 2 February 2023) (Davis et al., 2020). The *Vibrio* sp. J383 genome was submitted to the National Center for Biotechnology Information (NCBI) for public accessibility and re-annotated using the NCBI Prokaryotic Genome Annotation Pipeline. The *Vibrio* sp. J383 chromosomes and plasmid genomes were visually mapped using CG view software (https://cgview.ca/ (accessed on 20 June 2023).

2.3.3.3. Comparative Genomics Analysis

Average nucleotide identity (ANI) was calculated by whole genome alignments using the CLC genomic workbench whole genome analysis tool with default parameters. (Min. initial seed length = 15; Allow mismatches = yes; Min. alignment block = 100). A minimum similarity of 0.8 and a minimum length of 0.8 were used as parameters for CDS identity. A comparative heat map was made using CLC. Phylogenetic analysis was performed using two different software packages, CLC Genomic workbench v22.0 and MEGA11 (Tamura et al., 2021). Evolutionary history was estimated using the Neighbor-Joining method with a bootstrap consensus of 500 replicates (Saitou & Nei, 1987), and evolutionary distance was computed using the Jukes–Cantor method (Jukes &

Cantor, 1969). *Photobacterium damselae* 91-197 (AP018045/6) chromosomes were utilized as an outgroup (Teru et al., 2017). Whole genome dot plots between closely related strains were constructed using the whole genome analysis tool to visualize and analyze genomic differences. Comparative alignment analysis was conducted using the CLC genomic workbench (Qiagen, v22.0). This analysis was used to identify homologous regions (locally collinear blocks), translocations, and inversions within the two bacterial genomes for chromosomes 1 and 2.

2.3.3.4. Genomic Islands

The detection of genomic islands (GIs) was conducted using the Island Viewer v.4 pipeline (https://www.pathogenomics.sfu.ca/islandviewer/browse/; accessed on 1 February 2023), which integrates Island Path-DIMOB, SIGH-HMM, and Island Pick analysis tools into a single platform (Bertelli et al., 2017). Analysis was performed for both chromosomes and the plasmid. SecReT6 v3 web server was utilized to identify and annotate the type VI secretion system (T6SS) genes that share sequence homology with characterized T6SSs (Li et al., 2015; Zhang et al., 2023).

2.3.4. Statistical Analysis

Fish survival rates were transformed using an arc-sin (survival rate ratio) function. One-way ANOVAs were utilized to identify significant differences. GraphPad Prism 9 was used to conduct all statistical analyses (GraphPad Software, California, CA, USA).

2.4. Results

2.4.1. Phenotypic Characterization

Vibrio sp. J383 displayed substantial growth in TSB with 2% NaCl between 15°C and 4°C (Table 2-1). However, it did not grow well at 28°C and did not grow at 37°C. *Vibrio* sp. J383 grew well in LB with 1% and 2% NaCl at 15°C. However, it did not grow in LB supplemented with 0

and 0.5% NaCl at 15°C. These results indicated that *Vibrio* sp. J383 is psychotropic and halophilic. *Vibrio* sp. J383 showed hemolytic activity in sheep and salmon blood agar at 15°C (Supplementary Figure S2-1G, I). *Vibrio* sp. J383 was shown to be motile, oxidase and catalase-positive, and type I-fimbria-negative (Table 2-1).

To evaluate siderophore synthesis, *Vibrio* sp. J383 was grown under iron-enriched and iron-limited conditions, inoculated onto CAS agar plates, and incubated at 15°C for 48 h. Siderophore secretion was observed under iron-enriched (100 μM of FeCl3), iron limited (100 μM of 2,2 dipyridyl), and control conditions (TSB). Furthermore, there were no noticeable differences in the size of the halo for siderophore secretion between different groups (Supplementary Figure S2-1B).

The biochemical and enzymatic profiles indicated that *Vibrio* sp. J383 can synthesize alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine, valine, and cysteine arylamidase, acid phosphatase, naphthol-AS-BI phosphohydrolase, and galactosidase (Supplementary Table S2-1). *Vibrio* sp. J383 reduces nitrates and glucose, produces indole from tryptophan, produces esculinase and gelatinase, β-galactosidase and can utilize D-mannitol, D-glucose and D-amygdaline (Supplementary Table S2-1). The API 20NE profile 7474004 indicated that the isolate could be *V. vulnificus* or *V. alginolyticus* with 64.8% and 34.6% confidence, respectively (Supplementary Table S2-1).

Table 2-1. Phenotypic characteristics of *Vibrio* sp. J383

Characteristics (Growth at)	*Vibrio* J383
Gram Stain	Gram-Negative
Capsule stain	+
Hemolysin in Salmon blood agar (15°C)	+
Hemolysin in Sheep blood agar (15°C)	+
Hemolysin in Salmon blood agar (28°C)	–
Hemolysin in Sheep blood agar (28°C)	–
Type 1 fimbria	–
Growing in LB 0% NaCl (15°C)	–
Growing in LB 0.5% NaCl (15°C)	–
Growing in TSB 2% NaCl (4°C)	+
Growing in TSB 2% NaCl (15°C)	+
Growing in TSB 2% NaCl (28°C)	+
Growing in TSB 2% NaCl (37°C)	–
Motility Test	+
Catalase	+
Oxidase	+
Biofilm	+
Antibiogram using sensi-disk of:	**Halo diameter (mm)**
Vibriostatic agent (O-129)	25(Susceptible)
Tetracycline (10 μg)	30(Susceptible)
Oxytetracycline (30 μg)	30(Susceptible)
Ampicillin (10 μg)	23(Susceptible)
Sulfamethoxazole (25 μg)	25(Susceptible)
Chloramphenicol (30 μg)	30(Susceptible)
Colistin sulphate (10 μg)	0 (Resistant)
Oxalinic acid (2 μg)	24(Susceptible)

2.4.2. Antibiogram

The antibiogram analysis showed that *Vibrio* sp. J383 is colistin-sulphate-resistant, but susceptible to ampicillin, tetracycline, oxytetracycline, sulfamethoxazole, chloramphenicol, oxalinic acid, and the vibriostatic agent O-129 (Table 2-1). These results are similar to other *Vibrio* spp. strains (Machimbirike et al., 2023).

2.4.3. Infection Trials in Atlantic Salmon

We isolated a total of five strains, three from the head kidney, one from the liver, and one from the spleen, from different infected fish. An initial screening to determine the virulence of the five isolates was conducted in Atlantic salmon (200 g). The fish were transferred to the AQ2/3 biocontainment zone of the CDRF, acclimated for 1 week, and intraperitoneally (ip) injected with a very high dose (1×10^8 CFU/dose) of each isolate. Fish ip injected with PBS were used as a negative control, and fish ip injected with *M. viscosa* J311 were used as a positive control. As expected, *M. viscosa* killed all the animals quickly, and all the animals ip injected with PBS survived (Supplementary Figure S2-2). Only the strain J383 (SP6) caused mortality and clinical signs of ulcer disease. Mortality associated with J383 infection in Atlantic salmon indicates that it causes a chronic type of infection rather than an acute infection. This is consistent with the infection event from which these samples were obtained.

The first infection trial was conducted to evaluate Koch's postulates under rising temperature conditions (10°C for 4 weeks, 12°C for 4 weeks, and 16°C for 4 weeks). Approximately 22.5% mortality was recorded in the high-dose (10^8 CFU/dose) group, 5% mortality rate in the medium-dose (10^7 CFU/dose) group, and no mortality was observed in the low-dose (10^6 CFU/dose) group (Figure 2-2A). Mortality started at 10°C, peaked at 12°C, but was not reported at 16°C. Clinical signs and presence of *Vibrio* sp. J383 were observed in all moribund Atlantic salmon (Figure 2-2B). *Vibrio* sp. J383 was not detected in internal organs and blood samples at 2 wpi, but it was detected in blood in the high-dose infection group at 4, 6, 8 and 10 wpi. *Vibrio* sp. J383 was detected in the blood of fish infected with the medium dose at 6 and 10 wpi. However, at 12 wpi, no bacteria were observed in the collected samples from the different doses (Table 2-2). The isolation of *Vibrio* sp. J383 from blood samples confirmed Koch's postulates.

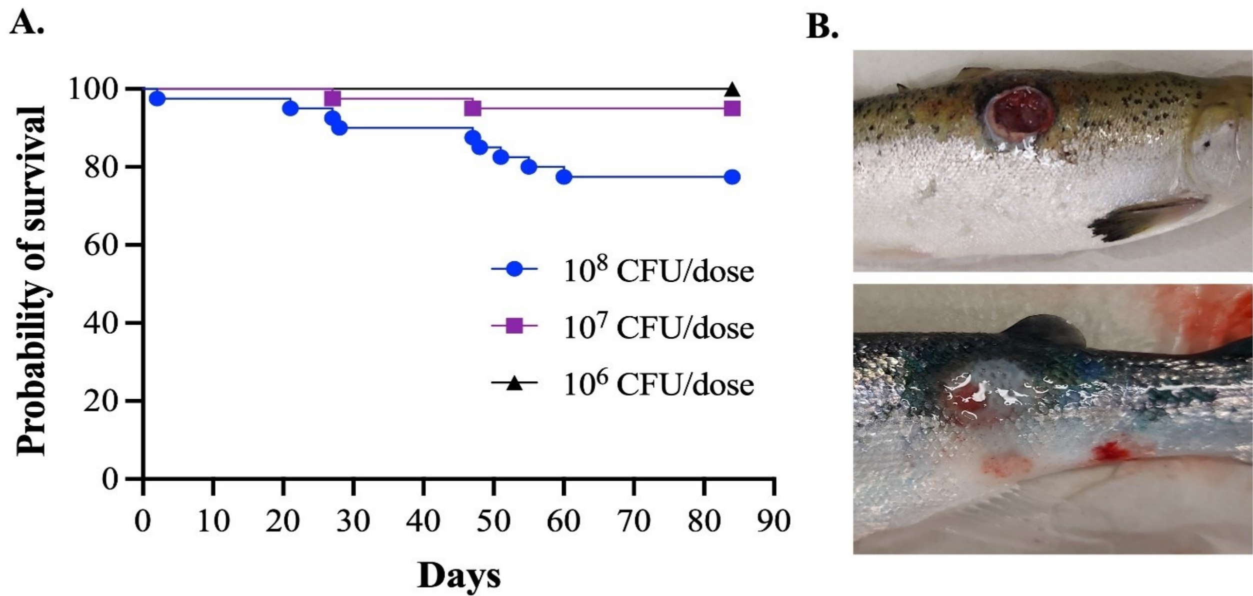

Figure 2-2. Survival and clinical signs of Atlantic salmon interperitoneally infected with *Vibrio* sp.: (A) Mortality of Atlantic salmon infected with *Vibrio* sp. J383; and (B) clinical sign of Atlantic salmon infected with *Vibrio* sp. J383.

Table 2-2. *Vibrio* sp. J383 isolated from blood samples at different time points post-infection (weeks post-infection: wpi).

Positive Samples for *Vibrio* spp. J383 (Total Positive/6 Fish)						
Temperature	**10°C**		**12°C**		**16°C**	
Dose	**2 wpi**	**4 wpi**	**6 wpi**	**8 wpi**	**10 wpi**	**12 wpi**
10^6	0/6	0/6	0/6	0/6	0/6	0/6
10^7	0/6	0/6	1/6	0/6	1/6	0/6
10^8	0/6	3/6	4/6	3/6	2/6	0/6

A second infection assay was performed at 12°C to determine the infection kinetics of *Vibrio* sp. J383, with 10^8 CFU/dose. Mortality started at 5 dpi and reached 20% by 90 dpi (Figure 2-3). *Vibrio* sp. J383 caused bacteremia in about 30–100% of the infected fish, and reached a peak at 6 wpi, which is consistent with the mortality levels (Figures 2-3 and 2-4A). Bacteremia started to decrease

by 8–10 wpi. No bacteria in the blood were detected after 12 wpi (Figure 2-4A), but *Vibrio* sp. J383 was detected in the spleen, liver, and head kidney at this sampling point (Figure 2-4B). Higher bacterial loads were observed in the spleen samples compared to the liver and head kidney (Figure 2-4B).

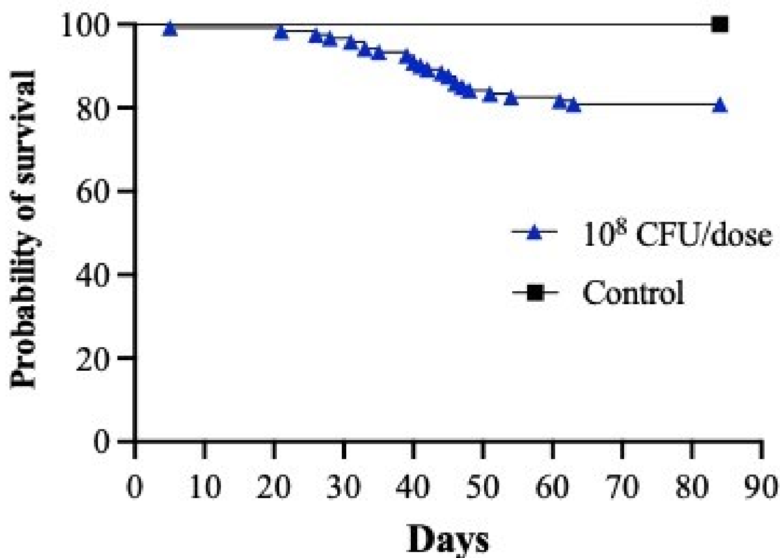

Figure 2-3. Survival percentage of Atlantic salmon interperitoneally infected with a high dose of *Vibrio* sp. J383 at 12°C.

2.4.4. *Vibrio* sp. J383 Genomics

Vibrio sp. J383 gDNA sequenced by PacBio and MiSeq revealed the presence of two chromosomes and one large plasmid. *Vibrio* sp. J383 chromosome 1 (NZ_CP097293.1) has 3,633,265 bp, chromosome 2 (NZ_CP097294.1) 2,068,312 bp, and the large plasmid pJ383 (NZ_CP097295.1) 201,166 bp (Figure 2-5A–C). The coverage assembly for chromosome 1, chromosome 2, and the parge plasmid was 306, 27, and 14 times, respectively. The plasmid profile agrees with the genomic analysis, supporting the theory that this *Vibrio* sp. possesses one large plasmid and no small plasmids. *Vibrio* sp. J383′ s genome was submitted to NCBI under the

BioProject (PRJNA836625) and BioSample (SAMN28165975). *Vibrio* sp. J383 genome has a total estimated length of 5.9 Mb and a G + C content of 44.3 and 44.1% for chromosomes 1 and 2, respectively. RAST pipeline annotation predicted a total of 309 subsystems and 3235 coding sequences (CDS) for chromosome 1, a total of 101 subsystems and 1866 CDSs for chromosome 2, and a total of 8 subsystems and 237 CDSs for the large plasmid p.J383 (Table 2-3). The NCBI Prokaryote Genome Annotation pipeline (PGAP) presented a total of 5288 genes predicted, a total of 16 (5S), 15 (16S), and 15 (23S) rRNAs, 138 tRNAs, and 5 ncRNAs for the whole genome (Table 2-4).

2.4.5. Genomic Islands (GIS)

Twenty-four putative GIs were identified within the chromosomes and plasmid: sixteen GIs in chromosome 1, seven GIs in chromosome 2, and one GI in the plasmid (Figure 2-6A–C). The GIs' size ranged from 6 kb to 50 kb, with a total of 929 genes (Supplementary Files S2-1–S2-3). Genes encoding for integrases, transposases, phage integrase, and multidrug-resistance transporters were found in GIs 6, 7, 9 and 12, respectively. Also, UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase, virulence factor VirK and alpha-galactosidase were in GIs 3, 7 and 15, respectively. Zonula occludes toxin (Zot)-like phage protein was found in chromosome 1 of *Vibrio* sp. J383. Glutaredoxin encoding gene was detected in genomic island 20 of chromosome 2.

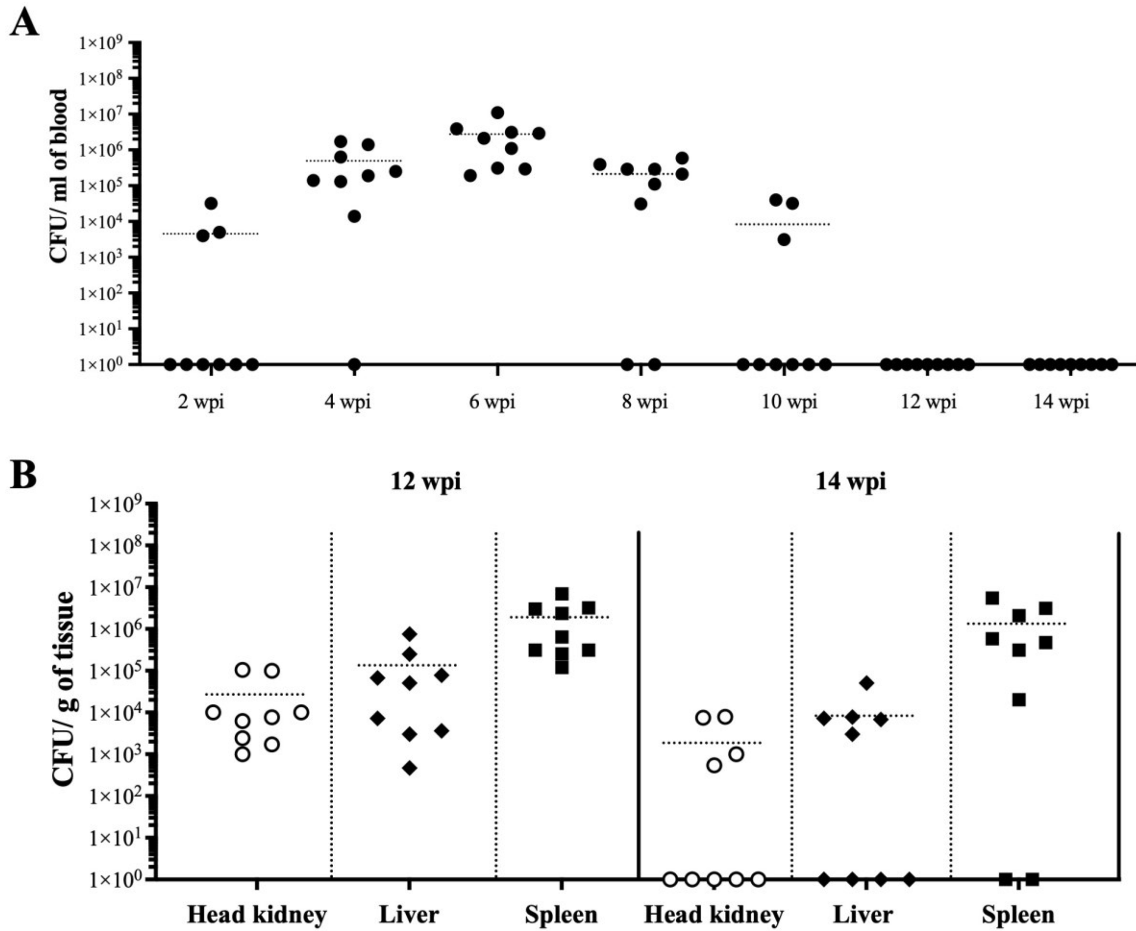

Figure 2-4. *Vibrio* sp. J383 blood and tissue colonization in vaccinated farmed Atlantic salmon. (A) *Vibrio* sp. J383 loads in blood of (n = 9) infected fish with the high dose (1×10^8 CFU/dose) at 2, 4, 4, 6, 8, 10, 12 and 14 wpi; and (B) *Vibrio* sp. J383 loads in head kidney, liver, spleen of (n = 9) infected fish with the high dose (1×10^8 CFU/dose) of *Vibrio* sp. J383 at 12 and 14 wpi. Full circle: blood; empty circle: head kidney; full rhomboid: liver; full square: spleen.

Table 2-3. Rapid annotation subsystem technology (RAST) *Vibrio* sp. J383 annotation.

Characteristics	Chromosome-1	Chromosome-2	Plasmid
Genome size (bp)	3,633,265	2,068,312	201,166
G + C content (%)	44.3	44.1	43.4
Number of subsystems	309	101	8
Number of coding sequences	3235	1866	237
Number of RNAs	163	21	0

Table 2-4. Prokaryotic genome annotation summary (*Vibrio* sp. J383).

Attribute	Data Provider
Annotation Pipeline	NCBI prokaryotic Genome Annotation pipeline
Annotation Method	Best-placed reference protein set; GeneMarkS-2+
Genes (total)	5288
CDSs (total)	5099
Genes (coding)	5031
CDSs (with protein)	5031
Genes (RNA)	189
rRNAs	16, 15, 15 (5S, 16S, 23S)
Complete rRNAs	16, 15, 15 (5S, 16S, 23S)
tRNAs	138
ncRNAs	5
Pseudo Genes (total)	68
CDSs (without protein)	68
Pseudo Genes (ambiguous residues)	0 of 68
Pseudo Genes (frameshifted)	29 of 68
Pseudo Genes (incomplete)	33 of 68
Pseudo Genes (internal stop)	21 of 68
Pseudo Genes (multiple problems)	13 of 68

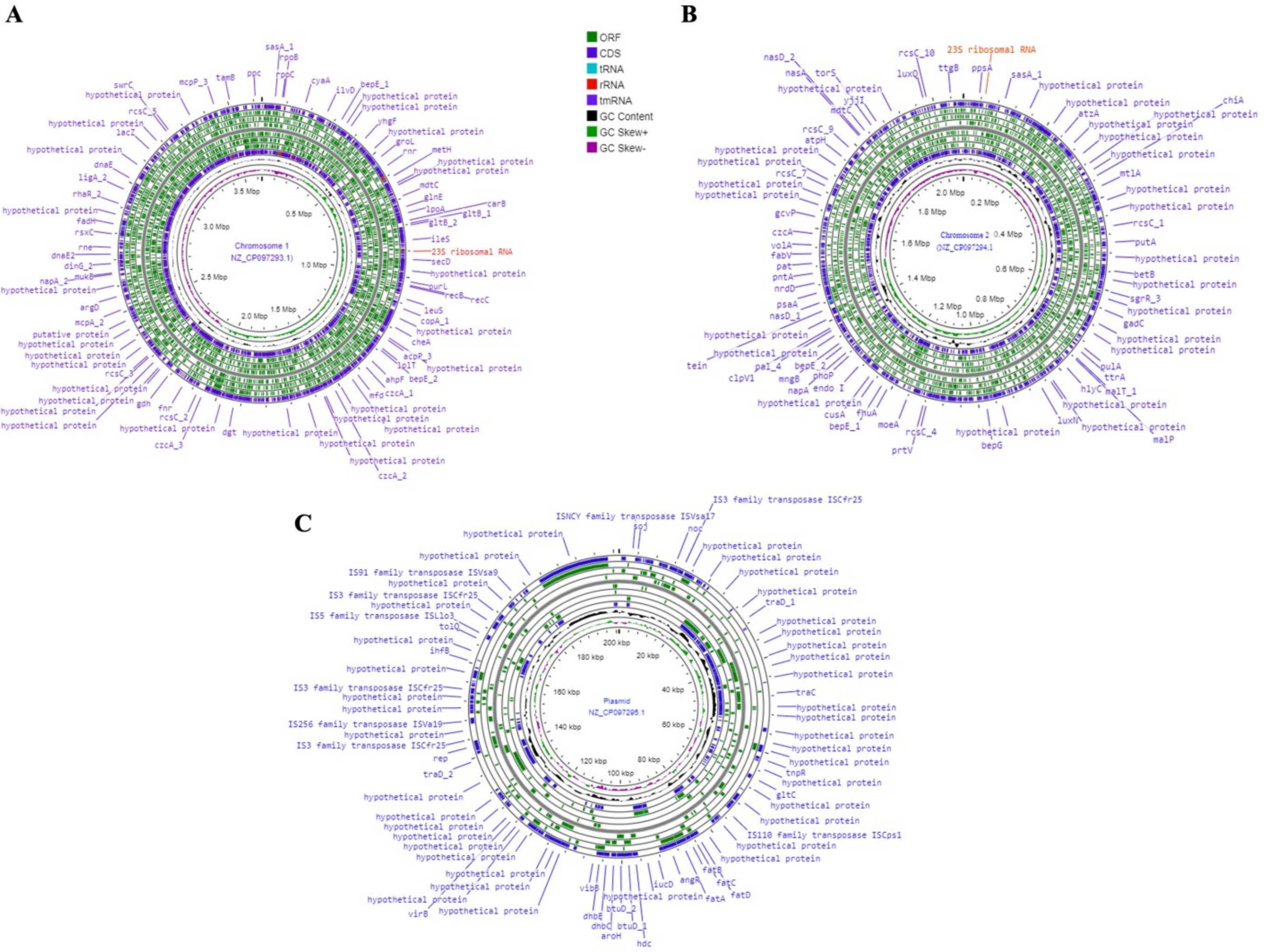

Figure 2-5. *Vibrio*. sp. J383 chromosomes. (A) *Vibrio*. sp. J383 chromosome 1 genome visualization; (B) *Vibrio*. sp. J383 chromosome 2 genome visualization; and (C) genome map representation of the large plasmid of *Vibrio* sp. J383. A circular graphical display of the distribution of the genome annotations is provided. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew.

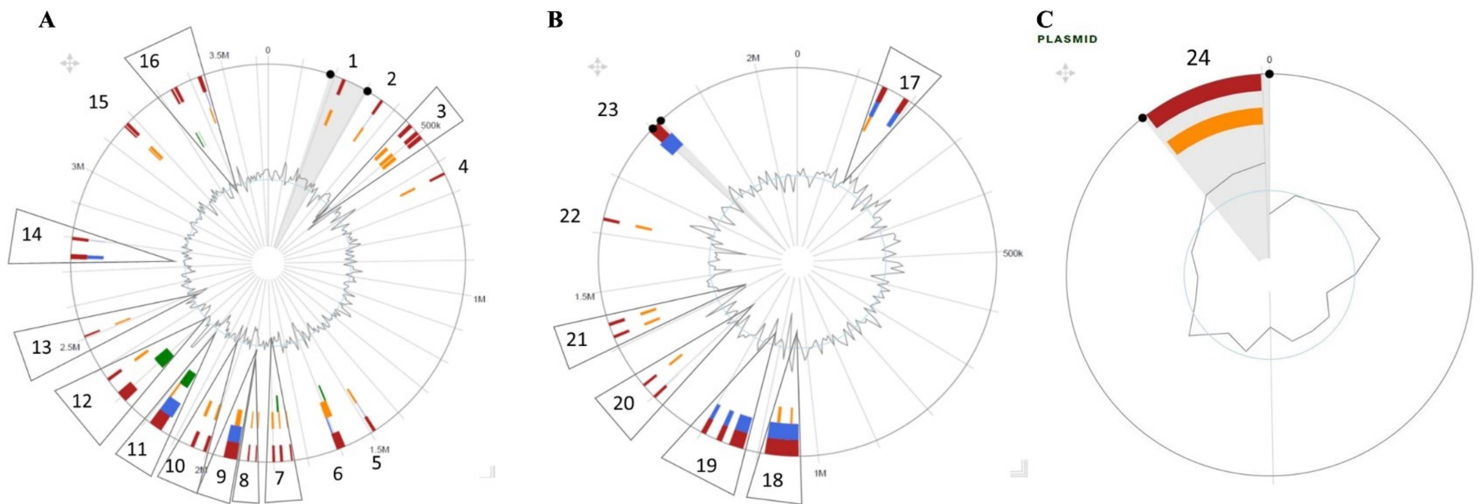

Figure 2-6. Genomic islands (GIs) detected in *Vibrio* sp. J383. (A) Chromosome 1 and (B) chromosome 2; and (C) genomic islands (GIs) detected in plasmid. Red bars represent GIs detected using 3 different packages; blue bars represent the GIs detected with SIGI-HMM package; orange bars represent the GIs detected with the Island Path-DIMOB package; green bars represent the GIs detected with the Island Pick package.

The type VI secretion system (T6SS) is an important virulence factor detected in *Vibrio* sp. J383. The SecReT6 v3 web server identified and annotated the T6SS genes that share sequence homology with characterized T6SSs and indicated that the *Vibrio* sp. J383 genome encodes two distinct T6SSs in GI 12 of chromosome 1 and GI 21 of chromosome 2 (Table 2-5, Figure 2-7, and Supplementary Files S2-4 and S2-5). The *Vibrio* sp. J383 secretion systems belong to the T6SSi family and are most closely related to the T6SS in *Vibrio coralliilyticus* OCN008. Some genes encoded within these two loci do not share sequence homology with known T6SS genes; however, predicting the structures of the encoded proteins with AlphaFold and comparison to solved structures in the Protein Data Bank (http://rcsb.org) (last accessed on 20 June 2023) allowed us to identify additional T6SS genes (Jumper et al., 2021). These analyses indicate that both T6SS loci

in the *Vibrio* sp. J383′ s genome encode for the full complement of components required for T6SS assembly and activity (Guillemette et al., 2020).

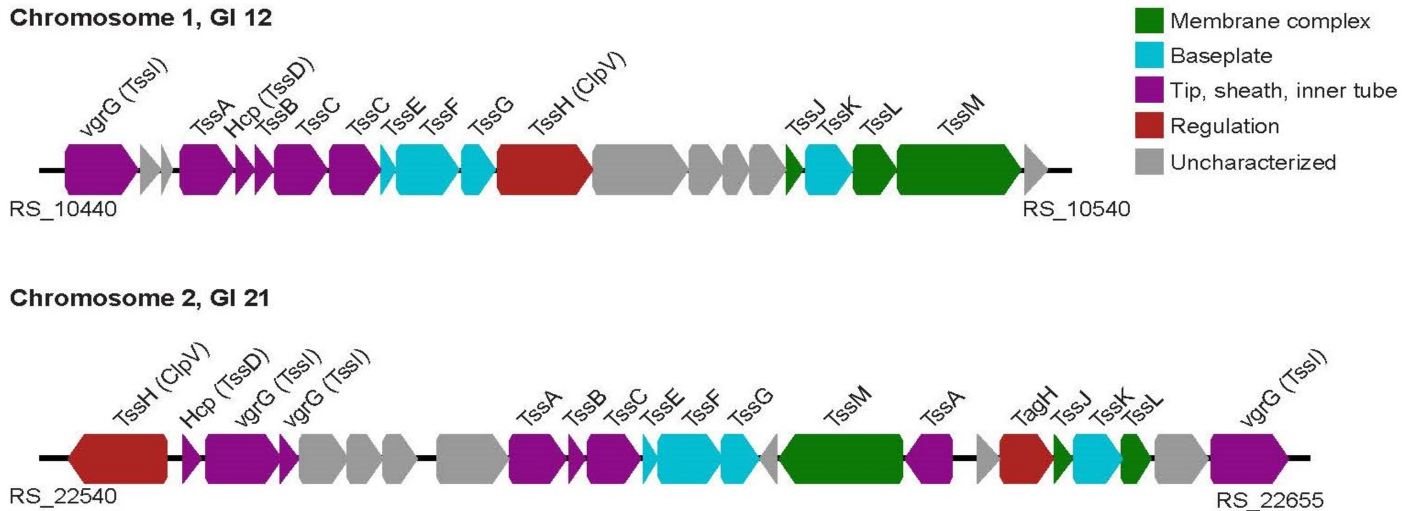

Figure 2-7. Type VI secretion system (T6SS) gene cluster in *Vibrio* sp. J383. The T6SS in chromosomes 1 and 2 are made from 13 Tss (Type Six Subunits) proteins that are known as the "core components". TssC gene duplicated in chromosome 1. Several uncharacterized genes are present in two T6SS loci and these may encode toxins secreted by the apparatus; however, further characterization is required to elucidate the role of these genes**.**

2.4.6. Comparative Genomic Analysis

The phylogenetic analysis of *Vibrio* sp. J383 chromosomes indicated that it was closely related to *V. splendidus* (Figure 2-8A–D). The average nucleotide identity (ANI) analysis between *Vibrio* sp. J383 and *V. splendidus* showed 95.75% identity for chromosome 1 (Supplementary Figure S2-3A) and 93.31% identity for chromosome 2 (Supplementary Figure S2-3B), which suggests that these two strains share a common ancestor.

Table 2-5. Genes of type VI secretion system (T6SS) in *Vibrio* sp. J383.

Gene	Locus Tag	Chromosome/GI	Location(nt)		Putative Function
vgrG	M4S28_RS10440	1/12	2,282,509	2,284,491	Tip of the T6SS apparatus
	M4S28_RS10445	1/12	2,284,552	2,285,112	Unknown
	M4S28_RS10450	1/12	2,285,122	2,285,421	Unknown
tssA	M4S28_RS10455	1/12	2,285,641	2,287,107	Cap of the T6SS sheath
hcp	M4S28_RS10460	1/12	2,287,140	2,287,661	Inner tube of the T6SS
tssB	M4S28_RS10465	1/12	2,287,681	2,288,184	T6SS sheath
tssC	M4S28_RS10470	1/12	2,288,184	2,289,662	T6SS sheath
tssC	M4S28_RS10475	1/12	2,289,701	2,291,095	T6SS sheath
tssE	M4S28_RS10480	1/12	2,291,095	2,291,517	T6SS baseplate
tssF	M4S28_RS10485	1/12	2,291,510	2,293,261	T6SS baseplate
tssG	M4S28_RS10490	1/12	2,293,326	2,294,258	T6SS baseplate
tssH	M4S28_RS10495	1/12	2,294,306	2,296,918	Disassembly of the T6SS apparatus
	M4S28_RS10500	1/12	2,296,928	2,299,495	MFS transporter
	M4S28_RS10505	1/12	2,299,492	2,300,490	ABC transporter protein
	M4S28_RS10510	1/12	2,300,477	2,301,205	Transporter protein
	M4S28_RS10515	1/12	2,301,209	2,302,159	FHA domain-containing protein
tssJ	M4S28_RS10520	1/12	2,302,156	2,302,644	T6SS membrane complex
tssK	M4S28_RS10525	1/12	2,302,686	2,304,017	T6SS baseplate
tssL	M4S28_RS10530	1/12	2,304,023	2,305,219	T6SS membrane complex
tssM	M4S28_RS10535	1/12	2,305,222	2,308,614	T6SS membrane complex
	M4S28_RS10540	1/12	2,308,694	2,309,347	AarF/UbiB family protein
tssH	M4S28_RS22540	2/21	1,384,843	1,387,536	Disassembly of the T6SS apparatus
hcp	M4S28_RS22545	2/21	1,387,991	1,388,509	Inner tube of the T6SS
vgrG	M4S28_RS22550	2/21	1,388,584	1,390,662	Tip of the T6SS apparatus
vgrG	M4S28_RS22555	2/21	1,390,662	1,391,147	Tip of the T6SS apparatus
	M4S28_RS22560	2/21	1,391,173	1,392,492	Unknown
	M4S28_RS22565	2/21	1,392,473	1,393,465	Unknown
	M4S28_RS22570	2/21	1,393,458	1,394,411	Unknown
	M4S28_RS22575	2/21	1,394,912	1,396,903	Unknown
tssA	M4S28_RS22580	2/21	1,396,905	1,398,479	Cap of the T6SS sheath
tssB	M4S28_RS22585	2/21	1,398,497	1,399,003	T6SS sheath
tssC	M4S28_RS22590	2/21	1,399,012	1,400,487	T6SS sheath
tssE	M4S28_RS22595	2/21	1,400,548	1,400,958	T6SS baseplate
tssF	M4S28_RS22600	2/21	1,400,969	1,402,717	T6SS baseplate
tssG	M4S28_RS22605	2/21	1,402,714	1,403,712	T6SS baseplate
	M4S28_RS22610	2/21	1,403,742	1,404,194	Lrp/AsnC transcriptional regulator
tssM	M4S28_RS22615	2/21	1,404,255	1,407,647	T6SS membrane complex
tssA	M4S28_RS22620	2/21	1,407,701	1,409,011	Cap of the T6SS sheath
	M4S28_RS22625	2/21	1,409,679	1,410,284	Unknown
tagH	M4S28_RS22630	2/21	1,410,294	1,411,790	Regulatory
tssJ	M4S28_RS22635	2/21	1,411,783	1,412,283	T6SS membrane complex
tssK	M4S28_RS22640	2/21	1,412,295	1,413,620	T6SS baseplate
tssL	M4S28_RS22645	2/21	1,413,617	1,414,408	T6SS membrane complex
	M4S28_RS22650	2/21	1,414,557	1,416,014	Unknown
vgrG	M4S28_RS22655	2/21	1,416,026	1,418,182	Tip of the T6SS apparatus

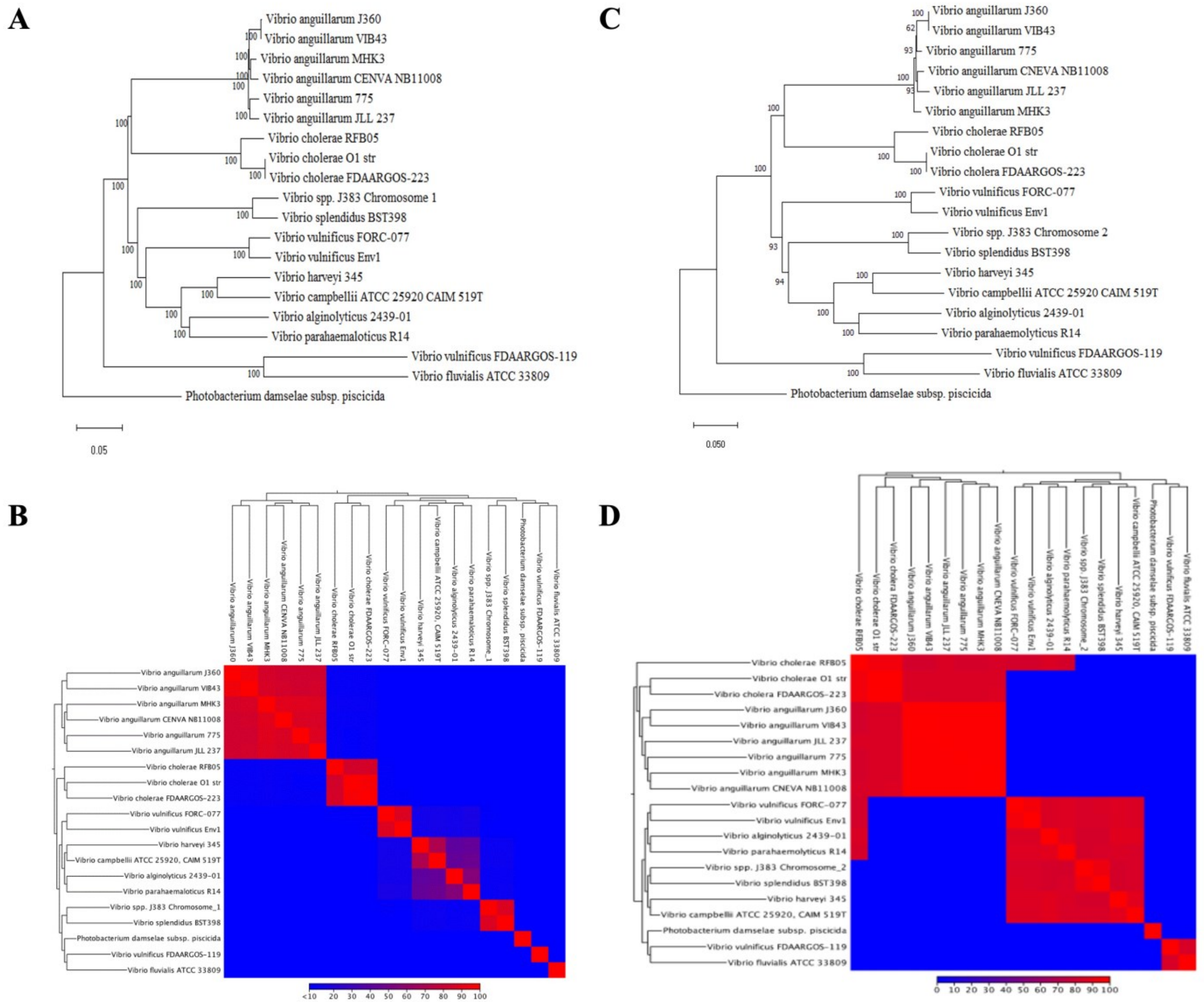

Figure 2-8. Phylogenetic history of *Vibrio* sp. J383 genome. (A) Chromosome 1 evolutionary history was inferred using the neighbor-joining method, with a bootstrap consensus of 500 replicates for taxa analysis in MEGA 11 software; (B) heat map visualization of aligned sequence's identities for *Vibrio* sp. J383 chromosome 1; genome alignment involved 20 *Vibrio* sp, analysis in CLC; and (C) chromosome 2 evolutionary history was inferred using the neighbor-joining method, with a bootstrap consensus of 500 replicates for taxa analysis in MEGA 11 software. (D) Heat map visualization of aligned sequences identified for *Vibrio* sp. J383 chromosome 2 genome alignment. This involved 20 *Vibrio* sp, and analysis in CLC.

However, the dot plot showed significant differences between *Vibrio* sp. J383 and *V. splendidus* in both chromosomes (Figure 2-9A, C), including genome gaps and one inversion event in chromosome 1 (Figure 2-9A, B).

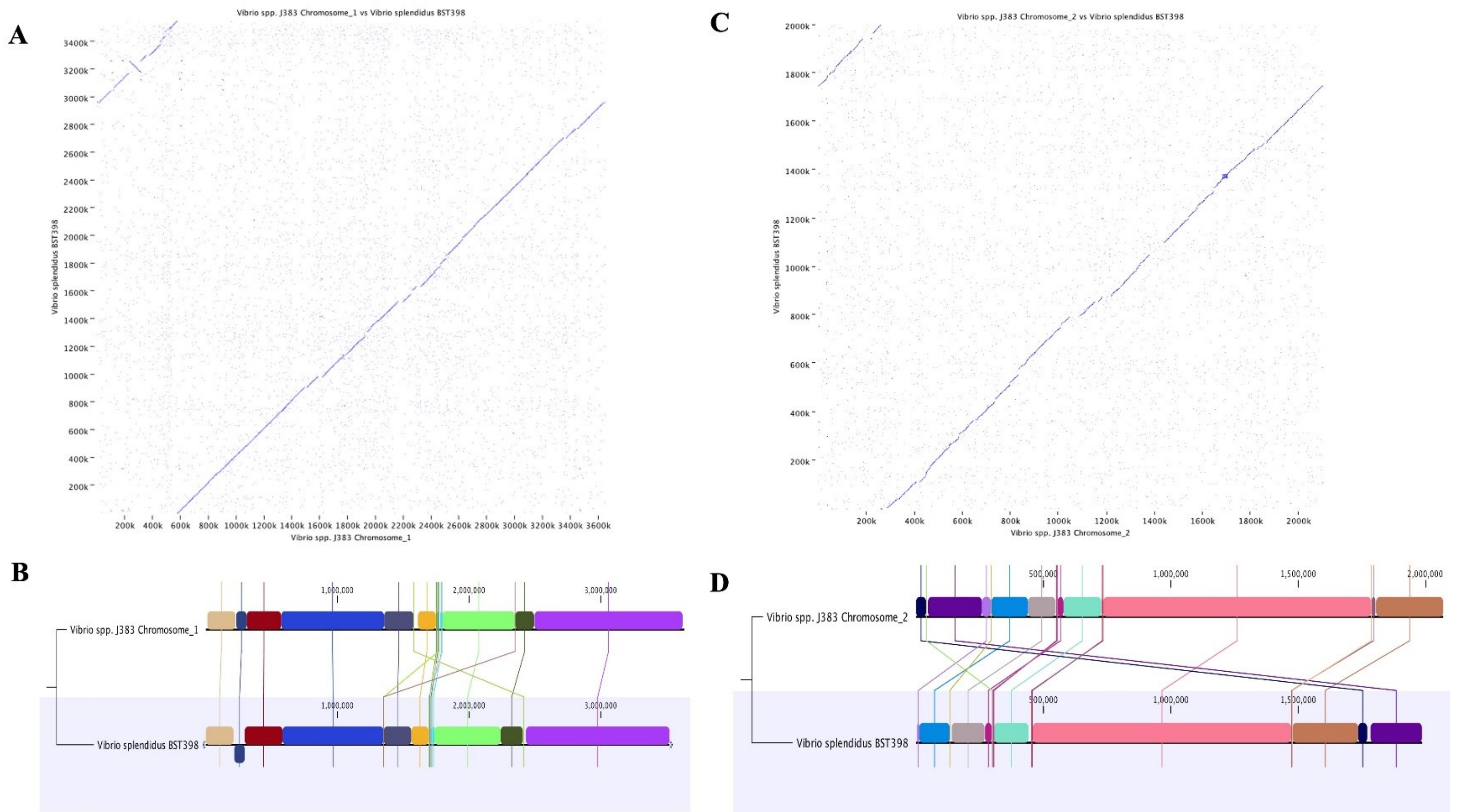

Figure 2-9. Comparative genome synteny between *Vibrio* sp. J383 and *V. splendidus* BST 398. (A) Dot plot analysis for chromosome 1; dot plots were computed using CLC Genomics Workbench v.20; blue arrow represents inversion. (B) Homologous regions identified as locally colinear blocks (LCBs) of chromosome 1. (C) Dot plot analysis for chromosome 2; Dot plots were computed using CLC Genomics Workbench v.20. (D) Homologous regions identified as locally colinear blocks of chromosome 2.

The whole genome alignment identified 18 locally collinear blocks (LCBs) in *Vibrio* sp. J383, which are conserved segments with no genomic rearrangements (Darling, et al., 2004). The comparative alignment analysis of each chromosome showed 9 LCBs in chromosome 1 (Figure 2-9B) and 9 LCBs in chromosome 2 (Figure 2-9D). Also, comprehensive genome analysis in PATRIC indicated that there were some pathogenesis-associated genes. Chromosomes 1 and 2 contain several transporter-related genes as well as genes linked to virulence or antibiotic resistance. RAST and PATRIC comprehensive genome analyses identified the presence or absence of specific genes in the chromosomes and the plasmid (Supplementary Table S2-2).

2.5. Discussion

The causes of ulcerative skin disease in Atlantic salmon are not fully understood. Several causative agents are being described in the North Atlantic rim, including *M. viscosa*, *Tenecebacillum* spp, and *A. wondandis* (Benediktsdottir et al., 1998; Olsen et al., 2011). However, differences in disease etiology indicate that several undescribed pathogens might also cause skin ulcers in Atlantic salmon. In the present study, we isolated *Vibrio* sp. strain J383 from the spleen of Atlantic salmon exhibiting skin ulcers (Figure 2-1). The phenotypic characterization indicates that this strain is marine and requires the presence of at least 1% of NaCl for survival (Table 2-1 and Supplementary Figure S2-1A). Also, *Vibrio* sp. J383 showed substantial growth between 4 and 15°C, but no growth at temperatures over 28°C, suggesting that this strain is adapted to cold temperatures. *Vibrio* sp. J383 also possesses several virulence factors, including hemolysins, siderophores, and LPS (Table 2-1, Supplementary Figure S2-1B, D, G, I), indicating that it has pathogenic properties. Finally, *Vibrio* sp. J383 constitutively synthesizes siderophores, indicating that this strain can scavenge essential iron within and outside the host. The synthesis of

siderophores is usually regulated (Miethke & Marahiel, 2007), but *Vibrio* sp. J383 possesses a natural constitutive expression that requires further study.

Infection assays indicated that *Vibrio* sp. J383 is a non-acute, chronic pathogen that can produce skin ulcers and kill fish, especially at 12°C (Figures 2-2 and 2-3). *Vibrio* sp. J383 triggered clinical signs of ulcer disease in vaccinated farmed Atlantic salmon, indicating that the generic vaccine utilized does not confer protection against this novel chronic pathogen (Figure 2-2B). Skin ulcer severity ranged from mild to severe in some of the fish that were infected with medium and high doses (10^7 and 10^8 CFU/dose, respectively) (Figure 2-2B). Although clinical signs of ulcer disease were evident in the infected fish, the low mortality rates indicated that this strain is not an acute pathogen. Around 5% to 22.5% mortality was recorded in the infected fish given the medium and high doses of *Vibrio* sp. J383 (Figure 2-2A). Our result is consistent with the literature, which indicates that the mortality during outbreaks in sea-cages with skin ulcers ranged from 0.01 to 23.32% (MacKinnon et al., 2019a). *Vibrio* sp. J383 caused bacteremia, and it was detected until 10 wpi in Atlantic salmon at 12°C. Bacteremia showed the same patterns in both infection assays (Figure 2-4A, Table 2- 2). This indicates that *Vibrio* sp. J383 can cause a systemic infection. *Vibrio* sp. J383 was detected at 6 wpi in most of the blood samples in the high-dose infection group (Table 2-2), and isolated from all the blood samples at 12°C (Figure 2-4A). Bacteremia was not recorded at 10°C at 2 wpi but was reported at 12°C in some blood samples (Table 2-2, Figure 2-4A). No bacteremia and mortality were recorded when the temperature increased to 16°C (Table 2-2). These results are consistent with previous field observations, which indicated that the water temperature during skin ulcerative disease outbreaks ranged from 10°C to 13°C (MacKinnon et al., 2019a). Our findings indicated that *Vibrio* sp. J383 becomes more invasive at 12°C compared to 10°C. Regardless of water temperature, *Vibrio* sp. J383 was not detected in the blood at 12 wpi

(Table 2-2, Figure 2-4A). However, *Vibrio* sp. J383 was detected in the spleen, head kidney, and liver at 12 and 14 wpi in Atlantic salmon infected with the high dose at 12°C (Figure 2-4B). Bacterial loads were significantly higher in the spleen compared to the liver and head kidney at 12 and 14 wpi. *Vibrio* sp. J383 loads decreased substantially from 12 wpi to 14 wpi (Figure 2-4B). Collectively, our findings suggest that *Vibrio* sp. J383 is a chronic pathogen.

The biochemical characterization and identification of environmental *Vibrio* species have been complicated because of their notable diversity (Alsina & Blanch, 1994; Sadok, et al., 2013). The biochemical profile attained using API 20NE showed that *Vibrio* sp. J383 was unable to reduce urea but reduces nitrates and produces indole, suggesting 64.8% and 34.6% similarity to *V. vulnificus* and *V. alginolyticus*, respectively (Supplementary Table S2-1). Phylogenetic and comparative analyses showed that *Vibrio* sp. J383 is closely related to *V. splendidus*, with 93% identity, and this suggests that these two strains share a common ancestor. However, phenotypical tests revealed significant differences between *Vibrio* sp. J383 and other *Vibrio* strains, indicating that *Vibrio* sp. J383 could be a novel species.

V. anguillarum J360, a virulent strain isolated from the North Atlantic, displayed thermo-inducible α-hemolysin activity at 28°C, but no hemolytic activity at 15°C (Vasquez et al., 2020a). In contrast, *Vibrio* sp. J383 showed hemolysin activity at 15°C but not at 28°C. *Vibrio* spp. utilizes hemolysins to lyse host erythrocytes to acquire nutrients, such as iron (Johnson, 2013). Hemolysins are crucial virulence factors for *V. anguillarum* and play a key role in boosting its pathogenicity (Hirono et al., 1996; Rock & Nelson, 2006). The presence of hemolytic activity in *Vibrio* sp. J383 indicates its potential pathogenicity. *Vibrio* sp. J383 showed growth at 4°C, optimal growth around 15°C, weak growth at 28°C and no growth at 37°C. Also, *Vibrio* sp. J383 produces catalase and oxidase. These results are consistent with most pathogenic marine *Vibrio* spp. (Lunder et al., 2000),

and the ability of *Vibrio* sp. J383 to grow at 4°C indicates that this novel pathogen is well adapted to cold environments.

The genotypic characterization of *Vibrio* sp. J383 indicated the presence of two chromosomes. The existence of two chromosomes is a basic characteristic of *Vibrio* spp. that developed as a survival means and allows for the rapid adaptation of the pathogen to different environments and hosts (Frans et al., 2011). *Vibrio* sp. J383 has a genome size of 5,902,734 bp, very similar to *V. splendidus* BST 398, (5,508,387 bp) (Table 2-3). The genome of *V. splendidus* BST 398 included 4700 predicted open reading frames, a G + C content of 44.12%, 137 tRNA genes and 46 rRNA genes (Park et al., 2018). In contrast, the whole genome of the novel strain *Vibrio* sp. J383 has a total of 16 (5S), 15 (16S), and 15 (23S) rRNAs, 138 tRNAs, and 5 ncRNAs (Table 2-4). These results suggest that although *V. splendidus* and *Vibrio* sp. J383 share a common ancestor, they are different strains with distinct genomic characteristics.

Some specific genes associated with virulence and antibiotic resistance were detected in the PATRIC comprehensive genome analysis. The tetracycline resistance subsystem was found in *Vibrio* sp. J383, which makes this strain resistant to antibiotics and toxic compounds. Hydroxyacylglutathione hydrolase was also found in *Vibrio* sp. J383, which is a virulence-related gene and contributes to the stress response in bacteria.

Cold shock proteins of the CSP family were found in chromosome 2 of *Vibrio* sp. J383 (Supplementary Table S2-2). Cold shock proteins help cells adapt by reducing some of the negative effects of temperature changes (Phadtare, 2004). They play a crucial role in the cold shock response, and recent data suggest that CSPs may have a greater role in bacterial stress tolerance (Derman et al., 2015; Schmid et al., 2009; Wang et al., 2014). Following the initial cold shock response, the production of CSPs declines while the production of other proteins increases. This

helps the cells to grow at a low temperature, but at a slower rate (Ermolenko & Makhatadze, 2002). This finding can explain why *Vibrio* sp. J383 has fast growth at 15°C and slightly slower growth at 4°C (Table 2-1).

Flagellum was detected in chromosome 1 of *Vibrio* sp. J383 (Supplementary Table S2-2), which plays a key role in bacteria motility, and can contribute to biofilm formation, protein export and adhesion (Haiko & Westerlund-Wikström, 2013). Chemotaxis present in chromosome 1 of *Vibrio* sp. J383 plays several roles, including biofilm formation, auto aggregation and swarming, as well as in bacterial interactions with their hosts (Colin et al., 2021). Toxin–antitoxin systems are common in bacterial genomes and found in chromosome 2 of *Vibrio* sp. J383 (Supplementary Table S2-2). They are normally made of two elements: a toxin that inhibits a vital cellular process and an antitoxin that hinders its cognate toxin (Jurėnas et al., 2022).

A total of 24 genomic islands were detected in *Vibrio* sp. J383. Genomic Islands have also been identified in the species *V. anguillarum*. For example, *V. anguillarum* J360 has 21 GIs (Vasquez et al., 2020a). Genes encoding for integrase and transposase were found in the mentioned *Vibrio* strains. Glutaredoxin was detected in chromosome 2 of *Vibrio* sp. J383 and performs a critical role in the protection against oxidative stress in bacteria (Laporte et al., 2012). VirK is a virulence factor present in several bacterial pathogens that has been shown to contribute to *Salmonella enterica* serovar Typhimurium and *Escherichia coli* virulence (Detweiler et al., 2003; Song et al., 2019; Tapia-Pastrana et al., 2012). It may contribute to *Vibrio* sp.'s pathogenicity as well. Also, Zot was detected in the genomes of *V. parahaemolyticus* (Pérez-Reytor et al., 2020), indicating a correlation between this gene and the cytotoxicity of bacteria (Castillo et al., 2018; Pérez-Reytor et al., 2020). In *V. cholerae*, Zot is an important toxin after the classical cholera toxin (CT), and it is encoded by the CTX prophage (Schmidt et al., 2007). It has been shown that Zot

has enterotoxic activity and it is hypothesized that it plays a role in the classic diarrhea symptom of *V. cholerae* infections (Fasano et al., 1991; Suzuki, 2013). The presence of Zot in the genome of *Vibrio* sp. J383 perhaps contributes to its virulence in fish hosts.

The T6SS is a contractile nanomachine that plays a role in interbacterial competition and bacterial pathogenesis by secreting toxic effector proteins into adjacent cells (Bingle et al., 2008; Crisan & Hammer, 2020; Hachani et al., 2011; Russell et al., 2011; Russell et al., 2014; Zong et al., 2019). The *Vibrio* sp. J383 genome includes two T6SSs on genomic islands 12 and 21 (Table 2-5, Figure 2-7, and Supplementary File S2-4). T6SS apparatuses are composed of 13 core components that form two parts, the membrane complex and phage-like tail (Boyer et al., 2009; Cherrak et al., 2019). Three membrane associated proteins comprise the membrane complex, TssJ, TssL, and TssM (Aschtgen et al., 2010; Bingle et al., 2008; Boyer et al., 2009; Cascales, 2008). Consistent with this, the *Vibrio* sp. J383 homologs are predicted to be lipidated or to contain transmembrane domains. The T6SS tail is evolutionarily related to the contractile tail of the T4 bacteriophage and is composed of several subassemblies. The baseplate is composed of tssE, tssF, tssG, and tssK (Cherrak et al., 2018; Taylor et al., 2016), and the *Vibrio* sp. J383 homologs share 60–80% identity with other *Vibrio* T6SS counterparts. tssB, tssC, and tssA comprise the sheath and cap of the T6SS and are encoded in both loci (Basler et al., 2012; Bönemann et al., 2010; Zoued et al., 2017). Of note, tssB and tssC share greater than 95% identity with other *Vibrio* T6SSs. Hcp and VgrG play a role in substrate recognition and are co-secreted with associated toxins, and both are encoded in the T6SS loci in *Vibrio* sp. J383 (Alteri & Mobley, 2016; Boyer et al., 2009; Cascales, 2008).

The genes that encode these T6SS apparatus subunits share high levels of homology between genomic islands 12 and 21. Based on homology with well-characterized T6SSs, both apparatuses

encoded by *Vibrio* sp. J383 belong to the T6SSi family. It is impossible to identify T6SS-exported toxins based on sequence alone; however, some of the hypothetical genes in these two loci encode hallmarks that suggest that they may be effector proteins. The presence of toxic effectors genes in close proximity to T6SS apparatus genes does not preclude other effectors encoded in distant genomic loci, and further research can identify these toxins in the future.

Gene duplications are essential prerequisites for gene innovation, which may assist in adaptation to changing environmental conditions (Gevers et al., 2004). In chromosome 1 of *Vibrio* sp. J383, tssC is duplicated (Figure 2-7). T6SSs have been strongly linked to a variety of biological processes, including biofilm formation, bacterial survival in the environment, virulence, and host adaptation; therefore, the duplication of tssC in *Vibrio* sp. J383 may be an important step toward increasing the fitness of this strain in the environment, amongst the microbiota, or as a pathogen (Chen et al., 2020; Masum et al., 2017; Yu et al., 2021).

In summary, *Vibrio* sp. J383 has several virulence factors and genes that are associated with pathogens.

Vibrio sp. J383 has one large plasmid, and an analysis based on PATRIC annotation found one subsystem, the MazEF toxin–antitoxin (program cell death) system (Table S2-2). Toxin–antitoxin (TA) systems, initially discovered in plasmids, were recognized as extra chromosomal genes responsible for post-segregationally killing, which protects plasmid integrity (Ramisetty et al., 2015). Toxin–antitoxin (TA) systems have been reported in many bacterial genomes and mediate program cell death (PCD) and are therefore attractive targets for new antimicrobial drugs since they are recognized to “kill from within” (Amitai et al., 2009; Engelberg-Kulka et al., 2009). The antitoxins neutralize the toxin using different mechanisms and play vital roles, including the maintenance of genomic stability, and assist in biofilm formation in some bacteria (Ramisetty et

al., 2015). The presence of the TA system in the plasmid of *Vibrio* sp. J383 indicates that it may play the same role and enable *Vibrio* sp. J383 to survive in different temperatures and maintain its virulence factors.

2.6. Conclusions

The biochemical profile showed that *Vibrio* sp. J383 is similar to *V. vulnificus*, with 64.8% identity. However, phylogenetic and comparative analyses showed that *Vibrio* sp. J383 is closely related to *V. splendidus*, with 93% identity. The isolation of *Vibrio* sp. J383 from blood samples confirmed Koch's postulates. Mortality was approximately 20% in the vaccinated fish infected with a 10^8 CFU/dose, but no mortality was observed in fish infected with a low dose (10^6 CFU/dose). *Vibrio* sp. J383 was detected for 10 wpi in the blood and at up to 14 wpi in the internal organs (spleen and kidney). The pathogenicity of this new strain is supported by Koch's postulates and the presence of pathogenic genomic islands (GIs 12 and 21) containing virulence factors such as type VI secretion system (T6SS) genes and multidrug-resistance transporter/family protein. *Vibrio* sp. J383 displays unique characteristics, and has notable differences, compared to other *Vibrio* strains. The results of this study revealed that *Vibrio* sp. J383 is potentially a new species that can trigger clinical signs of ulcer disease and cause chronic infections in vaccinated farmed Atlantic salmon. The impact of the co-infection of *Vibrio* sp. J383 with other etiological agents, like *Moritella viscosa*, on Atlantic salmon remains to be investigated.

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3. Chapter 3. Haemato-Immunological Response of Immunized Atlantic Salmon (*Salmo salar*) to *Moritella viscosa* Challenge and Antigens

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3.1. Abstract

Winter ulcer disease is a health issue in the Atlantic salmonid aquaculture industry mainly caused by *Moritella viscosa*. Although vaccination is one of the effective ways to prevent bacterial outbreaks in the salmon farming industry, ulcer disease related to bacterial infections is being reported on Canada's Atlantic coast. Here, we studied the immune response of farmed immunized Atlantic salmon to bath and intraperitoneal (ip) *M. viscosa* challenges and evaluated the immunogenicity of several *M. viscosa* cell components. IgM titers were determined after infection, post-boost immunization, and post-challenge with *M. viscosa*. IgM^+ (B cells) in the spleen and blood cell populations were also identified and quantified by 3,3 dihexyloxacarbocyanine (DiOC6) and IgM-Texas red using confocal microscopy and flow cytometry. At 14 days post-challenge, IgM was detected in the serum and spleen. There was a significant increase in circulating neutrophils three days after ip and bath challenges in the *M. viscosa* outer membrane vesicles (OMVs) boosted group compared to non-boosted fish. Lymphocytes also increased in the blood at 7 and 14 days after the ip and bath challenges, respectively, in the OMVs boosted group. Furthermore, a rise in IgM titers was detected in the OMVs boosted group. We determined that the commercial vaccine is effective against *M. viscosa* strain J311 (ATCC BAA-105) and OMVs are the most immunogenic component of the *M. viscosa* cell.

Keywords: Atlantic salmon, *Moritella visco*sa, haemato-immune, flow cytometry, outer membrane vesicles, antibody

3.2. Introduction

A recognized cause of winter ulcerative disease in Atlantic salmon is *Moritella viscosa* (Løvoll et al., 2009). Two main clades ('typical' and 'variant') have been recognized in this pathogen. Typical *M. viscosa* has been isolated from Atlantic salmon cultured in European

countries. Variant *M. viscosa* has been isolated from Atlantic salmon reared in Canada (Grove et al., 2010; Karlsen et al., 2017). There are a few published studies on skin ulcerative diseases in Atlantic salmon, but less is known about it on Canada's east coast (MacKinnon, et al., 2019a, b). The Atlantic salmon industry attempts to prevent or control bacterial outbreaks using vaccines (Mondal & Thomas, 2022). Although vaccination is an effective management strategy that decreases disease outbreaks and minimizes the use of antibiotics in the aquaculture industry (Shoemaker et al., 2009), ulcerative disease is frequently reported in vaccinated Atlantic salmon in eastern Canada (Ghasemieshkaftaki et al., 2023). To develop effective vaccines, an in-depth understanding of the immune response in the target species after immunization is required. However, there are many unknown aspects regarding fish immunology, and we are still far from understanding which immune mechanisms are responsible for protecting fish against some of these pathogens (Secombes, 2008; Tafalla et al., 2013; Yamaguchi, et al., 2019). Most licensed vaccines for finfish are inactivated microbes mixed with adjuvant, which contain either single or combined heat or formalin-killed pathogenic microorganisms (Kayansamruaj et al., 2020; Ma et al., 2019). An efficient vaccine stimulates the development of long-lived plasma cells that produce high-affinity antibodies and memory B-cells. Antibodies play a significant role in limiting or preventing infection and can neutralize and remove pathogens before an infection becomes severe (Evensen, 2016). The pathogen's capability to grow in an iron-restricted environment led to the synthesis of Iron-Regulated Outer-Membrane Proteins (IROMP), which have been proposed as important antigens that protect against bacteria (Durbin et al., 1999). Their interaction with the host immune system makes them suitable candidates for vaccine development (Björnsson et al., 2011). Additionally, bacterial outer membrane vesicles (OMVs) contain protective antigens in their

structures and can be used for vaccines as well (Anand & Chaudhuri, 2016; Balhuizen et al., 2021; Mehanny et al., 2021; Prior et al., 2021; Sartorio et al., 2021).

Several studies have investigated aspects of the fish immune system (Bengtén et al., 2006; Gomez et al., 2013; Koshio, 2016; Mokhtar et al., 2023; Mu et al., 2022; Smith et al., 2019; Solem & Stenvik, 2006; Yu et al., 2020), and examined hematological responses in vaccinated and infected fish species (Isla et al., 2022; Monir et al., 2020). However, there are no studies assessing blood cell populations in Atlantic salmon following *M. viscosa* infection. Hematological analyses are commonly utilized to assess a fish's physiological status and health (Fazio, 2019). For example, leukocytes are polymorphic and multifunctional immune cells, and carry out various physiological and immunological tasks. They help fish adjust to various biotic and abiotic factors and protect the body from foreign substances (Gordeev et al., 2017). Changes in the number of leukocytes and their differential count (e.g., lymphocytes, neutrophils, eosinophils, and monocytes) are crucial clinical indicators, as they are symptomatic of several conditions, including acute and chronic stress, and pathogen exposure / infection (Witeska et al., 2022).

Here, we evaluated the hematological and immune response of vaccinated and boosted farmed Atlantic salmon after *M. viscosa* challenge. Farmed Atlantic salmon immunized with ALPHA JECT micro IV (Pharmaq, Norway) containing *M. viscosa* bacterin were bath and intraperitoneally challenged with *M. viscosa*. The vaccine efficacy and immune response were examined. Also, Atlantic salmon were boosted by *M. viscosa* antigens to evaluate the antigenicity of different bacterial components (bacterin and exudates′ (complete bacterin), bacterin cell, IROMP, and OMVs). IgM titers, peripherical and spleen IgM^+ cell populations, were measured in vaccinated and challenged groups. White blood cells (WBCs) showed a significant increase after challenge, with a higher cell percentage in the OMVs boosted group compared to the non-boosted

fish. IgM titers also increased following the vaccination and challenge in the OMVs boosted group. Our results show that *M. viscosa* OMVs are highly effective at stimulating an immune response in farmed Atlantic salmon.

3.3. Materials and Methods

3.3.1. Fish Holding

Vaccinated farmed Atlantic salmon (~ 350 ± 50 g) provided by Cooke Aquaculture Inc were held at the Ocean Sciences Center [Memorial University of Newfoundland]. Fish were kept and fed according to standard protocols (Ghasemieshkaftaki et al., 2023) and ethical procedures #18-01-JS, #18-03-JS, and biohazard license L-01 were implemented for the study.

3.3.2. Infection Trials

3.3.2.1. Inoculum Preparation

One colony of *M. viscosa* J311 (ATCC BAA-105) was cultivated in 3 mL of Trypticase Soy Broth (TSB, Difco) supplemented with 2% NaCl and set in a drum roller (TC7, New Brunswick, MA, USA) at 15°C for 24 h. Then, 300 µL of the culture was mixed with 30 mL of TSB 2% NaCl and was kept at 15°C incubator for 24 h with aeration (180 rpm). Bacterial proliferation was measured by Genesys 10 UV spectrophotometer (Thermo Spectronic, Thermo Fisher Scientific, MA, USA) up to an OD ~ 0.7. Then, the bacterial culture was subjected to centrifugation at 4200 x g for 10 min to isolate the sediment. The pellet was washed twice with filtered-sterilized (0.22 µm) seawater and re-suspended in 300 µL of filtered-sterilized seawater and its concentration was determined by plating method (Connors et al., 2019; Leboffe & Pierce, 2015; Soto-Dávila et al., 2019).

3.3.2.2. Challenge Assay in Vaccinated Atlantic Salmon

Atlantic salmon were vaccinated with ALPHA JECT micro IV when they reached a weight of approximately 60-80 g on October 16, 2020, six months before challenge. This procedure was performed in the AQ3 biocontainment unit at the Cold-Ocean and Deep-Sea Research Facility (CDRF) at MUN. Fish were transferred to the AQ3-CDRF unit and acclimated for one week at 10°C. A total of 240 immunized Atlantic salmon (~350 ± 50 g) were evenly distributed into six 500 L tanks containing 40 fish each. Fish were divided into three groups (Supplementary Figure S3-1). Two tanks were anesthetized with 50 mg/L of MS-222 (Syndel Laboratories, Vancouver, BC, Canada) and intraperitoneally (ip) injected with 100 µL (1 x 10^6 CFU/dose) of *M. viscosa* J311; two other tanks were bath challenged (1 x 10^6 CFU/mL) for 30 min. The final two tanks were not infected and used as control group. The mortality was recorded daily until 30 days post-challenge (dpc).

3.3.3. Flow Cytometry

We performed flow cytometry, which is the most common technique used for single-cell analysis and isolation (Gross et al., 2015), to quantify the different blood cell populations in the blood, and IgM^+ cells in the spleen. Briefly, three fish from each group were netted at 3, 7, and 14 dpc and euthanized with an overdose of MS-222 (400 mg/L). Spleen and blood samples were aseptically collected. Spleens were homogenized using a 100 µm mesh strainer and resuspended in FACS media [PBS; 0.1% fetal bovine serum (FBS; Gibco, NY, USA)]. Blood samples were heparinized (100 mg/mL. Pfizer Inc, NY, USA). All suspensions were preserved on ice until performing flow cytometry. The procedures and analyses were conducted according to previously described method with modifications (Inoue et al., 2002). Briefly, fresh blood or spleen cell suspensions (20 µL) were diluted in FACS media (1:100 µL). Then, they were stained with

40 µL of 3,3 dihexyloxacarbocyanine (DiOC6) (1 µg/mL) and 2 µL of Texas red-IgY anti-salmon IgM fresh solution. After staining, blood cell populations and IgM^+ cells in the spleen were analyzed using a BD FACS Aria II flow cytometer (BD Biosciences, San Jose, CA) and BD FACS Diva v7.0 software.

3.3.4. Immune Confocal Microscopy

Three healthy fish were euthanized with an overdose of MS-222 (400 mg/L), and their spleens were aseptically collected, homogenized using a 100 µm mesh strainer, and re-suspended in FACS media. Biotinylated chicken IgY anti-salmon IgM (1 mg/mL, Somru BioSciences, Charlottetown, PEI, Canada) was labeled with Texas red-avidin (2.5 mg/mL, Thermo Fisher Scientific, MA, USA) by mixing in a 1:1 volume for 30 min at room temperature. Then 50 µL of the spleen cell suspensions were gently mixed with 3 µL of Texas red-IgY anti-salmon IgM, and 1 µL of 4,6-diamidino-2-phenylindole (DAPI, 5 mg/mL concentration) (Thermo Fisher Scientific, MA, USA) in a 1.5 mL centrifuge tube. A 3 µL aliquot of the stained cell suspension was then added onto mountain media (Thermo Fisher Scientific, MA, USA) on a microscope slide, covered with a cover slide, and sealed. These samples were visualized using a confocal microscope (Nikon AX/AX R, NY, USA) for image acquisition. This study was conducted to verify the flow cytometry results.

3.3.5. IgM Titer Determination Using Indirect Enzyme-linked Immunosorbent Assay (ELISA)

Six fish from each group were netted at 3, 7 and 14 dpc and anesthetized with 50 mg/L of MS-222. Blood samples were taken, and centrifuged at 4200 x g for 5 min and preserved at -80°C. The samples were thawed in ice, and the complement system was inactivated by heating the samples to 56°C for 30 min. Then, lipids were removed by adding 100 µL of chloroform (Sigma-

Aldrich, St. Louis, USA), and the samples centrifuged at 4200 x g for 10 min at room temperature. Following this, the supernatant of each sample was collected and stored at -80°C until analysis. Indirect ELISAs were conducted to quantify serum antibodies against *M. viscosa* according to published methods (Erkinharju et al., 2017; Rønneseth et al., 2017), with modifications. Briefly, the ideal antigen concentration (Formalin-killed *M. viscosa* cell antigen) to react with salmon serum was determined by a checkerboard titration method (Crowther, 2008). The anti-*M. viscosa* antibodies in the serum were measured at six distinct antigen concentrations (4, 2, 1, 0.5, 0.25 and 0.125 µg/mL) to interact with the pooled serum of Atlantic salmon (1:2 to 1:64). Antigen concentrations were coated onto polystyrene 96-well microtiter plates (Thermo Scientific, MA, USA), and the plates were then incubated at 4°C overnight. The wells were then blocked with 150 µL of ChonBlock™ (Chondrex Inc, Woodinville, WA, USA) for 1 h at 37°C, washed three times with PBS-Tween, and then incubated with various dilutions of control and *M. viscosa* infected salmon serum. Based on the results of these assays, 4 µg /mL was chosen.

Finally, 96-well microtiter plates were coated with 100 µL (4 µg/mL) of *M. viscosa* in coating buffer (0.015 mM Na_2CO_3; 0.035 mM $NaHCO_3$; pH 9.8) and were incubated overnight at 4°C. The antigen-coated plates were washed with 100 µL of PBS 0.1% Tween-20 (PBS-T) three times, and blocked with 150 µL of Chondrex buffer for 1 h at 37°C. Then, the 96 well-plates were washed with 100 µL of PBS-T 0.1% three times, and 100 µL of Atlantic salmon serum from the infected or control group were added to the first well row, serially diluted 2-fold until the last row, and incubated at 37°C for 1 h. In the next step, the wells were washed with 100 µL of PBS-T five times, and 100 µL of IgY anti-salmon IgM diluted with PBS-T (1: 10000) was added and the plates were incubated for 1 h at 37°C. Afterward, the plates were washed five times with 100 µL of PBST, and incubated with 100 µL of Streptavidin-HRP (Southern Biotech, Birmingham, AL,

USA) diluted with PBST (1: 10000) for 1 h at 37°C. Thereafter, the plates were washed three times with PBST. Finally, 50 µL of TBM buffer (3,3', 5,5-tetramethylbenzidine; (Thermo Scientific, MA, USA) was added to each well, and the plates were incubated for 15 min at room temperature. The chemical interaction was stopped by adding 50 µL of 2M H_2SO_4, and absorbance was determined at 450 nm using a microplate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). To find the ideal cut-off, the specific antibody titer was determined by identifying the maximum serum dilution at which the O.D. values exhibited an increasing trend with the dilution factor. This number was then normalised to a logarithmic scale (log 2) (Crowther, 2008).

3.3.6. *M. viscosa* Antigenicity in Farmed Atlantic Salmon

3.3.6.1. Vaccine Preparations

Four different vaccines (bacterin cell, bacterin with exudates' (complete bacterin), IROMP and OMVs) were used in this study. For preparing the bacterin cell, one colony of *M. viscosa* was cultivated in a tube containing 3 mL of TSB supplemented with 2% NaCl for 48 h at 15°C with aeration (180 rpm). Then, 500 µL of the tube's content was transferred to a 250 mL flask containing 50 mL of TSB 2% NaCl and grown at 15°C up to an OD ~ 0.6-0.7 at 600 nm, and these bacteria were harvested by centrifugation (4200 x g, 10 min, 4°C) and washed two times with seawater and resuspended in 500 µL of seawater. The bacterial cell concentration was measured after 72 h of incubation at 15°C (Leboffe & Pierce, 2015). After counting, the bacterial cells were inactivated by adding 6% (v/v) of formalin (Sigma Chemical, USA), and incubated at 4°C for 48 h. Formaldehyde was removed by centrifugation, the cells were washed three times with PBS 1X, and then the cells were dialyzed (Spectrum™ Spectra/Por™ dialysis membrane; 12-14,000 Dalton molecular weight cut-off; ThermoFisher, USA) in 1 L PBS 1X at 4°C for 24 h with agitation in an

orbital shaker (Dang et al., 2021). *M. viscosa* inactivation was confirmed by plating onto TSA 2% NaCl for 24 h at 15°C. The final concentration of bacterin (4.1x 10^8 CFU/mL) was determined by a flow cytometer (Supplementary Figure S3-2A) (Eslamloo, et al. 2020; Vasquez, et al. 2020). *M. viscosa* bacterin was kept at 4°C until use. For vaccine preparation, 10% carbigen carbomer-based (Carbopol 934P) adjuvant was mixed with bacterin (4.1x 10^8 CFU/mL) and buffered to pH 7 according to the manufacturer's protocols (MVP Adjuvants®, Phibro Animal Health Corporation, Teaneck, NJ, USA).

For preparing bacterin with exudates' (complete bacterin), *M. viscosa* was grown in a flask containing 50 mL of TSB supplemented with 2% NaCl up to an OD ~ 0.6-0.7 at 600 nm, as previously described. Then, 5 mL of this *M. viscosa* culture was grown in 500 mL of TSB 2% NaCl up to an OD ~ 0.9 at 600 nm. The bacteria were then inactivated with 0.4% formaldehyde for 3 days with gentle shaking at room temperature. Then, the final concentration of cells was calculated by flow cytometer as outlined previously (Supplementary Figure S3-2B), and the formalin- killed bacterin (4.2×10^7 CFU/mL) was mixed with 10% carbigen adjuvant and kept at 4°C prior to immunization.

Laboratory preparations of *M. viscosa* outer-membrane proteins (OMPs), IROMP and OMVs were obtained by established protocols (Burdal et al., 2015; Hirst & Ellis, 1994; Sambrook & Russell, 2001; Santander et al., 2012). Then, 100 µL of IROMP containing 50 µL of IRON (1504.4 µg/mL) and 50 µL of *M. viscosa* OMP (1440 µg/mL) were added to 4450 µL of PBS and mixed with 450 µL of carbigen adjuvant according to the manufacturer´s instructions up to a concertation of 1 mg/mL (1:1 OMPs and IROMPs). For *M. viscosa* OMVs vaccines, 50 µL of OMVs (1546.3 µg/mL) were added to 4.5 mL of PBS and mixed with 450 µL of carbigen adjuvant.

3.3.6.2. Antigenicity of M. viscosa Components in Farmed Atlantic Salmon

This experiment was performed at the Dr. Joe Brown Aquatic Research Building (JBARB) in the Ocean Science Center, MUN. A total of 270 vaccinated Atlantic salmon with an average weight of ~ 380 ± 50 g were Passive Integrated Transponder- (PIT)- tagged and divided equally into six 500 L tanks, each containing 45 fish. The Atlantic salmon were anesthetized with 50 mg/L of MS-222 and individually injected with 100 µL of the vaccine preparation (e.g., *M. viscosa* bacterin, *M. vicosa* cell, IROMP, OMVs). In each tank, 45 fish were intraperitoneally boosted with their respective vaccine, and one group were injected with PBS mixed with carbigen adjuvant and used as the control group (Supplementary Figure S3-3). After immunization, blood samples from 6 fish were taken from each treatment every two weeks (i.e., 2, 4, 6, and 8 wpi). The serum of each sample was collected and stored at -80°C until IgM titer was determined by indirect ELISA.

3.3.6.3. M. viscosa Challenge in Atlantic Salmon

Atlantic salmon boosted with 4 vaccines and PBS mixed with carbigen-adjuvant were transferred from the JBARB to 6 tanks in the AQ3-CDRF and acclimated for 1 week at 10°C under the described optimal conditions. At 12 weeks after being boosted, the fish were challenged with *M. viscosa*. Briefly, two tanks containing 90 fish and 6 treatments were bath challenged with *M. viscosa* (1 x 10^6 CFU/mL) for 30 min without seawater flow through. The seawater flow through was re-established after the challenge. The fish in two tanks containing the different treatments were anesthetized with 50 mg/L of MS-222 (Syndel Laboratories, Vancouver, BC, Canada) and individually injected with 100 µL (1 x 10^6 CFU/dose) of *M. viscosa*. Two tanks were not challenged and remained as the control group (Supplementary Figure S3-4). Blood and spleen samples were aseptically collected at 3, 7, and 14 dpc to analyze the cell populations. IgM titers in

the blood were measured using indirect ELISA. Mortalities were recorded for 30 days post-challenge (Supplementary Figure S3-5).

3.3.7. Statistical Analysis

The data were analyzed using GraphPad Prism 10 (California, CA, USA). An arc-sin (survival rate ratio) function was utilized to calculate fish survival rates. The Shapiro-Wilk test was conducted to assess the normality of the data. All the data was not normally distributed, and thus one-way ANOVAs (non-parametric) followed by a Kruskal-Wallis's test were utilized to determine differences ($p \leq 0.05$) between cell populations. A two-way ANOVA (with Dunnett's multiple comparison post-hoc tests) was performed to analyze the ELISA data.

3.4. Results

3.4.1. Susceptibility of Vaccinated Farmed Atlantic Salmon to *M. viscosa* Challenge

Mortality was not observed in the control (non-infected) group. In contrast, at 30 dpc, mortality in the bath and ip-challenged fish was 2.82% and 5.64 %, respectively (Figure 3-1).

3.4.2. Cell Populations in the Challenged Atlantic Salmon

Blood cell populations were analyzed by flow cytometry (Supplementary Figure S3-6A, B). The IgM^+ (B cell) population was defined in the spleen (Supplementary Figure S3-7A-D). The cell populations were divided into two parts. Red blood cells (RBCs) were shown in P1. WBCs were highlighted in P2 (Supplementary Figure S3-6A). Then, P2 was divided into three distinct populations (Supplementary Figure S3-6B). P3 exhibited IgM^+ (B cells), P4 contained neutrophils and basophils, and P5 was monocytes (Supplementary Figure S3-6B). Neutrophils and basophils increased significantly at 3 days post ip and bath challenge as compared to the non-infected salmon. Also, lymphocytes were considerably higher than in the control group at 7 and 14 days

after the ip and bath challenges, respectively. Monocytes did not show meaningful differences between the groups (Figure 3-2).

We found no significant differences between the spleen IgM$^+$ cell populations of control and challenged groups at 3 and 7 dpc. However, at 14 dpc, the proportion of IgM$^+$ cells reached 15.4% in the ip challenged group, and this was significantly higher as compared to the non-infected fish (Figure 3-3).

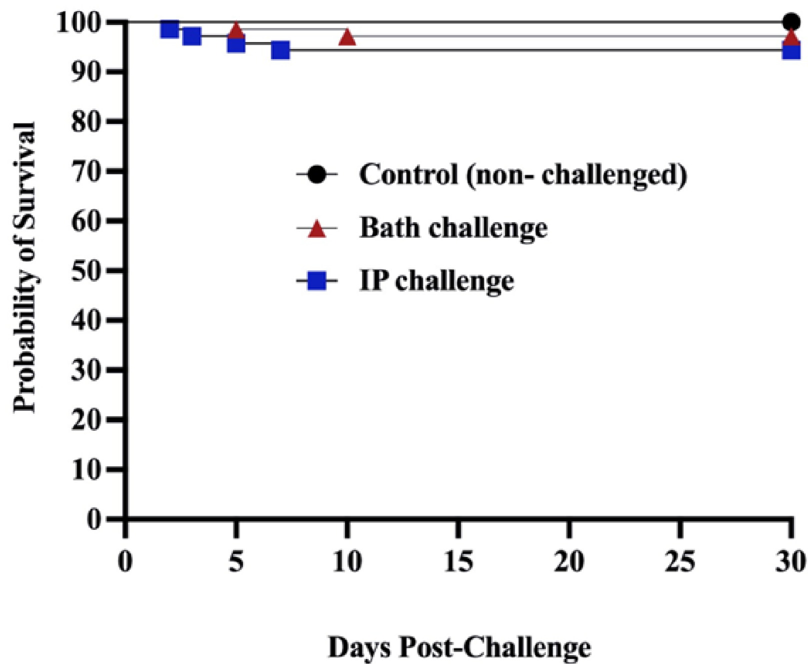


Figure 3-1. Survival of ALPHA JECT vaccinated farmed Atlantic salmon after being challenged with *M. viscosa*. The experimental fish were ip and bath challenged with 10^6 CFU/dose and 10^6 CFU/mL of *M. viscosa*, respectively. The control group was not infected.

3.4.3. Confocal Microscopy Analysis

Immunostaining of white blood cells (WBCs) using DAPI, DiOC6, and Texas red IgY anti-salmon IgM revealed that peripheral IgM^+ leukocytes were present in the spleen (Figure 3-4). This result validated the flow cytometry findings.

3.4.4. IgM Titer Levels in the Challenged Atlantic Salmon

IgM titers did not change with time in the control group (non-infected) salmon. However, they were higher at 14 dpc in the ip challenged group. Bath-challenged animals showed slightly higher IgM titers than the control group at all the examined time points. However, this difference was not significant (Figure 3-5).

3.4.5. Detection of Antibody After Booster Dose by Prepared Vaccines

Immunized fish boosted with the OMVs vaccine had the highest IgM levels at all the examined time points, and these values were significantly greater than measured in the non-boosted fish. In contrast, the IROMP-boosted group only had greater IgM titers at 4, 6 and 8 weeks post-boost immunization compared to the non-boosted group, while that of the bacterin-cell boosted group was only higher than the non-boosted group at 6 weeks following the boost. Finally, the salmon boosted with the complete bacterin did not have IgM titers higher than measured in the non-boosted (i.e. PBS-adjuvant injected) group (Figure 3-6).

3.4.6. Cell Populations After Challenge in the Boosted Groups

Neutrophils and basophils increased in the OMVs boosted group at 3 dpc (bath and ip) compared to the non-boosted one (Figures 3-7 and 3-8, respectively). Furthermore, lymphocytes were statistically higher in the OMVs boosted animals compared to the non-boosted group at 7 and 14 days post-ip and bath challenge, respectively. A slight rise of leukocytes was observed in

other boosted groups compared to the non-boosted; however, the non-parametric test revealed no significant differences between them (Figures 3-7 and 3-8). IgM^+ (B cell) increased from 3 to 14 dpc in the spleen and was slightly higher in the OMVs-boosted group compared to other treatments (Figure 3-9).

3.4.7. Detection of Antibody Levels After Challenge in the Boosted Groups

The antibody titer increased slightly from 3 to 14 days post-challenge (dpc) (Figures 3-10A, B). Fish given the OMVs vaccine generally had the highest antibody (IgM) level at all the studied time points, and IgM levels in these fish were statistically higher than measured in the non-boosted animals at 14 dpc (Figure 3-10B). The IROMP-boosted group displayed slightly higher antibody titer than the bacterin cell, complete bacterin, and control groups. However, statistical analysis indicated no significant difference between them. Antibody levels were slightly higher in the ip-challenged animals compared to the bath-challenged group. Also, no mortality was observed in the boosted animals until 30 dpc. This investigation indicated a positive correlation between survival proportions and antibody titers. All the vaccines induced the fish immune response, and the survival rate was 100% in all the treatments after the challenge (Supplementary Figure S3-5).

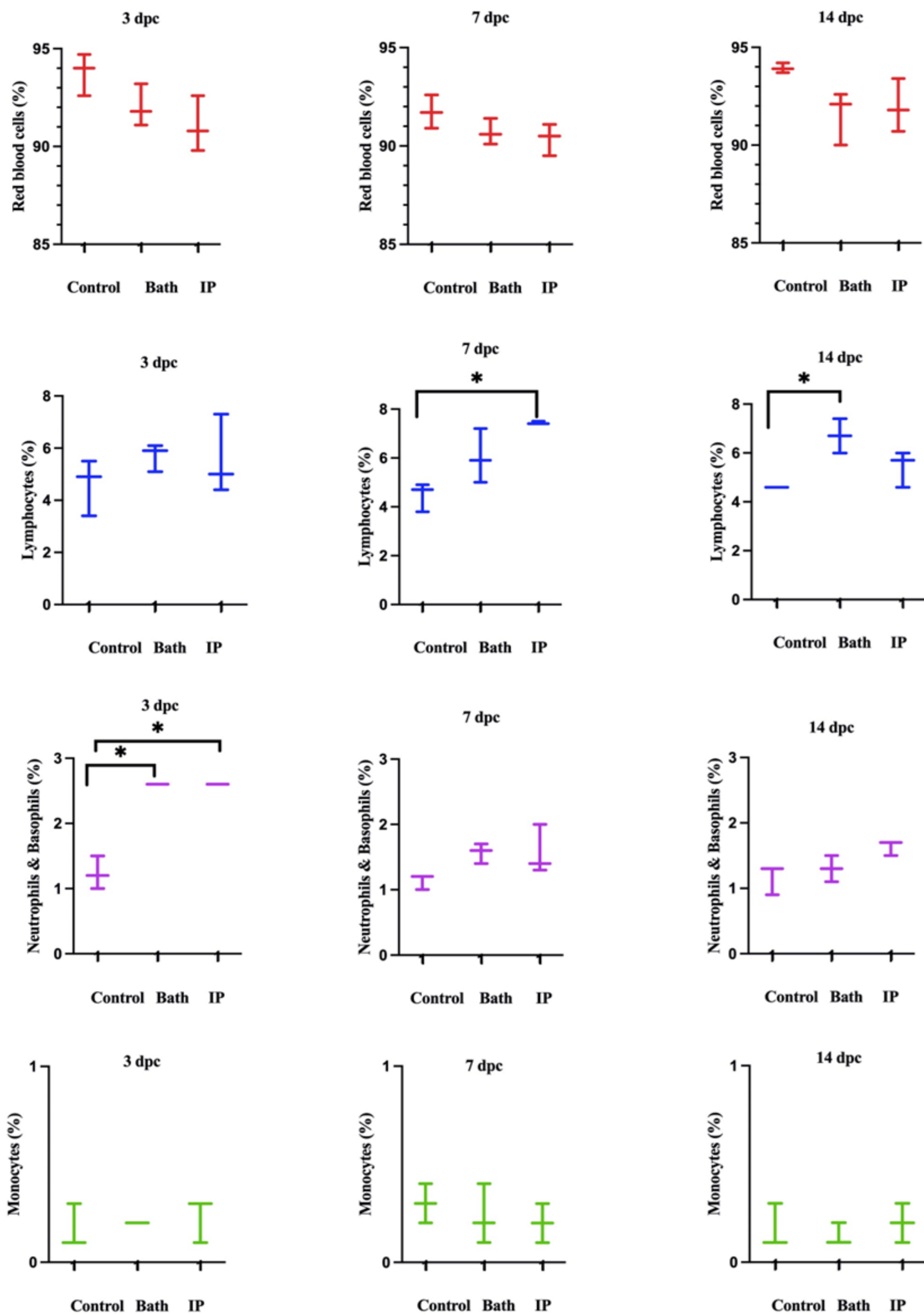


Figure 3-2. Atlantic salmon blood cell populations were identified by flow cytometry at 3, 7, and 14 dpc with *M. viscosa*. Differences were identified using one-way ANOVA (non-parametric) followed by Kruskal-Wallis tests at each sampling point. Asterisks (*) indicate a significant difference between groups ($p \leq 0.05$).

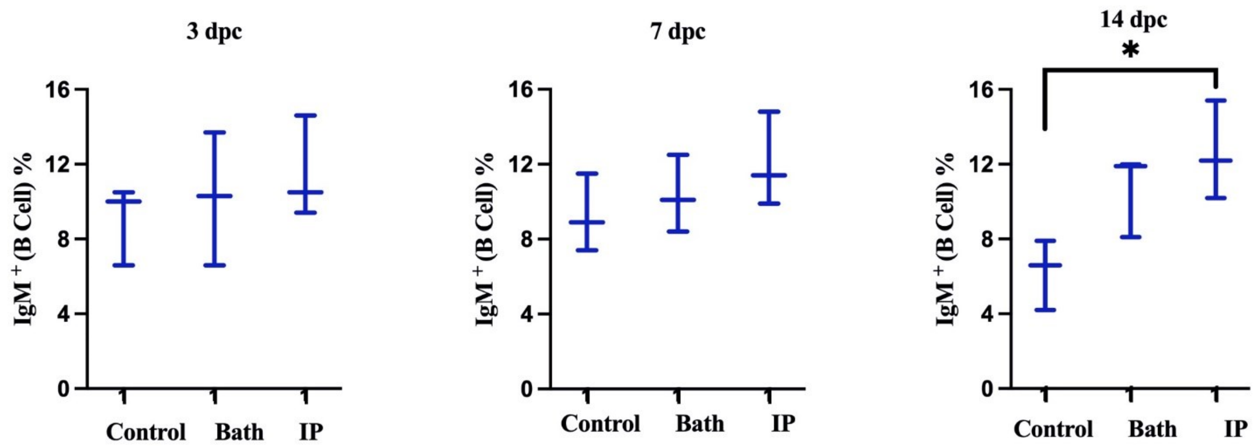


Figure 3-3. Spleen IgM^{+} (B cells) were detected by flow cytometry in ALPHA JECT-vaccinated Atlantic salmon at 3, 7, and 14 dpc with *M. viscosa*. Significant differences ($p \leq 0.05$) were identified using one-way ANOVA (non-parametric) followed by Kruskal-Wallis post-hoc tests. At each time point, challenged groups are compared versus control (non-infected) fish.

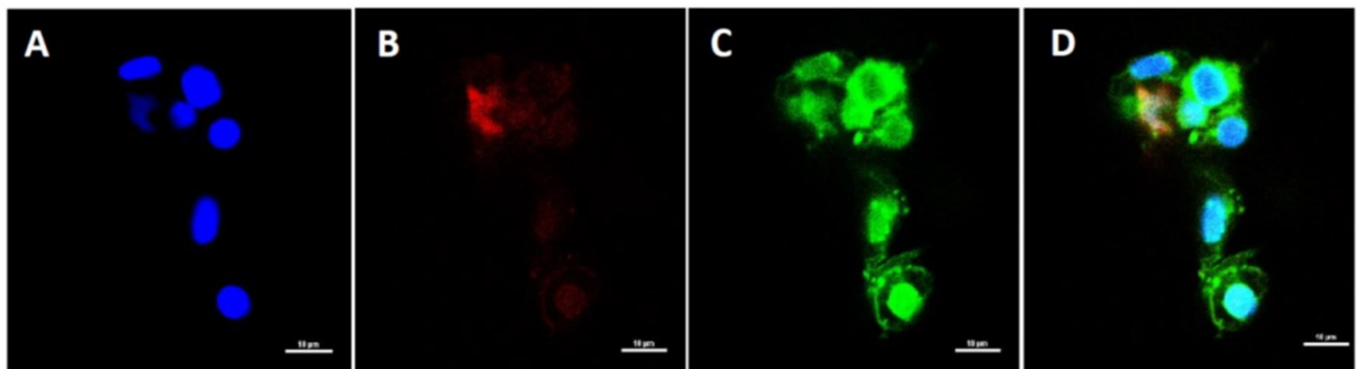


Figure 3-4. Confocal microscopy of Atlantic salmon leukocytes from the spleen; (A) Cell nuclei were stained blue with DAPI; (B) Immunostaining of WBCs using chicken anti-salmon-IgM/ IgY and avidin-Texas Red (TX-R), revealing an IgM^{+} cell with red fluorescence; (C) Green fluorescence indicates cells that were stained with FITC; (D) Overlay of all colors used in confocal imaging.

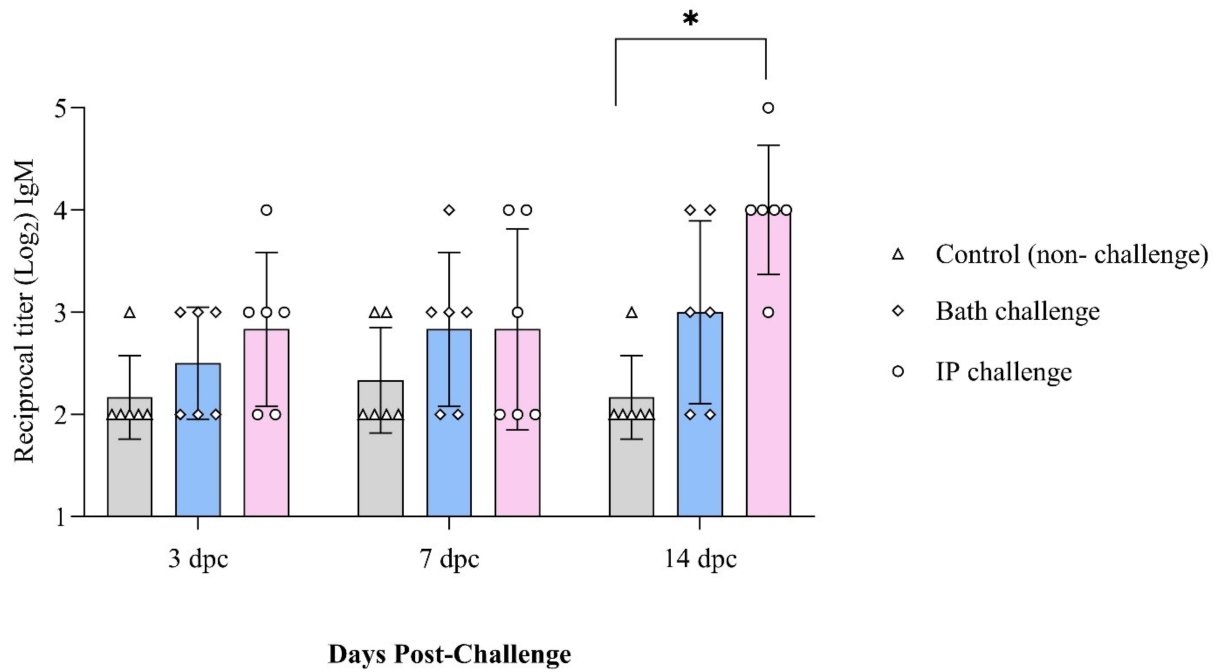


Figure 3-5. IgM serum titers in Atlantic salmon at 3, 7, and 14 dpc with *M. viscosa* were determined by indirect ELISA. Significant differences were identified using two-way ANOVA followed by Dunnett's multiple comparisons test. The asterisk (*) indicates a significant difference ($p \leq 0.0001$) between the treatment and the control group at a particular sampling point.

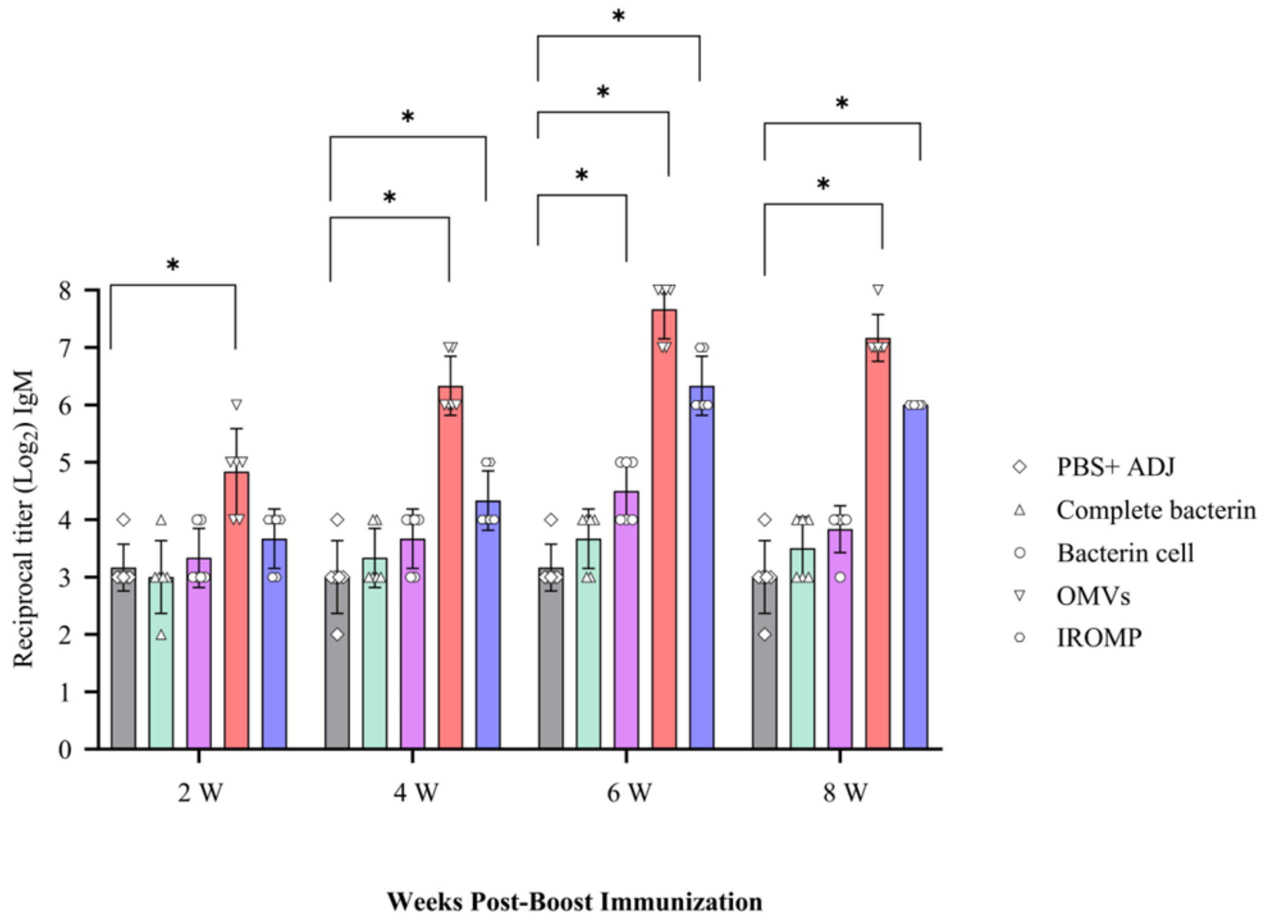


Figure 3-6. IgM serum titers in Atlantic salmon at 2, 4, 6 and 8 weeks post-boost immunization were determined by indirect ELISA. Significant differences were identified using two-way ANOVA (Dunnett's multiple comparisons) post hoc tests. Asterisks (*) demonstrate meaningful differences ($p \leq 0.0001$) between the reciprocal titers.

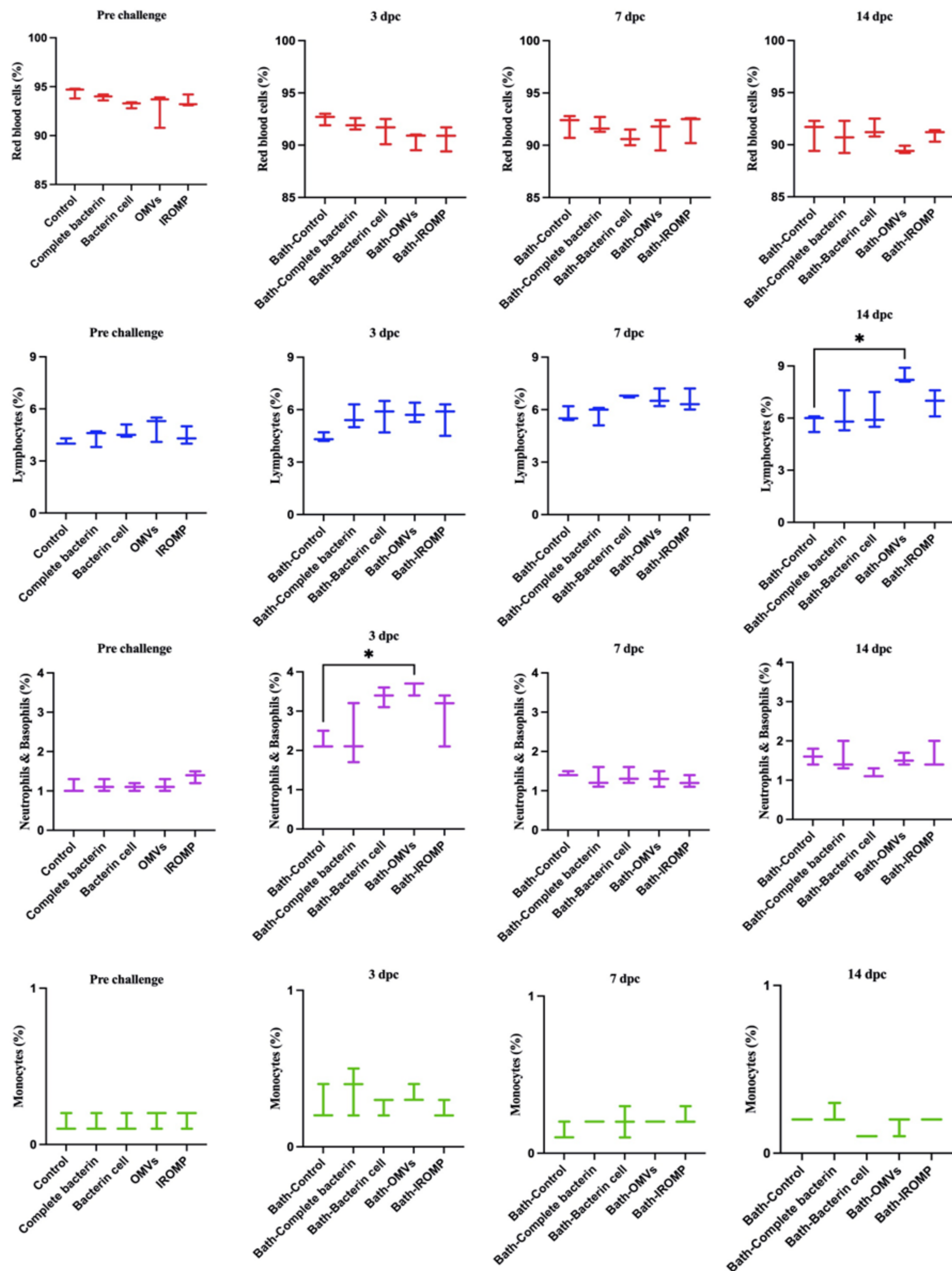


Figure 3-7. Blood cell populations in boosted Atlantic salmon at 3, 7 and 14 days post-bath challenge with *M. viscosa*. Significant differences were identified using one-way ANOVA (non-parametric) followed by Kruskal-Wallis post-hoc tests. Asterisks (*) reveal meaningful differences ($p \leq 0.05$) between boosted and non-boosted fish (i.e. given a PBS injection with adjuvant).

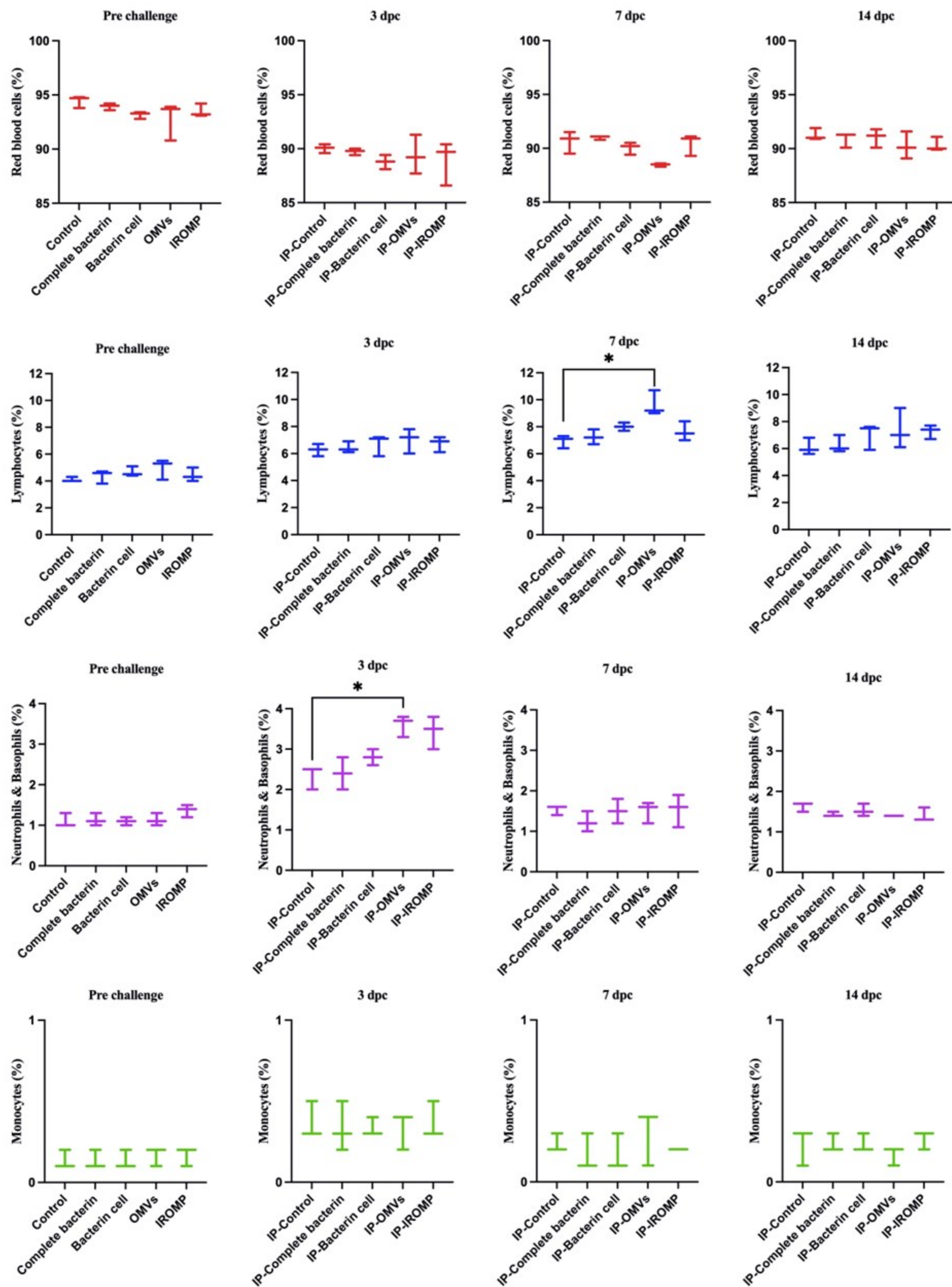


Figure 3-8. Blood cell populations in boosted Atlantic salmon were identified by flow cytometry at 3, 7, and 14 days post ip challenge with *M. viscosa*. Significant differences were identified performing one-way ANOVA (non-parametric) followed by Kruskal-Wallis post-hoc tests. Asterisks (*) reveal meaningful differences ($p \leq 0.05$) between boosted and non-boosted fish (i.e. given a PBS injection with adjuvant).

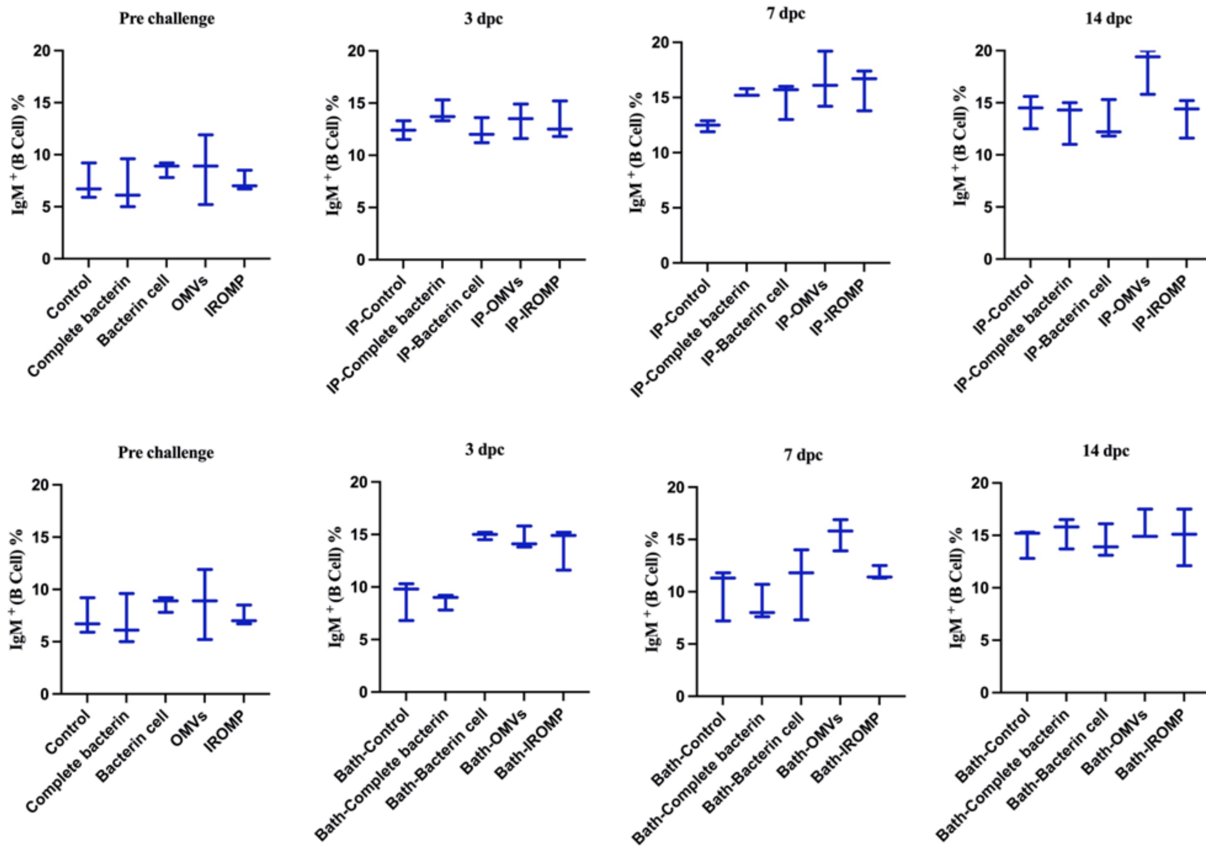
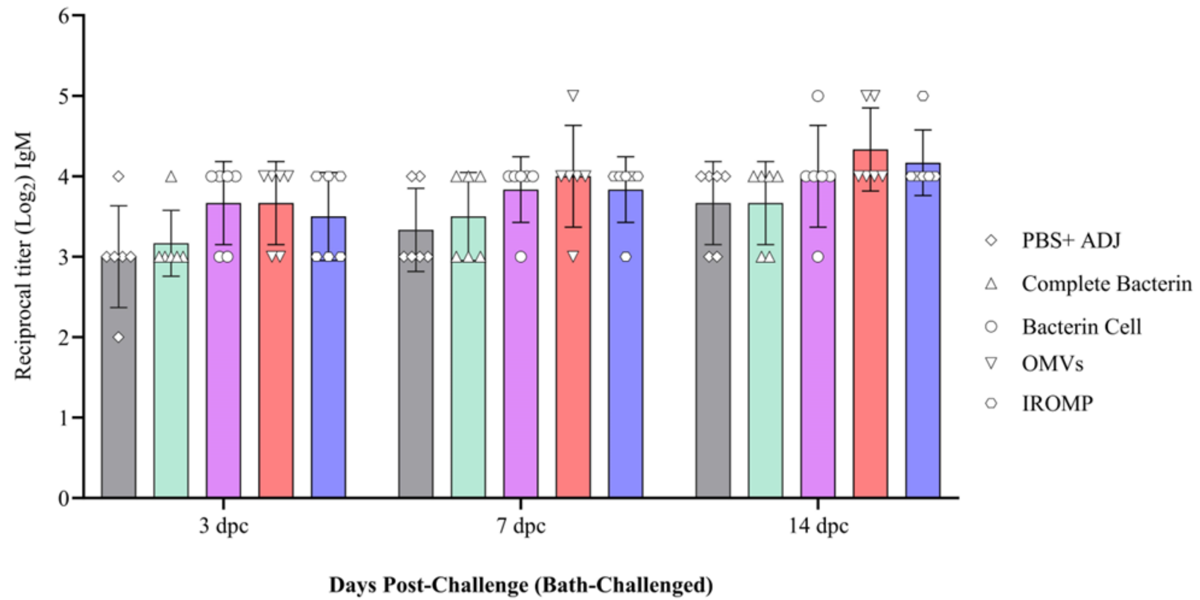
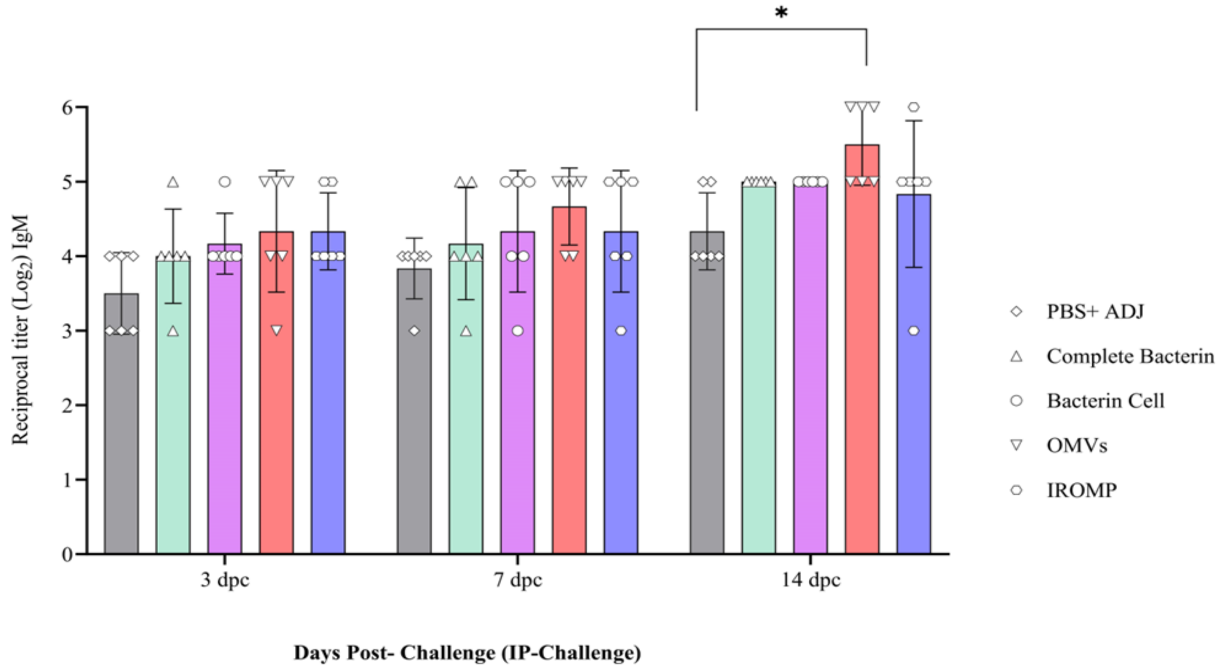


Figure 3-9. IgM^+ (B cells) in the spleen of boosted Atlantic salmon analysed 3, 7 and 14 days after ip (top panel) and bath challenges (bottom panel) with *M. viscosa*. No significant differences ($p \leq 0.05$) were identified between the non-boosted and boosted treatments using one-way ANOVA (non-parametric) followed by Kruskal-Wallis post-hoc tests.



A



B

Figure 3-10. Serum IgM titers in Atlantic salmon at 3, 7 and 14 days (A) after bath and (B) ip challenges. Two-way ANOVA (Dunnett's multiple comparisons tests) were used to detect significant differences. The asterisk (*) indicates a significant difference ($p \leq 0.05$) between boosted and non-boosted groups.

3.5. Discussion

Winter ulcer disease is mainly, but not solely, caused by *M. viscosa* (Karlsen et al., 2017; Karlsen et al., 2014; Løvoll et al., 2009). Winter ulcers cause mortality, downgrading at slaughter, and animal welfare concerns (Poppe et al., 2007). Bacterin based vaccines are usually used to control bacterial disease in the Atlantic salmon industry (Adams, 2019). This study assessed the farmed Atlantic salmon's immunological response and susceptibility to *M. viscosa* challenge. ALPHA JECT micro IV- vaccinated Atlantic salmon were bath and ip challenged. Only 2.82% and 5.64 % mortality were recorded in the bath and ip challenge, respectively. All the control (non-infected) groups survived and the survival portions in the bath- and ip-challenged animals were 97.18% and 94.36%, respectively. The high survival percentage showed an appropriate immune response of infected fish and confirmed the effectiveness of the ALPHA JECT micro IV vaccine in protecting Atlantic salmon from *M. viscosa* J311 challenge (Figure 3-1). The virulence of this *M. viscosa* strain has been previously verified in Atlantic salmon (Ghasemieshkaftaki et al., 2023). This result was expected since Norwegian strains are used as a vaccine component, such as the *M. viscosa* type strain used in this study. Efficacy against local strains of Atlantic Canada might be valuable for further study.

Karlsen et al (2017) showed that vaccination against *M. viscosa* significantly reduced the clinical effects of this pathogen, including mortality and skin ulceration. Relative protection was 91% and 65% as compared to saline controls and the vaccine formulation lacking *M. viscosa* antigen, respectively. Exposure to *M. viscosa* antigens generally resulted in protective immunity. The survivors' group did not exhibit the clinical symptoms of winter ulcer disease (Karlsen et al., 2017). This result was consistent with our findings. In this study, no clinical signs of winter ulcer disease were observed after the challenge in the vaccinated Atlantic salmon.

Rozas-Serri et al. (2022) reviewed the circulating blood cells of farmed Atlantic salmon. They indicated lymphocytes had the largest proportion of white blood cells (WBCs) in all the age ranges in farmed Atlantic salmon, consistent with our current study results. Lymphocytes comprised most of the WBC populations, followed by neutrophils and monocytes (Figures 3-2, 3-7, and 3-8).

The hematological analysis showed that there was an increase in the leukocyte population after *M. viscosa* challenge. Neutrophils and basophils increased significantly at 3 days post ip and bath challenges (Figure 3-2). This suggests that there is a leukocyte adhesion cascade (Chavakis et al., 2009; Ley et al., 2007), that is crucial for early defense against infection (Janeway, 1997).

Lymphocytes considerably increased at 7 and 14 days after the ip and bath challenges. Lymphocytes, including B cells and T cells, have essential roles in the adaptive immune response (Mutoloki et al., 2014). Our results showed that lymphocytes peaked earlier in the ip challenge compared to the bath route. This relatively early peak might indicate that adaptive immune response was triggered more rapidly in the ip challenge. The immune response in the ip route may have been accelerated, leading to the earlier recruitment and activation of lymphocytes. The delayed peak (at 14 days) in the bath challenge suggested that adaptive immune response took longer to develop in the bath route.

An increase in leukocytes after bacterial infections has been observed in other fish species (Afiyanti et al., 2018; Harikrishnan et al., 2003; Isla et al., 2022; Martins et al., 2008), which is consistent with our finding after infection with *M. viscosa* (Figure 3-2). For example, an increase in WBCs has been reported in Atlantic salmon following the infection with *Piscirickettsia salmonis* (Isla et al., 2022), which supported our results following the infection with *M. viscosa* (Figure 3-2). In another study, Common carp (*Cyprinus carpio*) were infected with *Aeromonas*

hydrophila. WBCs in the infected fish increased compared to the control (non-infected) group (Harikrishnan et al., 2003), and there was a significant increase in neutrophils in carp three days after infection with *Aeromonas salmonicida*, (Afiyanti et al., 2018). However, these same authors also reported that there was no significant change in monocytes following doses of 10^5 and 10^6 cell/mL (Afiyanti et al., 2018). It is possible that their dose was not high enough to induce monocytes proliferation in an attempt to control the infection, or that monocyte levels peak earlier than 3rd day (Afiyanti et al., 2018). These latter findings are consistent with our study on Atlantic salmon, and it might explain why we did not detect significant differences in the monocytes after the challenge (Figure 3-2). Nile tilapia infected with 1 x 10^6 CFU/mL of *Enterococcus* sp. had increased levels of WBCs and lymphocytes as compared to non-injected control (Martins et al., 2008), and this finding is consistent with our data (Figure 3-2). Finally, Monir et al. (2020) reported an increase in granulocytes and neutrophils in vaccinated hybrid red tilapia (*Oreochromis mossambicus O. niloticus*) after infection with *Streptococcus iniae* and *Aeromonas hydrophila.* This result supports the findings of the current study (Figures 3-2, 3-7 and 3-8).

IgM has been associated with mucosal and systemic immunity (Mu et al., 2022; Sheng et al., 2012; Sheng, et al., 2018). Teleost fish membrane-bound IgM^+ (B lymphocytes) are a B cell subset implicated in innate and adaptive immune responses (Li et al., 2006). In this study, IgM^+ (B cell) increased after the challenge and were statistically higher than measured in non-infected animals at 14 days after the ip challenge in the spleen. B cells secrete antibodies against invading infections and are at the center of humoral immunity (Wu et al., 2022). B lymphocytes that have undergone terminal differentiation and can secrete antibodies are plasma cells (Wu et al., 2022). Lymphocytes were not statistically higher at 14 days ip challenge in the blood; however, a great volume of

antibodies was observed in the serum (Figure 3-5). This finding suggests that B cells may differentiate into plasma cells and produce antibodies.

An increase in IgM levels after infection has also been reported in the spleen of fish (van der Wal, et al., 2021). In our study, IgM increased at 2 weeks following the ip challenge (Figure 3-3). The early appearance of IgM^+ cells in the spleen suggests that vaccination quickly induced the immune response after infection. The pre- and post-infection serum IgM titers had been reported significantly greater in vaccinated fish compared to the unvaccinated. This result indicated an active immune response and phagocytic activity (Monir et al., 2020), which matched our results in this study (Figures 3-6 and 3-10B).

Most licensed aquaculture vaccines are inactivated pathogenic microbes mixed with adjuvant, which contain either single or combined microorganisms (Kayansamruaj et al., 2020; Ma et al., 2019). These vaccine preparations not only contain immune protective antigens, but also immune suppressive molecules, and identifying the most immunogenic bacterial component that can contribute to improved vaccine efficacy. This study used four different vaccines to boost the animals and reviewed the immunogenic response of *M. viscosa* antigen in vaccinated Atlantic salmon after the challenge to determine the immunogenicity of these vaccine preparations.

In this investigation, one group of salmon was boosted with the bacterin vaccine containing killed *M. viscosa* and exudates, such as the inactivated commercial vaccines that are commonly used in aquaculture. Also, one group of salmon was boosted with a bacterin cell vaccine containing only *M. viscosa* cells.

IROMP, a vaccine utilized in this investigation, is the combination of iron and the OMPs of *M. viscosa*. Gram-negative bacteria's OMPs represent a significant portion of the cell surface. They are crucial structural elements of the membrane, acting in virulence-related processes like

adhesion, invasion of host tissues, and reducing the host immune response. They are also highly antigenic (Björnsson et al., 2011; Dumetz et al., 2008). Since they interact with the host immune system, they are important vaccine candidates (Björnsson et al., 2011).

OMVs, a vaccine preparation used in this study, are spherical lipid vesicles released from the outer membranes of Gram-negative bacteria to facilitate communication among the organisms as they grow in various environments and control the host immunological response (Ellis & Kuehn, 2010; Jan, 2017). OMVs are composed of OMPs, phospholipids, peptidoglycan, lipopolysaccharides (LPS), proteins, nucleic acids, ion metabolites, and signaling molecules (Jan, 2017; Koeppen et al., 2016). OMVs have been classified and used in many ways. They contain adhesion, autolysins, cytotoxins, virulence factors, and toxin delivery that elude host defense mechanisms, among many more biomolecules (Jan, 2017). In primary infection, OMVs are critical for delivering highly virulent factors to host cells, where they dramatically degrade the host cells' enzymes and cause cell death. A crucial candidate for a new vaccine formulation is bacterial outer membrane vesicles carrying antigens. They efficiently phagocytize antigen-presenting cells due to the surface-associated antigens they transport. They also bring a variety of PAMPs (pathogen-associated molecular patterns) that trigger and support immune system responses (Furuyama & Sircili, 2021). It supported our findings in this study, which demonstrated a high capacity of OMVs to induce an immunological response in Atlantic salmon (Figures 3-6, 3-7, and 3-8). Our results revealed a significant increase in IgM level and WBCs in the OMVs-boosted group compared to the non-boosted Atlantic salmon following the challenge (Figures 3-7, 3-8 and 3-10B). This suggests that the salmon's immune cells were more strongly activated in fish that received the OMVs booster compared to the non-boosted group. The heightened levels of neutrophils and basophils indicate that OMVs resulted in an enhanced (more robust) immune response, and this

could potentially contribute to a more effective defense against pathogens. In the post-challenge cell analysis, the OMVs-boosted group exhibited a significant increase in lymphocytes, reflecting the successful stimulation of memory B and T cells by the booster vaccination. The heightened lymphocyte response suggests an accelerated activation and proliferation of these key immune cells, contributing to robust adaptive immune response upon re-exposure to the pathogen (Figures 3-7 and 3-8). These findings highlight the effectiveness of OMVs booster in enhancing the adaptive immune system's functionality.

OMVs have demonstrated the specific nature of the protective antibody and significant antigens that induce an adaptive memory immune response and possess self-adjuvant characteristics (Schwechheimer & Kuehn, 2015). Our findings were in line with this. According to our research, adaptive immune response quickly triggered following the challenge, and OMVs-boosted group had higher lymphocytes and IgM titers than the other treatment (Figures, 3-7, 3-8 and 3-10B).

It has been demonstrated that OMVs can induce an immunological response and protect fish (Tandberg et al., 2017). Also, *P. salmonis* OMVs cause the generation of IgM in Atlantic salmon. IgM was produced against *P. salmonis* proteins in the serum taken from immunized fish after 14 days (Oliver, et al. 2023). This was consistent with our finding in Atlantic salmon boosted with *M. viscosa* OMVs. IgM was detected at 14 days post-boost immunization (Figure 3-6).

The OMVs vaccines have been evaluated in developing fish vaccines against *Francisella noatunensis* in zebrafish, *P. salmonis* in salmonids, and *V. anguillarum* in Japanese flounder (Brudal et al., 2015; Hong et al., 2009; Oliver et al., 2023). These findings suggested that OMVs effectively induce stable immune responses, confer protection against bacteria, and are efficient antigens to produce vaccines (Gerritzen et al., 2017; Kashyap et al., 2022). In this study, OMVs

were extracted from *M. viscosa* and used as a vaccine component. This is the first research that utilized the OMVs vaccine to boost the farmed Atlantic salmon against *M. viscosa*. The results of our experiment showed *M. viscosa* OMVs are the most immune-stimulating antigens compared to other vaccine elements in Atlantic salmon (Figures 3-6, 3-7, 3-8, and 3-10B).

Our findings indicated that antibody titer in the boosted group with OMVs significantly differed from the control group at all the examined time points (2, 4, 6, and 8 weeks). Antibody titers (IgM) peaked at 6 weeks in all the treatments and reached the highest in the OMVs boosted group. The IROMP and bacterin cell antibody levels were in second and third place, respectively, and they differed significantly from the control group at 6 weeks post boost immunization (Figure 3-6). Several studies have been conducted on Atlantic salmon and reviewed antibody titer by ELISA after vaccination. Romstad et al. (2012) indicated that Atlantic salmon immunized with A-layer positive vaccines demonstrated an increased mean antibody response with rising antigen dose. According to Liu et al. (2020), all the immunized groups had noticeably higher antibody levels post-vaccination in comparison to the control group. Increasing the antibody levels aligned with our observations in this study following the boost (Figure 3-6).

Also, in other fish species, significant rise of antibody levels was observed after vaccination. Japanese flounder was vaccinated against *P. fluorescens* and *A. hydrophila*. In all cases, specific antibodies were found at 5 weeks post vaccination (wpv) and persisted for up to 8 wpv (Wang et al., 2009). Similar results were obtained in this study in Atlantic salmon. At 6 weeks post boost-immunization, a significant difference in IgM titers was observed between the boosted groups (OMVs, IROMP, bacterin cell) and non-boosted animals. The IgM titers remained relatively high in OMVs and IROMP boosted animals compared to the non-boosted group to the end of the experiment at 8 weeks post boost immunization (Figure 3-6). A considerable rise in IgM titers

after the challenge was reported in rainbow trout (Raida et al., 2011), similar to our results (Figure 3-5). High IgM titers were detected in the boosted animals compared to the non-boosted group (Figure 3-6). IgM titers were noticeably higher in the OMVs boosted group at 14 days following ip challenge (Figure 3-10B). Villumsen et al. (2012) reported that experimental vaccine produced noticeably greater IgM titers in each of the three intervals between immunization and challenge compared to the unvaccinated controls, which is similar to our results (Figures 3-6 and 3-10B).

Hematological analysis revealed a considerable rise in the neutrophils and basophils in the OMVs boosted group compared to the non-boosted fish at 3 dpc (Figures 3-7 and 3-8). Lymphocytes increased at 7 and 14 days-post ip and bath challenge, respectively, in the OMVs boosted group (Figures 3-7 and 3-8). A tendency of the rise was observed in the IgM^+ cells in the spleen of OMVs boosted salmon compared to the non-boosted group (Figure 3-9). *M. viscosa* OMVs might contain several immunogenic molecules and perhaps fewer immunosuppressors, thus boosted salmon may have a faster activation of immune cells, including neutrophils, upon *M. viscosa* infection. Further studies on the *M. viscosa* OMVs protein profile might provide an antigenic profile for vaccine development against *M. viscosa*.

3.6. Conclusions

This is the first comprehensive study using flow cytometry to analyze the hematological cells of vaccinated and boosted farmed Atlantic salmon before and after *M. viscosa* challenge. This investigation evaluated the immune response of Atlantic salmon and reviewed the antibody titers after vaccination and challenge. Our research showed that the booster dose of OMVs vaccine had a strong capacity to trigger the farmed Atlantic salmon's immune response following immunization and challenge with *M. viscosa*. Leukocytes were statistically higher in the OMVs boosted group, which indicated the appropriate haemato-immune response of the vaccine to

control the infection. *M. viscosa* OMVs could be used as vaccines, heightened antibody response and potentially enhanced immune cell activity. These advantages contribute to the overall effectiveness of the vaccination strategy in conferring immunity against the specific pathogen.

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4. Chapter 4: Summary, Research Limitations and Perspectives

4.1. Summary

The key points of the investigation and summary of my thesis findings are highlighted in Chapter 4. Additionally, it offers suggestions for further study.

The main goals of this thesis were to: 1) use genomics and phenotypic characteristics to identify the pathogen that caused a skin ulcer outbreak in Atlantic salmon held at Memorial University; 2) evaluate this bacteria's pathogenicity; 3) evaluate the effectiveness of ALPHA JECT at preventing salmon mortality caused by *M. viscosa* infection; and 4) to examine whether, and to what extent, a number of *M. viscosa* vaccine preparations (cells and exudate, the bacterin cells, IROMPS and OMVs) are effective at stimulating the salmon's immune system.

In Chapter 2, published in *Microorganisms* (Ghasemieshkaftaki et al., 2023), I was able to determine the pathogen that caused the ulcer outbreak at MUN was a new *Vibrio* sp. (J383). It has a genome size of 5,902,734 bp with two chromosomes and one plasmid. This bacterium shares a common ancestor, and 93% similarity at the genomic level, with *Vibrio splendidus*, and has a biochemical profile that is most similar (i.e., at 64.8%) to *V. vulnificus*. However, in contrast to all other *Vibrio* species which can grow in warm seawater, *Vibrio* sp. J383 showed minimal growth at 28°C and was not able to grow at 37°C. *Vibrio* sp. J383's hemolytic activity showed its pathogenicity.

The mortality rates were reported 12 weeks post infection (wpi). In the group infected with high-dose (10^8 CFU/dose), 22.5% mortality, and in the medium-dose (10^7 CFU/dose), 5% mortality were recorded; however, the low-dose (10^6 CFU/dose) group experienced no mortality. This investigation indicated that this strain is not highly virulent. My findings indicated that clinical signs of fish mostly presented at 5 and 6 wpi when *Vibrio* sp. J383 was isolated from all the collected blood samples. It was no longer present in the blood after 12 wpi. However, this new

strain was isolated from all and some of the collected tissue samples at 12 and 14 wpi, respectively. Our results showed that the spleen had considerably higher bacterial loads than the liver and head kidney samples.

Koch's postulates and the presence of pathogenic genomic islands (GIs 12 and 21), including virulence factors such as type VI secretion system (T6SS) genes and multidrug-resistant transporter/family protein, supported the hypothesis that this novel strain is pathogenic. When compared to other *Vibrio* strains, *Vibrio* sp. J383 has unique characteristics, suggesting that J383 is a new *Vibrio* species adapted to cold temperatures.

The results of this research revealed that *Vibrio* sp. J383 can cause clinical signs and chronic infection in farmed Atlantic salmon. Minor lesions to severe wounds were observed in the skin of immunized Atlantic salmon after ip infection with *Vibrio* sp. J383. Although the mortality rate was not high, reducing the fillet quality due to the clinical signs is one of the negative impacts of this new pathogen on the salmon farming industry.

To summarize, Chapter 2 of this thesis analyzed the phenotypic and genomic characteristics of *Vibrio* sp. J383, a new pathogenic strain isolated from vaccinated farmed Atlantic salmon. The isolation of this strain indicated that other undescribed bacteria could cause ulcerative disease in vaccinated Atlantic salmon.

Chapter 3, published in *Vaccines* (Ghasemieshkaftaki et al., 2024), evaluated the susceptibility of farmed Atlantic salmon reared in Atlantic Canada to *M. viscosa* challenge. Also, hemato immune response of vaccinated and boosted Atlantic salmon were analyzed after the challenge. An increase in blood leukocytes has been reported after bacterial infections and challenge in other fish species (Afiyanti et al., 2018; Harikrishnan et al., 2003; Isla et al., 2022; Martins et al., 2008; Monir et al., 2020). However, this is the first study that analyzed the

hematological cells of vaccinated and boosted farmed Atlantic salmon before and after the challenge with *M. viscosa.* The findings indicated that ip administration of 10^6 CFU/dose or bath challenge of 10^6 CFU/mL with *M. viscosa* caused immunological response and low mortality in the vaccinated farmed Atlantic salmon. ELISA's results revealed that antibody titers (IgM) were higher in the ip-challenged fish compared to the bath and control (non-infected) groups.

Analysis of the collected spleen and serum samples showed that IgM^+ (B cell) and antibody titers were significantly higher than the non-infected group at 14 days post ip challenge. I found that challenged group had higher WBCs than the non-challenged ones. My findings demonstrated a considerable rise in neutrophils 3 days after the ip and bath challenges compared to the non-infected group. Lymphocytes significantly increased at 7 and 14 days post ip and bath challenges, respectively. At the comparable time points, the same type of leukocytes statistically increased in the boosted group by OMVs vaccine compared to the non-boosted one.

This study also evaluated the immune response of vaccinated farmed Atlantic salmon after injection of the booster dose with in-house vaccines. The results indicated that antibody titers peaked at 6 weeks post boost immunization for all the treatments. OMVs boosted group had the highest antibody levels. The IROMP and bacterin cell boosted groups had the second and third highest antibody levels, respectively, and they were substantially different from the non-boosted group at 6 weeks following the boost. The control group exhibited the lowest antibody levels compared to the fish that received booster doses of prepared vaccines.

The IgM^+ (B cell) levels in spleen samples from the boosted groups were also measured following the challenge. The findings indicated that the B cell percentage in the OMVs boosted group was higher than the non-boosted group. However, it was not statistically different. Antibody titers (IgM) in the serum reached a peak at 14 days post ip challenge, and it was significantly higher

than the non-boosted group. Compared to other treatments, the boosted group by OMVs had a greater antibody level in all the examined time points following the bath and ip challenges.

This investigation evaluated the immune response of Atlantic salmon and reviewed the antibody titers after vaccination and challenge. Our results showed that the non-boosted animals experienced 97.18% and 94.36% survival post-bath and ip challenges. The boosted group revealed 100% survival, and no clinical signs were recorded after the challenge with *M. viscosa*. The high survival percentage indicated the vaccine's efficacy and the appropriate immune response of fish after the challenge.

I observed a significant rise in antibody levels after boosted with OMVs vaccine compared to the non-boosted Atlantic salmon. This finding suggests that the booster dose effectively enhanced the immune system to make more antibodies, which are crucial for neutralizing pathogens and preventing infection. The higher antibody levels induced by the booster dose indicate that the immune system is better prepared to respond to the specific pathogen. This translates to improved protection against infection, as the immune system can determine and eliminate the pathogen more effectively. The booster dose promotes a more durable immune memory. This demonstrates that the immune system may continue to respond to the pathogen quickly and efficiently over time and providing ongoing protection. With higher antibody levels and a more robust immune response, the Atlantic salmon that received the OMVs booster are likely more capable to fend off infections. This could lead to a reduced risk of breakthrough infections even when exposed to a higher dose of pathogen. Also, boosting the immune response with a second vaccine can create a stronger and more lasting immune memory. This memory helps the immune system to react more rapidly and effectively upon re-exposure to the pathogen.

The effectiveness of a vaccine is often determined by its ability to induce a strong and protective immune response. The significant rise in antibody levels after the OMVs vaccine suggests that the booster dose was successful in enhancing vaccine efficacy. It has the highest antigenicity compared to other *M. viscosa* vaccines (IROMP, bacterin cell, and complete bacterin).

In summary, the OMVs vaccine in our experiment has provided several advantages, including a heightened antibody response, longer-lasting immunity, and potentially enhanced immune cell activity. These advantages contribute to the overall effectiveness of the vaccination strategy in conferring immunity against the specific pathogen.

In conclusion, this research described the characteristics of a new *Vibrio* strain that causes ulcer disease and chronic infection in vaccinated Atlantic salmon. Also, vaccination offered strong protection against *M. viscosa*. Although *M. viscosa* is thought to be a principal causative agent of winter ulcer disease, the results of this study highlighted the role of unknown bacterial infections that lead to ulcerative disease and mortality in farmed Atlantic salmon. Further studies are required to determine the other ulcerative disease causative agents in Eastern Canada and provide the salmon farming industry with effective vaccines against those pathogens. This investigation also revealed the high immunogenicity of outer membrane vesicles (OMVs) and highlighted how the booster dose of this vaccine can strengthen the immune response and defend farmed Atlantic salmon against *M. viscosa*.

4.2. Research Limitations and Future Studies

4.2.1. Chapter 2

There are a few published studies on skin ulcerative diseases in Atlantic salmon, but less is known about it on Canada's east coast (MacKinnon, et al., 2019a, b; MacKinnon, et al., 2020). The study on *Vibrio* sp. J383 in Atlantic salmon has introduced a novel pathogen in Eastern Canada.

While this study provides valuable insights, it naturally focuses on this single bacterial strain. This specific focus, although crucial for in-depth understanding, is just a part of the broader spectrum of pathogens that could contribute to ulcer diseases in fish. Furthermore, the research was carried out under controlled conditions, which, while offering profound knowledge may not fully capture the complexity of natural aquatic environments. Additionally, this study did not involve developing a specific vaccine for *Vibrio* sp. J383, since it was conducted with Atlantic salmon that were already vaccinated, which made the development of a vaccine for this new pathogen unfeasible in the given context.

While my research provided new insights into an ulcerative disease causative agent in Eastern Canada and highlighted the role of other pathogenic bacteria in this disease, it is unclear how the co-infection of *Vibrio* sp. J383, with other etiological agents, such as *Moritella viscosa*, would affect Atlantic salmon. Therefore, further investigation is required to discover different aspects of this new strain. Future studies should broaden the scope by including a wider range of pathogen strains. This would provide a more comprehensive understanding of the microbial dynamics in fish ulcerative diseases. Research should also focus on exploring the interaction of these pathogens with various environmental factors within natural aquatic ecosystems. Investigating the impact of different water conditions, host species, and microbial communities could provide crucial information for effective ulcerative disease management in aquaculture. To further this line of research, future studies on non-vaccinated Atlantic salmon are necessary to develop an effective vaccine against *Vibrio* sp. J383, which was shed light on by this work.

4.2.2. Chapter 3

Although this study comprehensively focused on analyzing the hemato immunological response of vaccinated Atlantic salmon after the *M. viscosa* challenge, there are opportunities for

future research to explore additional hematological parameters, such as a detailed analysis of cytokine profiles. While these aspects were not the central focus of my investigation, their inclusion can provide valuable information, thereby contributing to a deeper comprehension of the immunological responses in Atlantic salmon.

Moreover, future research should explore the use of OMVs as a vaccine, without the additional boosting, and compare its efficacy with an industrial vaccine. This approach would provide valuable insights into the effectiveness of OMVs as a standalone vaccine candidate for Atlantic salmon. Direct comparison with a standard industrial vaccine allows researchers to assess the effectiveness of OMVs more accurately in inducing an immune response, and thus providing a more comprehensive understanding of their role in fish health management.

4.3. References

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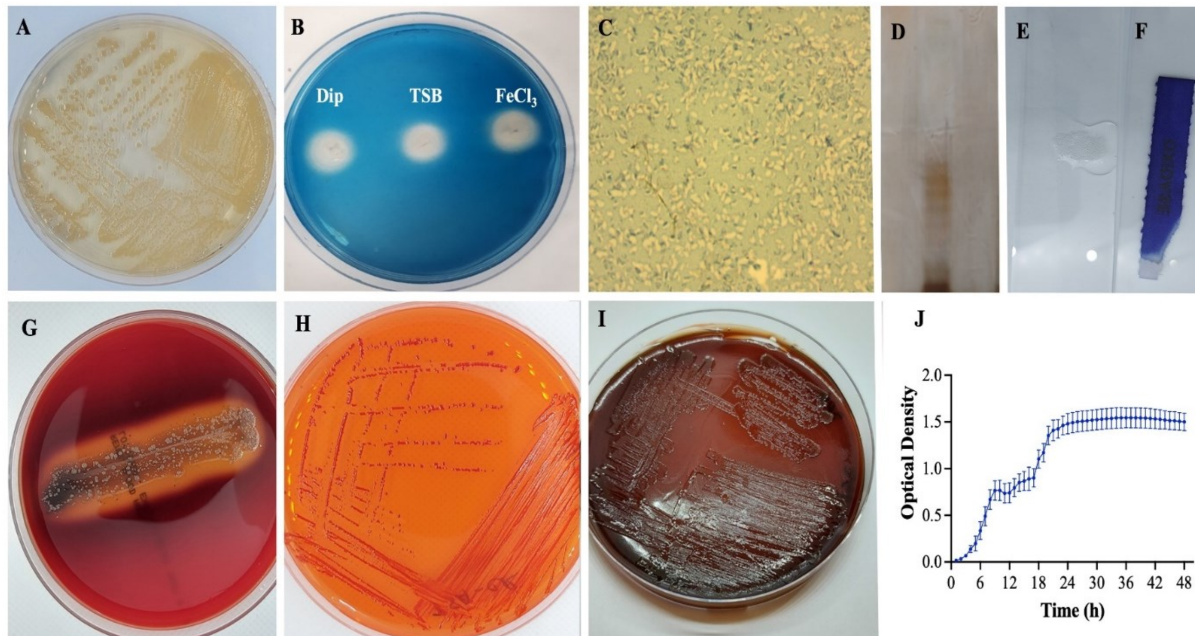
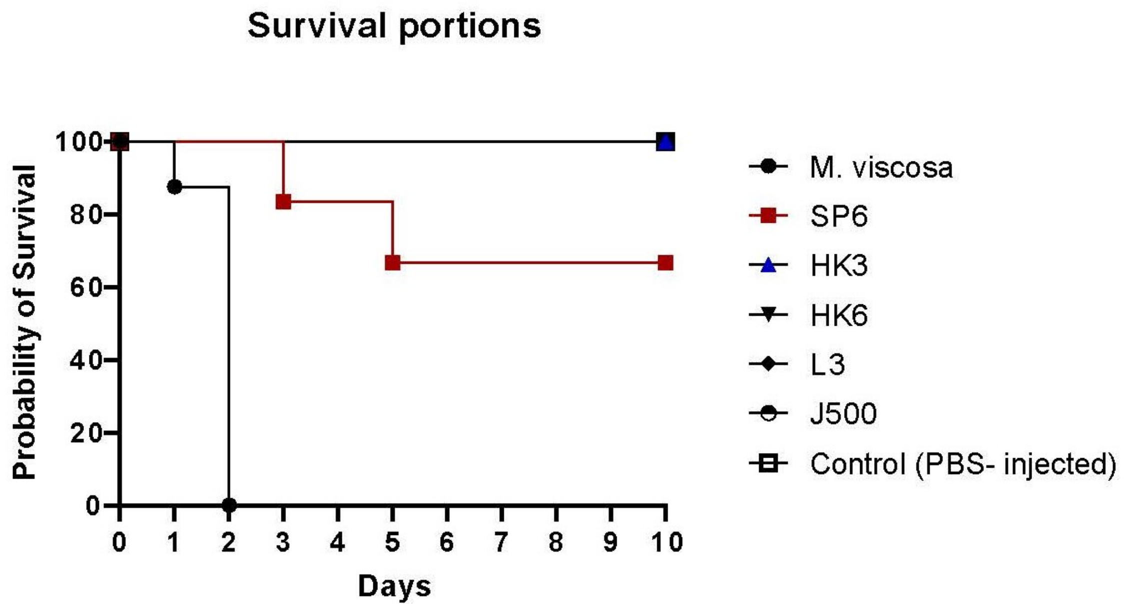
5. Supplementary Materials

The supplementary files can be downloaded at:

https:// www.mdpi.com/article/10.3390/microorganisms11071736/s1



Supplementary Figure S2-1. Phenotypic characteristics of *Vibrio* sp. J383. (A) Growth on TSA with 2% NaCl. (B) Siderophore synthesis on chrome azurol S (CAS) agar plates from *Vibrio* sp. J383 grown under iron-enriched conditions (TSB supplemented with 100 µm of $FeCl_3$), standard culture conditions (TSB), and iron limited conditions (TSB supplemented with 100 µm of 2,2-diprydil) at 15ºC. Yellow reaction around *Vibrio* sp. J383 colony indicative of being positive for siderophore synthesis. (C) Capsule stain of *Vibrio* sp. J383. (D) LPS profile for *Vibrio* sp. J383. (E) Positive catalase test. (F) Positive Oxidase test. (G) B hemolysin production in sheep blood agar. (H) Growth on congored agar. (I) Growth on salmon blood agar and B hemolysin production. (J) Growth in TSB with 2% NaCl at 15°C. Culture was aerated with aeration (180 rpm) and grown in a 12 well plate for 48 h.



Supplementary Figure S2-2. Acute mortality of Atlantic salmon infected with different bacterial strains isolated from fish exhibiting 'winter ulcer'-like clinical signs. *Moritella viscosa* J311 was obtained from American Type Culture Collection, NHI. The animals were injected with 100 μl of pathogen (10^8 CFU/ dose) or PBS."

A

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Vibrio vulnificus FDAARGOS-119	1		84.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio fluvialis ATCC 33809	2	9.36		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio harveyi 345	3	0.10	0.10		82.74	0.00	83.11	0.00	86.38	0.00	0.00	0.00	87.11	0.00	0.00	0.00	0.00	0.00	85.15	88.38	85.88
Vibrio spp. J383 Chromosome_2	4	0.08	0.05	2.69		0.00	93.31	0.00	84.15	0.00	0.00	0.00	83.36	0.00	0.00	0.00	0.00	0.00	83.58	84.07	84.11
Vibrio cholerae O1 str	5	0.63	0.20	0.10	0.15		0.00	0.00	0.00	85.15	85.08	100.00	0.00	85.60	85.23	85.46	85.15	94.10	0.00	0.00	0.00
Vibrio splendidus BST398	6	0.17	0.13	2.58	76.36	0.19		0.00	84.67	0.00	0.00	0.00	83.71	0.00	0.00	0.00	0.00	0.00	84.19	83.82	84.52
Photobacterium damselae subsp. piscicida	7	6.29E-5	3.16E-4	0.07	2.15E-4	6.37E-3	2.90E-4		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio vulnificus FORC-077	8	0.05	0.32	5.44	1.14	0.85	1.14	1.50E-4		0.00	0.00	0.00	85.35	0.00	0.00	0.00	0.00	82.55	86.09	84.92	94.80
Vibrio anguillarum J360	9	0.24	0.11	0.03	0.02	2.23	0.08	8.11E-4	0.31		98.54	85.16	0.00	98.41	98.40	98.35	100.00	82.15	0.00	0.00	0.00
Vibrio anguillarum MHK3	10	0.29	0.06	0.01	0.10	2.82	0.09	9.25E-5	0.38	71.56		85.21	0.00	98.64	98.49	98.40	98.54	83.76	0.00	0.00	0.00
Vibrio cholera FDAARGOS-223	11	0.62	0.19	0.10	0.15	98.21	0.19	6.30E-3	0.84	2.21	2.82		0.00	86.00	85.24	85.47	85.17	94.10	0.00	0.00	0.00
Vibrio alginolyticus 2439-01	12	0.04	0.15	20.60	2.40	0.18	2.08	0.08	2.47	0.16	0.17	0.18		0.00	0.00	0.00	0.00	85.43	88.47	86.62	85.11
Vibrio anguillarum CNEVA NB11008	13	0.32	0.07	0.03	0.02	2.70	8.99E-3	9.19E-5	0.39	74.32	77.04	2.87	0.09		98.47	98.41	98.40	83.84	0.00	0.00	0.00
Vibrio anguillarum 775	14	0.20	0.07	0.02	0.03	2.74	0.10	0.00	0.37	75.48	80.48	2.71	0.18	80.26		98.38	98.39	83.42	0.00	0.00	0.00
Vibrio anguillarum JLL 237	15	0.44	0.06	0.01	0.09	2.73	0.09	9.04E-5	0.24	74.29	73.17	2.70	0.19	75.93	77.28		98.35	84.60	0.00	0.00	0.00
Vibrio anguillarum VIB43	16	0.28	0.11	0.03	0.02	2.25	0.08	8.17E-4	0.31	90.96	72.19	2.22	0.16	74.85	76.00	74.82		82.15	0.00	0.00	0.00
Vibrio cholerae RFB05	17	0.46	0.24	0.63	0.07	70.70	0.08	5.89E-3	1.62	1.62	2.14	69.90	1.01	2.19	2.22	2.24	1.63		83.49	0.00	81.87
Vibrio parahaemolyticus R14	18	0.09	0.25	19.34	1.95	0.17	1.83	0.08	4.20	0.18	0.32	0.16	42.46	0.09	0.18	0.09	0.18	1.32		84.24	85.98
Vibrio campbellii ATCC 25920, CAIM 519T	19	0.17	0.16	50.29	3.63	0.60	2.24	0.08	3.38	0.09	0.18	0.59	20.77	0.18	0.10	0.09	0.09	0.52	17.20		85.22
Vibrio vulnificus Env1	20	0.05	0.23	4.26	1.15	0.89	1.18	7.66E-5	86.10	0.27	0.35	0.87	2.44	0.35	0.30	0.35	0.27	1.04	4.14	2.86	

B

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Vibrio vulnificus FDAARGOS-119	1		84.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio fluvialis ATCC 33809	2	9.36		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio harveyi 345	3	0.10	0.10		82.74	0.00	83.11	0.00	86.38	0.00	0.00	0.00	87.11	0.00	0.00	0.00	0.00	0.00	85.15	88.38	85.88
Vibrio spp. J383 Chromosome_2	4	0.08	0.05	2.69		0.00	93.31	0.00	84.15	0.00	0.00	0.00	83.36	0.00	0.00	0.00	0.00	0.00	83.58	84.07	84.11
Vibrio cholerae O1 str	5	0.63	0.20	0.10	0.15		0.00	0.00	0.00	85.15	85.08	100.00	0.00	85.60	85.23	85.46	85.15	94.10	0.00	0.00	0.00
Vibrio splendidus BST398	6	0.17	0.13	2.58	76.36	0.19		0.00	84.67	0.00	0.00	0.00	83.71	0.00	0.00	0.00	0.00	0.00	84.19	83.82	84.52
Photobacterium damselae subsp. piscicida	7	6.29E-5	3.16E-4	0.07	2.15E-4	6.37E-3	2.90E-4		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio vulnificus FORC-077	8	0.05	0.32	5.44	1.14	0.85	1.14	1.50E-4		0.00	0.00	0.00	85.35	0.00	0.00	0.00	0.00	82.55	86.09	84.92	94.80
Vibrio anguillarum J360	9	0.24	0.11	0.03	0.02	2.23	0.08	8.11E-4	0.31		98.54	85.16	0.00	98.41	98.40	98.35	100.00	82.15	0.00	0.00	0.00
Vibrio anguillarum MHK3	10	0.29	0.06	0.01	0.10	2.82	0.09	9.25E-5	0.38	71.56		85.21	0.00	98.64	98.49	98.40	98.54	83.76	0.00	0.00	0.00
Vibrio cholera FDAARGOS-223	11	0.62	0.19	0.10	0.15	98.21	0.19	6.30E-3	0.84	2.21	2.82		0.00	86.00	85.24	85.47	85.17	94.10	0.00	0.00	0.00
Vibrio alginolyticus 2439-01	12	0.04	0.15	20.60	2.40	0.18	2.08	0.08	2.47	0.16	0.17	0.18		0.00	0.00	0.00	0.00	85.43	88.47	86.62	85.11
Vibrio anguillarum CNEVA NB11008	13	0.32	0.07	0.03	0.02	2.70	8.99E-3	9.19E-5	0.39	74.32	77.04	2.87	0.09		98.47	98.41	98.40	83.84	0.00	0.00	0.00
Vibrio anguillarum 775	14	0.20	0.07	0.02	0.03	2.74	0.10	0.00	0.37	75.48	80.48	2.71	0.18	80.26		98.38	98.39	83.42	0.00	0.00	0.00
Vibrio anguillarum JLL 237	15	0.44	0.06	0.01	0.09	2.73	0.09	9.04E-5	0.24	74.29	73.17	2.70	0.19	75.93	77.28		98.35	84.60	0.00	0.00	0.00
Vibrio anguillarum VIB43	16	0.28	0.11	0.03	0.02	2.25	0.08	8.17E-4	0.31	90.96	72.19	2.22	0.16	74.85	76.00	74.82		82.15	0.00	0.00	0.00
Vibrio cholerae RFB05	17	0.46	0.24	0.63	0.07	70.70	0.08	5.89E-3	1.62	1.62	2.14	69.90	1.01	2.19	2.22	2.24	1.63		83.49	0.00	81.87
Vibrio parahaemolyticus R14	18	0.09	0.25	19.34	1.95	0.17	1.83	0.08	4.20	0.18	0.32	0.16	42.46	0.09	0.18	0.09	0.18	1.32		84.24	85.98
Vibrio campbellii ATCC 25920, CAIM 519T	19	0.17	0.16	50.29	3.63	0.60	2.24	0.08	3.38	0.09	0.18	0.59	20.77	0.18	0.10	0.09	0.09	0.52	17.20		85.22
Vibrio vulnificus Env1	20	0.05	0.23	4.26	1.15	0.89	1.18	7.66E-5	86.10	0.27	0.35	0.87	2.44	0.35	0.30	0.35	0.27	1.04	4.14	2.86	

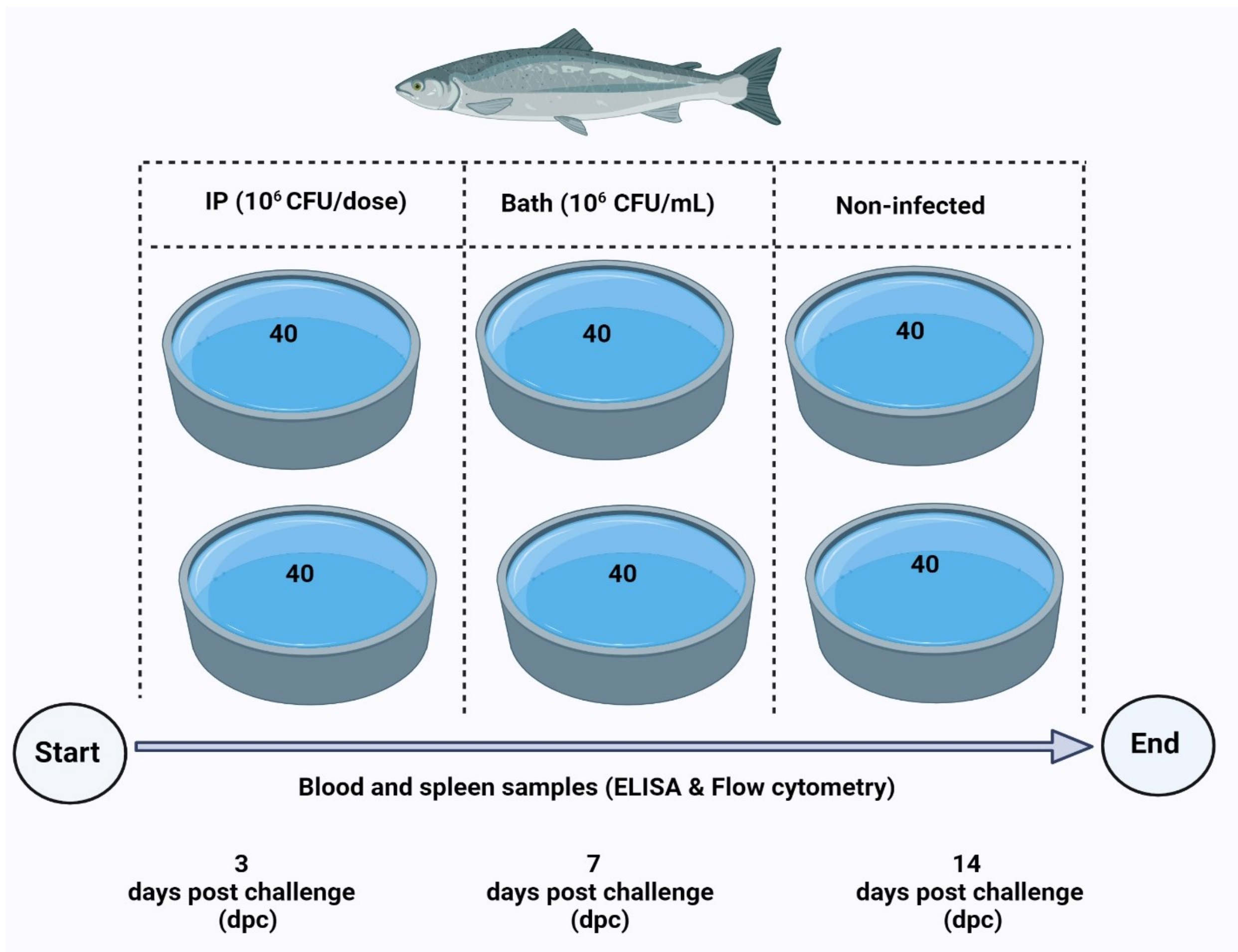
Supplementary Figure S2-3. Average nucleotide identity in *Vibrio* sp. J383. (A) Chromosome 1; (B) Chromosome 2. *Vibrio*. sp. J383 identity percentage parameters for annotated genes were set up as minimum similarity of 0.8 and minimum length of 0.8. Analyses were performed using CLC Genomic Workbench v20 (CLC Bio). Genome alignment involved 20 *Vibrio* sp.

Supplementary Table S2-1. Enzymatic profile of *Vibrio*. sp. J383 using commercial biochemical test kits (API system).

APIZYM		API20E		API20NE	
Alkaline phosphatase	+	β-Galactosidase	+	Reduction of nitrates to nitrites	+
Esterase (C4)	+	L-Arginine	-	Indole production	+
Esterase lipase (C8)	+	L-Lysine	-	Glucose fermentation	+
Lipase	+	L-Ornithine	-	Arginine	-
Leucine arylamidase	+	Citrate utilization	-	Urease	-
Valine arylamidase	+	H2S production	-	Esculin	+
Cystine arylamidase	+	Urease	-	Gelatin	+
Trypsin	+	L-Tryptophane	+	β-galactosidase	+
Chymotrypsin	+	Indole production	+	D-Glucose	+
Acid phosphatase	+	Acetoin production	-	L-Arabinose	-
Naphthol-AS-BI-phosphohydrolase	+	Gelatinase	+	D-Mannose	-
Galactosidase	+	D-Glucose	+	D-mannitol	+
B- galactosidase	-	D-Mannitol	+	N-acetyl-glucosamine	-
Glucuronidase	-	Inositol	-	D-maltose	-
B-glucuronidase	-	D-Sorbitol	-	Potassium Gluconate	-
Glucosidase	-	L-Rhamnose	-	Capric acid	-
B-glucosidase	-	D-Saccharose	-	Adipic acid	-
N-acetyl-B-glycosaminidase	-	D-Melibiose	-	Malic acid	-
Mannosidase	-	D-Amygdaline	+	Trisodium citrate	-
Fucosidase	-	L-Arabinose	-	Phenylacetic acid	-

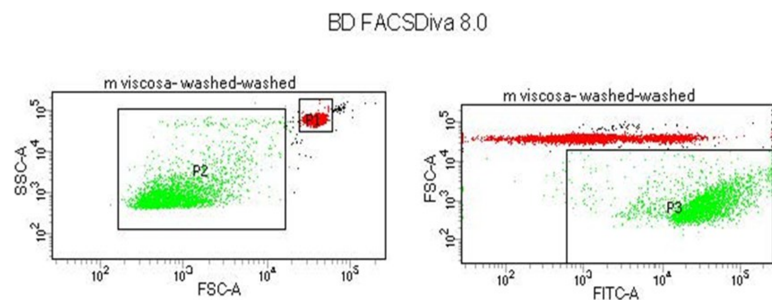
Supplementary Table S2-2. Genes associated with subsystems in chromosomes and plasmid.

Predicted genes associated with subsystems pathogenesis and environmental adaptations			
Gene Subsystem Category or Gene Name	**Presence/Absence of Gene in *Vibrio* sp. J383**		
	Chromosome 1	**Chromosome 2**	**Plasmid**
MazEF toxin-antitoxing			+
Flagellum	+		
Fatty acid biosynthesis	+		
Ferrichrome-iron receptor	+		
fur	+		
mdtL		+	
fabG	+		
Vibrio Ferrin synthesis	+		
Lipoic acid metabolism	+	+	
Polyamine metabolism	+	+	
Cold shock protein of CSP family		+	
Lactose utilization		+	
DNA repair, bacterial photolyase	+	+	
Cell division cluster	+		
Antibiotic targets in DNA processing	+		
Resistance to chromium compounds	+		
Xanthine dehydrogenase subunit		+	
Thiamin, thiazole, hydroxyethyl pyrimidine uptake		+	
Bacterial checkpoint control related cluster	+		
Proteasome bacterial	+		
Chemotaxis	+		
Antibiotic targets in cell wall biosynthesis	+	+	
Heme transport		+	
Iron ABC transport permease		+	
Toxin antitoxin system		+	
Antibiotic target in transcription	+		



Supplementary Figure S3-1. Challenge assay with *Moritella viscosa.* Blood and spleen samples were collected at 3, 7 and 14 days post challenge (dpc).

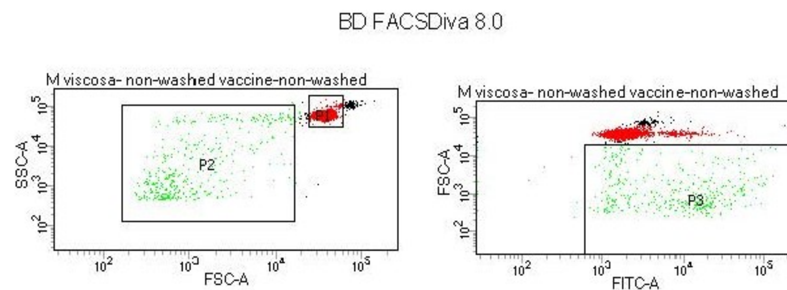
A



Experiment Name: Experiment_127
Specimen Name: m viscosa- washed
Tube Name: washed
Record Date: 11-Aug-2021 3:19:11 PM
$OP: Administrator
GUID: c90d4da0-0d42-4a4f-83e3-18c...

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	10,000	####	28,197	42,348
P1	7,035	70.4	38,954	58,222
P2	2,889	28.9	1,207	2,603
P3	2,872	28.7	1,213	2,485

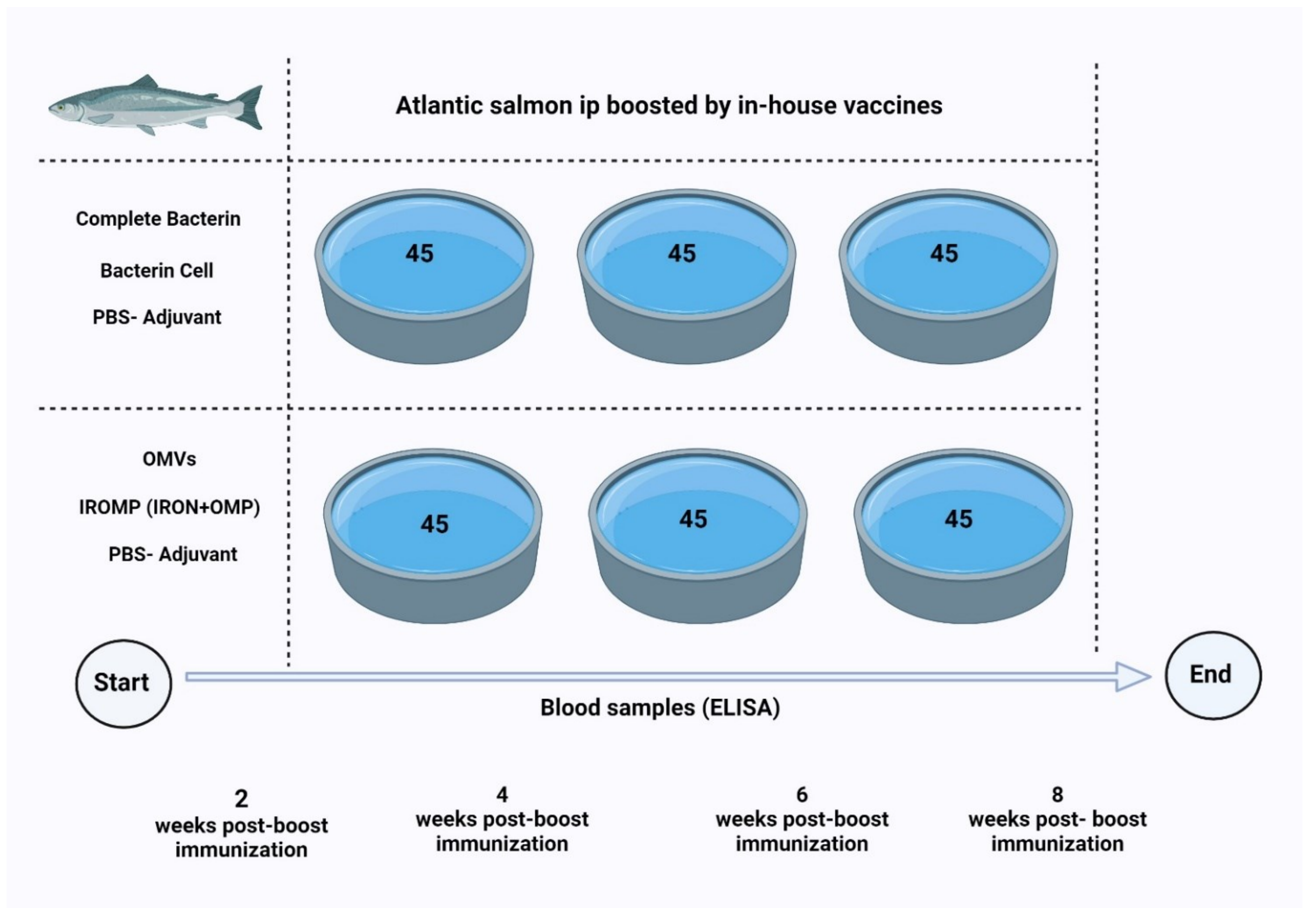
B



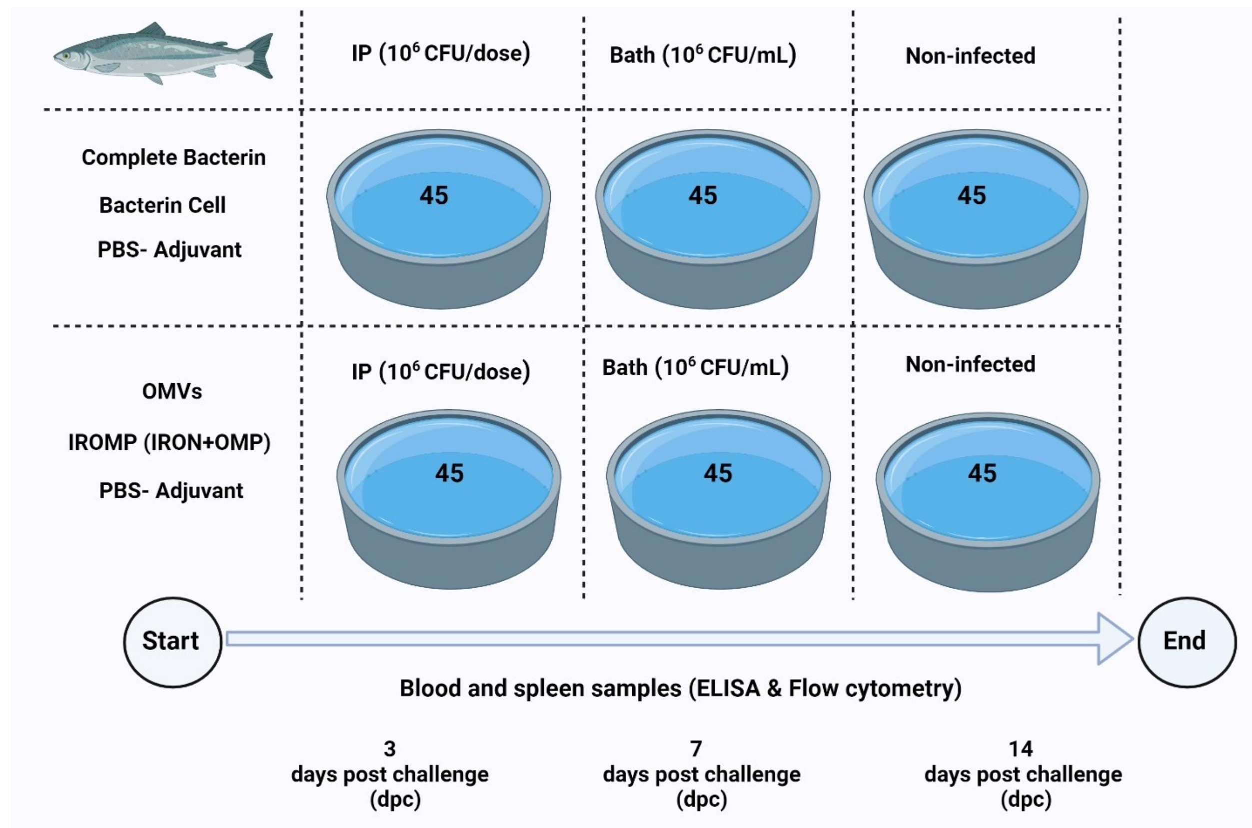
Experiment Name: Experiment_127
Specimen Name: M viscosa- non-washed vaccine
Tube Name: non-washed
Record Date: 11-Aug-2021 3:14:30 PM
$OP: Administrator
GUID: 38397b63-1d53-4f19-8086-285...

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean
All Events	10,000	####	37,609	57,260
P1	9,487	94.9	38,742	58,596
P2	399	4.0	2,571	13,695
P3	394	3.9	2,756	15,008

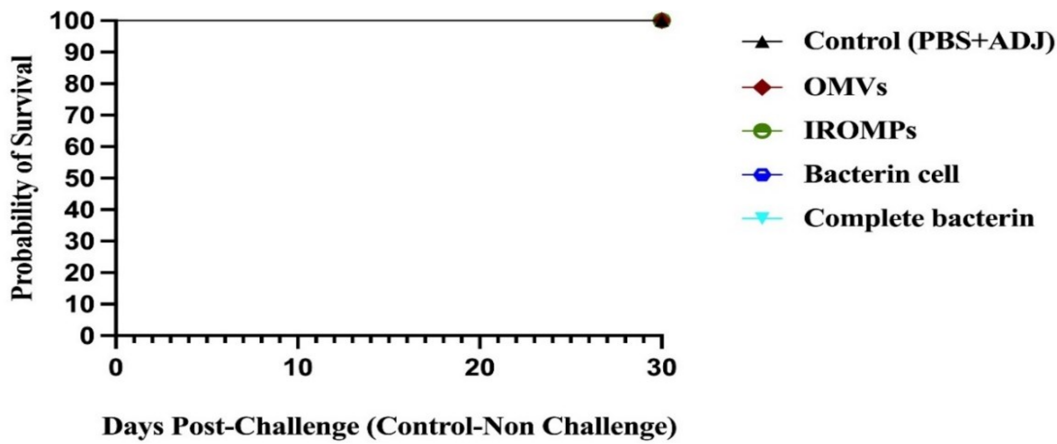
Supplementary Figure S3-2. Vaccine concentration. The concentration of (A) bacterin cell and (B) complete bacterin vaccines were calculated based on the defined formula by flow cytometry (bacterial counting cell: (No of bacterial cell ($p2$))/ (No of beads ($p1$)) $\times 10^6$ $\times$ dilution (10^3) in flow cytometry.

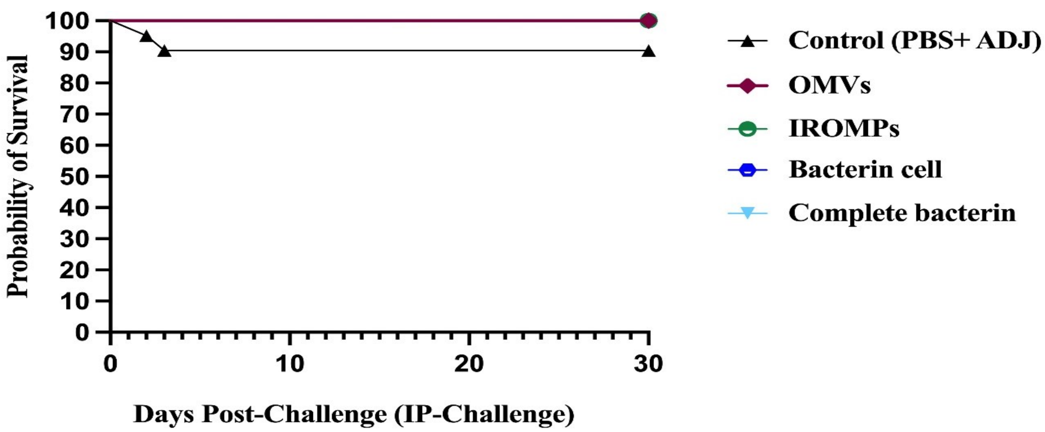


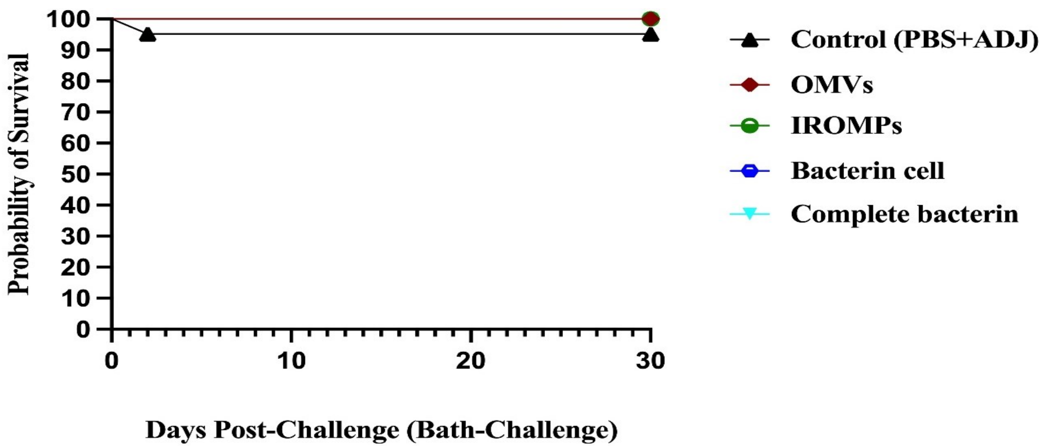
Supplementary Figure S3-3. Post-boost immunization design using in house vaccines. Blood samples were collected at 2, 4, 6 and 8 weeks following the boost.



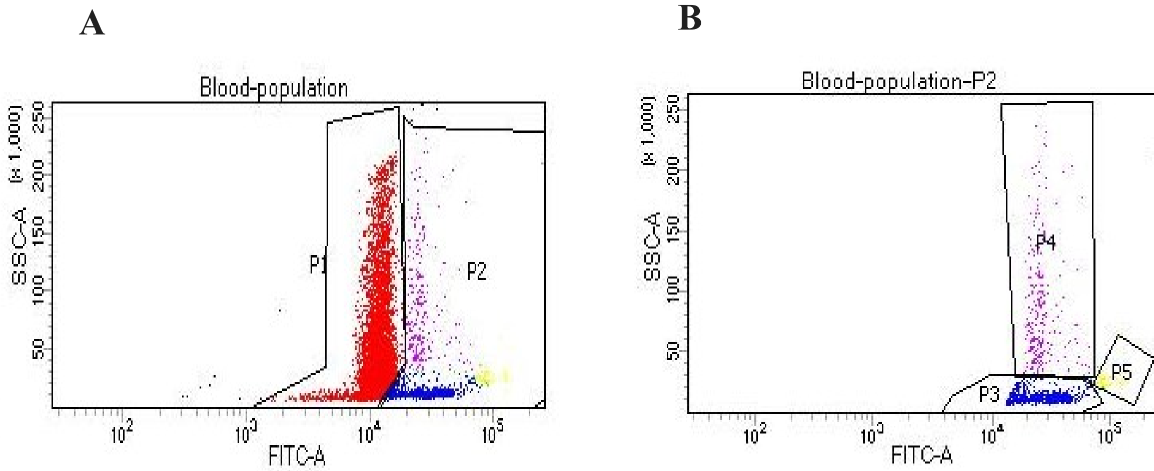
Supplementary Figure S3-4. Challenge assay with *M. viscosa* at 12 weeks post boost immunization. Blood and spleen samples were collected at 3, 7 and 14 days post challenge (dpc).



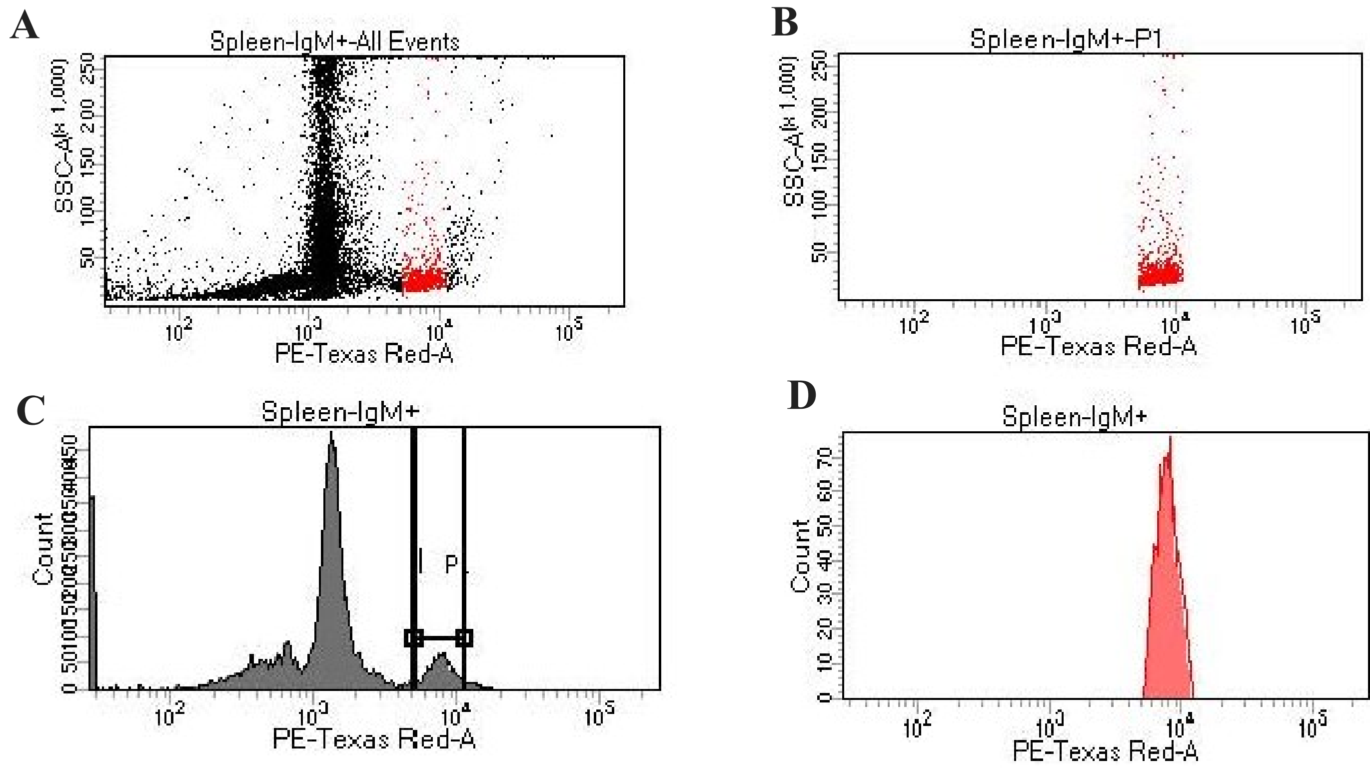




Supplementary Figure S3-5. Survival proportions were compared between boosted and non-boosted (PBS+ADJ) Atlantic salmon after ip and bath challenges with *M. viscosa*. A group of animals was not subjected to any challenges.



Supplementary Figure S3-6. The flow cytometry algorithm for dividing blood populations. (A) P1 shows red blood cells (RBCs) and P2 shows white blood cells (WBCs) in vaccinated farmed Atlantic salmon. (B) P3 shows lymphocytes. P4 indicates neutrophils and basophils and P5 displays monocytes. The value of the threshold operator was 5000 for FSC and SSC in flow cytometry. The voltages for FSC, SCC and FITC were 247, 231, and 313 respectively.



Supplementary Figure S3-7. The flow cytometry technique for defining IgM+ (B cells) in spleen. (A) The red color shows IgM in the spleen. (B) Texas red is a marker to show IgM+ population. (C) Based on the DIOC6 the population of RBCs and WBCs were separated. (D) The peak demonstrates IgM+.