Investigation into the Cytotoxicity of Soluble Extracellular Products Secreted by *Tenacibaculum maritimum* and other *Tenacibaculum* species

by

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Abstract

Atlantic salmon (*Salmo salar*) aquaculture in British Columbia (BC), Canada is the province's largest agricultural export and is a significant contributor to its economy. As with any food production industry, aquaculture faces challenges regarding disease. One disease of interest is tenacibaculosis, locally known as mouth rot or yellow mouth. Antibiotics are the only treatment for this infection, which results in mouth rot being responsible for the majority of antibiotic use in BC aquaculture. Further, there is no available vaccine for preventing infections of Atlantic salmon and the pathogenesis mechanism of the disease is unknown. Current evidence supports *Tenacibaculum maritimum* as an opportunistic pathogen capable of causing mouth rot disease. However, it is unclear as to how this bacterium may establish infection, when it is most likely to cause an outbreak, and the mechanisms by which other related bacterial species could be involved? These questions won't be answered in their entirety, but my goal is to advance our functional understanding of the associated disease bacteria by probing these overarching questions.

Previous studies have demonstrated that extracellular products (ECP) from *T. maritimum* are capable of causing fish muscle damage and cell death, implicating that these secreted products could be involved in disease pathogenesis. However, the mechanism by which these ECP work is not fully known. Our study uses a validated flow cytometry cell death quantification assay to determine the mechanism of salmonid cellular death after exposure to *T. maritimum* ECP. Cell lines originating from salmonid mucosal epithelial tissue were used as relevant models in cytotoxicity trials. When treated with *T. maritimum* ECP, these cells underwent programmed host cell death (apoptosis) due to the exposure. The impact of environmental conditions, such as salinity and temperature, on bacterial growth and the production of cytotoxic ECP were also examined. When investigated *in vitro*, both conditions appear to be involved in cytotoxicity, aligning with

recent literature and industry field reports. The bacterial ECP underwent inactivation treatments of heat denaturation, protease inhibitors, and a metalloprotease inhibitor to narrow down the responsible product. Overall, this work pushes forward the knowledge on *T. maritimum* secreted ECP; however, there is still much to follow up with regarding these products and their involvement in field infections.

Mouth rot infections occur in seawater and on the skin of fish where there are plenty of other bacteria that may contribute to disease pathogenesis. Related *Tenacibaculum* species are known to be capable of causing tenacibaculosis infections in Atlantic salmon. I hypothesize that the contributions from different bacteria at the site of infection result in the disease symptomatology differences seen internationally. Herin, two mouth rot isolated *Tenacibaculum* species, *Tenacibaculum dicentrarchi* (known pathogen) and *Tenacibaculum ovolyticum* (unknown if pathogenic), were compared to *T. maritimum* regarding their various potentially pathogenic characteristics, such as biofilm production and ECP cytotoxicity. In this study, *T. maritimum* was the only isolate that demonstrated an ability to form biofilms. Surprisingly, when cells from a salmonid immune cell line were treated with soluble ECP from the various bacteria, *T. dicentrarchi* and *T. ovolyticum* had the highest cytotoxicity. Further, after the various ECP underwent inactivation trials (heat-treatment, protease inhibitors, and metalloprotease inhibitors), there were varied results between the potential pathogens, thereby suggesting the production of distinct virulence factors between the *Tenacibaculum* species.

Overall, this study contributes to the functional understanding of cytotoxic extracellular products secreted by *T. maritimum* and other *Tenacibaculum* species. While the degree of involvement of these secreted products remains unknown, the mechanism of their cytotoxicity on

a cellular level is now uncovered. Further, the potential for cytotoxic contributions from related species was demonstrated in the current study and adds to the complexity of this disease.

Preface

This thesis is an original work by Matthew L. Michnik. My research was supported by the Natural Sciences and Engineering Research Council of Canada (RGPIN-2018-05768) and funding from Grieg Seafood BC Ltd. (RES0045719). I was supported throughout my research with a Graduate Teaching Assistantship from the Department of Biological Sciences, University of Alberta.

Dedication

I would like to dedicate this thesis to the many family members that were quintessential and imperative supporters of my journey to this point in my life. Specifically, my grandparents (Yvonne, Don, Elaine, and Brian), my parents (Brenda and Derek), my brothers (Braiden and Spencer), and my aunts/uncles (Adrian, Karina, Vance, Laura, Dean, Dale, and Deanna). This accomplishment is just as much yours as it is mine.

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Ever since I was little, I wanted to be as smart as my dad and as kindhearted as my mom. This has not changed. They never rolled their eyes when I said that I wanted to do more school. They were amiable and calming when I was frustrated (often science things that they likely didn't fully understand). They wanted me to tread my own path and push my limits. They also instilled in me the resiliency and belief that I could do it. If there is one thing that I am certain of, it is that I would not be where I am today without my endlessly supportive parents.

I must say a special thanks to my partner Apolline. She made dinners for me when my evenings in lab were long. She dropped me off at university when the parking passes got too expensive. The support I received from her on an everyday basis was unmatched and unwavering. This thesis would not have been completed without her support and passionate belief in my abilities, which gave me the confidence and motivation needed to persevere through failure.

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List of Abbreviations

ECP – Extracellular Products

BC – British Columbia

HK – Housekeeping

CFU – Colony Forming Unit

MPN – Most Probable Number

PCR – Polymerase Chain Reaction

PI – Propidium Iodide

AnnV – Annexin V

FITC - Fluorescein isothiocyanate

PB – Pacific blue

FMM – *Flexibacter maritimus* media

T9SS – Type IX secretion system

AB – Alamar blue

CFDA – 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (5-CFDA-AM shortened further)

CTD – C-terminal domain

P.I. – Protease inhibitors

EDTA – Ethylenediaminetetraacetic acid

HT – Heat-treatment

MIC – Minimum inhibitory concentration

ppt – Parts per thousand

OD – Optical density

FBS – Fetal bovine serum

DMSO – Dimethyl Sulfoxide

SBF – Specific biofilm formation

CV – Crystal Violet

DFO – Department of Fisheries and Oceans (Government of Canada)

Chapter 1: Introduction and literature review

1.1 General introduction

When envisioning the food production methods of the future, something that should come to mind more than it currently does is aquaculture. It is an internationally viable food production strategy used to meet fish protein demands and reduce harvesting stress on native fish stocks (FAO, 2018). This is relevant because one of many drivers endangering ocean fish populations is the over-exploitation of wild stocks, which aquaculture (with the implementation of the right policies) could prevent (Cottrell et al., 2021; Nahuelhual et al., 2019). Critics of aquaculture often cite the potential for disease transmission as harmful to the native fish populations (Murray et al., 2005; Aly et al., 2014; Krkošek et al., 2007). In British Columbia (Canada), concerns around disease in aquaculture resulted in the non-renewal of farming licenses along key Pacific salmon (*Oncorhynchus spp.*) spawning routes (DFO, News release). One disease cited as a concern to the dwindling wild Pacific salmon stocks in the Pacific Ocean is the salt-water ulcerative disease referred to as tenacibaculosis (Jones, 2022; Bateman et al., 2022; Di Cicco et al., 2023). A recent study, used infection trials to demonstrate that *T. maritimum* can infect Chinook salmon (*Oncorhynchus tshawytscha*) with tenacibaculosis disease (Kumanan et al., 2024a). Although, there have been risk assessments that state that tenacibaculosis is likely not a threat to native salmon populations (DFO, 2020). Nevertheless, due to tenacibaculosis infections in BC Atlantic salmon farms, there still is a great deal of industry financial costs (opportunity losses, antibiotics, etc.) and a potential to lose the industry's social licenses to operate (Hewison & Ness, 2015). Herin, disease research that leads to an improved understanding and preventative measures associated

with more sustainable aquaculture would be valued by industry, environmentalists, and government officials alike.

Due to the symptomatology of Atlantic salmon tenacibaculosis infections in the Canadian region, the disease is referred to as mouth rot (or yellow mouth) by the industry. The leading proposed causative agent for this infection is the gram-negative bacteria *Tenacibaculum maritimum*, which was identified to be disease causing (Frisch et al., 2018a; Frisch et al., 2018b). The mechanism of action for this pathogenic bacterium is currently unknown but a type IX secretion system (T9SS) and multiple potential toxins (with T9SS CTDs) were recently discovered in the *T. maritimum* genome (Pérez-Pascual et al., 2017). Further, there is evidence of *in vivo* salmonid death (Van Gelderen et al., 2009) and *in vitro* cyprinid cell death (Escribano et al., 2023) due to exposure to soluble extracellular products (ECPs) secreted by *T. maritimum*. However, the mechanism of cell death and the extent of *T. maritimum* ECP involvement in natural infection remains unknown.

Tenacibaculosis disease is also impacted by, although to an unknown extent, environmental conditions (temperature and salinity) (Mabrok et al., 2023; Nowlan et al., 2021b). In Canadian waters, mouth rot has been documented in the literature to occur at temperatures between 8–12°C (Frisch et al., 2018c), which is different from tenacibaculosis infections in Chilean waters occurring above 15°C (Avendaño-Herrera et al., 2006a). An increase in salinity is widely associated with increased tenacibaculosis infections (Nowlan et al., 2021b; Avendaño-Herrera et al., 2006a), whereas a decrease in salinity is associated with lower disease mortality (Handlinger et al.,1997). An additional complexity of the disease is the involvement of both *Tenacibaculum* species and non-*Tenacibaculum* species at the site of infection (Wynne et al., 2020). Recent scientific opinions have shifted towards other *Tenacibaculum* species having more of a shared role

in causing tenacibaculosis in salmonids. Further, *Tenacibaculum dicentrarchi* and *Tenacibaculum finnmarkense* have both been shown to be capable of causing tenacibaculosis in Atlantic salmon bath-immersion trials, supporting this hypothesis (Nowlan et al., 2021a). When examined altogether, very little is known about the mechanism of pathology, the effects of various environmental conditions, which bacteria are involved, and the extent of multiple bacterium involvement in the disease.

1.2 Objective of thesis

1.2.1 *Thesis aims*

My project aims to further the understanding of *T. maritimum* secreted products, and the potential mechanisms of pathology. Through direct work with industry partners, I used an isolate of *T. maritimum* obtained directly from an Atlantic salmon mouth rot infection. I examined the soluble extracellular secreted products (ECP) from the isolate under various relevant environmental conditions (including salinity and temperature). Further, I examined a number of mouthrot associated bacteria (*T. dicentrarchi*, *Tenacibaculum ovolyticum*, and *Cellulophaga baltica*), through a comparison of their biofilm forming capacities and characteristics of their soluble ECP.

1.2.2 *Thesis outline*

My masters project used flow cytometry to identify salmonid cell death mechanisms upon exposure to *T. maritimum* ECP. It highlighted the effects of relevant environmental conditions on *T. maritimum in vitro* growth and ECP cytotoxicity. The project also attempted functional

knockdowns of proteinaceous ECP components to narrow down the suspected product(s) of interest. This work was performed through two relevant cell line models, ASimf20 and RTgutGC (mucosal epithelial tissue origin). Additionally, the secreted products from other *Tenacibaculum* species were also compared to highlight any potential differences between the related fish disease-associated bacteria. This was assessed using methods established with previous *T. maritimum* ECP examination except with an immune cell line (SHK-1).

1.3 Literature review

1.3.1 *West coast Canadian aquaculture*

Atlantic salmon are responsible for over 30% of total global marine fish production, while also being the industry's most valuable commodity (FAO, 2022). They are the most farmed fish in Western Canada, responsible for almost 700 million dollars in production value for 2021 (Government of Canada, Aquaculture production and value). Further, the 84 thousand tonnes of Atlantic salmon harvested from British Columbia (BC) farms in 2021 was greater than the entire finfish production in the rest of Canada (65 thousand tonnes) (Government of Canada, Aquaculture production and value). A report from the Canadian aquaculture industry alliance (CAIA) (2020), states that salmon farming industry has shown a tremendous capacity for export growth in 2015 through to 2019 and is the largest agricultural export in BC (CAIA, 2020). This illustrates that the Atlantic salmon aquaculture industry is a critical component of the provincial economy. Furthermore, Western Canadian aquaculture has the potential to be a model for sustainable industry practices, which may lead to improved food production strategies internationally.

1.3.1.1 *Risk of disease*

Despite the benefits associated with BC farmed Atlantic salmon, there are some groups critical of aquaculture regarding its potential impact on the environment. Some Canadian researchers have proposed that there may be negative aquaculture impacts due to disease spread from Atlantic salmon net pen sites to wild salmonids (Bateman et al., 2022). As such, the health of the net pen salmonids is continually monitored by the industry and the sites are audited by the DFO (Department of Fisheries and Oceans) for disease (Government of Canada, aquaculture audit activities). Disease in net pens cost farms production (Behringer et al., 2020) and can have a negative effect on the social license to operate (public associates disease with risk to the environment) (Kraly et al., 2022). Further, higher salmonid stocking densities likely increases the disease transmission within the net pens, resulting in these populations being a higher disease risk (Behringer et al., 2020; Krkošek, 2010). When disease does occur, antibiotics are used to limit infectious disease spread, morbidity, and mortalities. However, antibiotics can often find their way into the environment leading to an increased risk of antimicrobial resistance (aligns with the idea of "One Health" sustainability and industry responsibility) (Velazquez-Meza et al., 2022). Additionally, there has been an industry evolution towards novel, accessible, and cost-effective strategies for earlier detection and treatment of disease in net pens (Bohara et al., 2024; MacAulay et al., 2022). Specifically, the Canadian industry has improved animal welfare practices (including fish husbandry, fish stocks, disease monitoring, disease management, etc.) leading to an overall reduction antibiotic use (Jonah et al., 2024). Although, despite the significant impact disease has on aquaculture, the main cause of fish mortality is actually from harmful algal blooms (oxygen depletion, toxin production, etc.) (Behringer et al., 2020; Haigh and Esenkulova, 2014).

1.3.2 *Tenacibaculosis*

A global problem for the aquaculture industry is tenacibaculosis, which affects salt-water pens in many countries worldwide (Japan, Spain, Australia, etc.) and across different genera of farmed fish (sea bream (*Pagrus major*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon, etc.) (see reviews by Avendaño-Herrera et al. (2006a) and Mabrok et al. (2023)). The symptomatology of tenacibaculosis is consistent with ulcerative damage along the fins, body, gills, and/or mouth of the fish and a lack of inflammation at the site of infection (Avendaño-Herrera et al., 2006a; Frisch et al., 2018b; Nowlan et al., 2021a; Handlinger et al.,1997). Behavioral symptoms associated with the disease include lethargy and a lack of eating (which may reduce the effectiveness of antibiotic feed treatments) (Kent & Poppe, 2002; Nowlan et al., 2020). Although, tenacibaculosis symptomology can differ between geographical regions, with mouth rot (also known as yellow mouth) being the predominant disease form in BC, Canada (Nowlan et al., 2020). Why these disease differences occur is currently unknown, but it is possibly due to the interactions between pathogenic bacterium genetics, involvement of different bacterial species, environmental conditions, and host responses.

Tenacibaculosis refers to the involvement of bacteria from the *Tenacibaculum* genus, one of which being *T. maritimum* (will be discussed in detail later). Interestingly, *T. maritimum* and other related *Tenacibaculum* species are described more as opportunistic pathogens due to their wide distribution in the natural environment, including on healthy salmon (before and post mouth rot symptoms) (Wynne et al., 2020). It is not currently clear how these tenacibaculosis infections begin in net pens, which leads to numerous theories from researchers and industry. In studies by Mabrok et al. (2016) and Magarinos et al. (1995), it was shown that *T. maritimum* is resistant to the bactericidal mucus activity of multiple hosts, supporting the idea that this bacterium can live

on the skin of fish. In fact, a study by Llewellyn et al. (2017) showed that *Tenacibaculum* was the most dominating genus in the microbial community from Atlantic salmon mucus, and interestingly, in water as well. A study by Wynne et al. (2020) demonstrated that *T. maritimum* was abundant in healthy salmonids (as well as mouth rot infected salmonids) but was not a dominant species in the water column. Differences between these water-related conclusions likely were from different bacteria collection methods, and/or Llewellyn et al. (2017) using tanks to house experimental fish whereas Wynne et al. (2020) collected water samples from open ocean net pen sites. A study by Avendaño-Herrera et al. (2006b), showed that the opportunistic bacterial pathogen (*T. maritimum*) does not survive for extended periods of time in seawater, likely indicating that it comes from hosts, persistent biofilms, or sediments (Avendaño-Herrera et al., 2006b). Other tenacibaculosis observations and hypotheses have involved jellyfish causing damage to the salmonid skin which progresses infections (Småge et al., 2017), or that jellyfish may be a mobile reservoir for *T. maritimum* (Delannoy et al., 2011; Ferguson et al., 2010). Overall, tenacibaculosis is likely caused by a persistent *Tenacibaculum* bacteria, originating from ocean-dwelling animals, sediment, or structures as an opportunistic pathogen.

1.3.2.1 *Mouth rot disease*

Atlantic salmon undergo smoltification, which is the transition of anadromous fish from freshwater to saltwater (Stefansson et al., 2020). In aquaculture, this occurs during the transfer from the freshwater hatcheries to the open ocean net pens. The government of Canada documents that the highest incidence of mouth rot (regional presentation of tenacibaculosis) and its associated mortality, occur a few weeks after smolt seawater entry (during this smoltification period) (Government of Canada, Mouth rot). Atlantic salmon smolts are vunerable and have a suppressed

immune system, likely due to the high energy costs (Johansson et al., 2016) and stress (Barton and Iwama, 1991) associated with the smoltification and handling. Further, these fish could be susceptible to skin abrasions due to handling during the transfer to sea-water (Kent and Poppe, 2002; Spilsberg et al., 2022), from changes to the fish skin microbiota due to the new salty environment (Lokesh and Kiron, 2016), and/or from a dysbiosis caused by physiological stress (Boutin et al., 2013). It is also proposed by Spilsberg et al. (2022) that the faces and sides of Atlantic salmon could come in contact with net pens (alowing for an opportunistic pathogen to take hold), which is exasterbated during the transfer to ocean net pens through a panicing behaviour of farmed salmonids. Further, the teeth in Atlantic salmon undergo continual replacement (Huysseune et al., 2007), which may give the opportunistic *Tenacibaculum* bacteria a place to enter and cause damage. Lastly, it should also be considered that Atlantic salmon strengthen their skin immune protection mechanism and barrier integrity as post-smolts the longer they spend in seawater (Karlsen et al., 2018), which aligns with the reduced suceptibility timeline for mouth rot infections. A combination of the above factors likely details why mouth rot disease may target Atlantic salmon at this stage in their life. Overall, the causes of mouth rot disease are still ill-defined, likely involving multiple factors, bacteria, and the correct circumstances to induce outbreaks.

1.3.2.1.1 Financial impact and mortality rates

According to the aquaculture industry, outbreaks of mouth rot in BC Atlantic salmon net pen sites caused financial losses of 1.6 million dollars annually (accounting for losses due to mortality, antibiotic treatment costs, and biomass losses in healthy fish) (Hewison & Ness, 2015). Atlantic salmon mortality associated with mouth rot symptoms is well documented, but often

variable between years. An article by Frelier et al. (1994) reports that mortality from ulcerative bacterial stomatitis (mouth rot) reached 10% of the total Altantic salmon in the netpens during the 1990–1991 out break; however, the mortality ranged between 1–20% at each individual site. Further, an anonymous source in an article by Ostland et al. (1999) said that mouth rot disease neared 15% mortality in net pens. Other aquaculture producers have seen 11% mortality as the high mark at a site, with an overall yearly total of 6% in 2015 (Hewison and Ness, 2015). It should be noted that the yearly total in 2007 was 1% mouth rot mortality (Hewison and Ness, 2015). Mortality rates associated with mouth rot disease appear to be quite variable and likely depend on environmental conditions, farm location, effectiveness of treatment, health or density of the stock, etc. This results in an added complexity associated with researching mouth rot disease in net pen infections.

1.3.2.1.2 Pathogenesis

The pathology of mouth rot is characterized by a plaque of filamentous bacteria on the mouth (oral cavity) of the fish (Frisch et al., 2018a; Ostland et al., 1999). The lesions, lined with yellow bacteria, are 1–5 mm in length and are round (Frelier et al., 1994). The bacteria are also described as creating a thick, but unicellular biofilm on the teeth of the infected fish (Frelier et al., 1994). Other ulcers, such as gill and skin lesions, have also been identified in Atlantic salmon infection trials (Frisch et al., 2018b). There were also behavioral symptoms of the disease, such as: a loss of host equilibrium (Frisch et al., 2018b), loss of appetite (anorexia) (Kent and Poppe, 2002), lethargy (Kent and Poppe, 2002), and even head shaking (Kent and Poppe, 2002). Interestingly, there is also a lack of overt inflammation seen in mouth rot infected salmonids (Frisch et al., 2018a). It is worth mentioning that a sub cutaneous injection of *T. maritimum* (the

likely mouth rot pathogen) was able to induce an inflammatory response in turbot (Faílde et al., 2013), however this was neither a native infection, the natural dose, or the same host species.

1.3.2.1.3 Antibiotic use

Currently, the only treatment to prevent mouth rot outbreaks is with the use of commercial antibiotics, administered with the fish feed. In an article by Morrison and Saksida (2013), they stated that mouth rot (referred to as stomatitis) was the reason for 98% of the antibiotics perscribed to some BC aquaculture sites in 2011. The most common antibitoic used is florfenicol, which requires multiple treatments (2–5) to effectively combat the disease (Morrison and Saksida, 2013). Further, there are only a handful of antimicrobial drugs approved for use in salmonid Canadian aquaculture (Government of Canada, List of Veterinary Drugs in Food-Producing Aquatic Animals). Oxyetracycline, one of these approved drugs, has been shown to have resistance genes developed against it in various Canadian and international *Tenacibaculum* species (Nowlan et al., 2023). No Canadian *Tenacibaculum* species have yet developed florfenicol resistant genes or changes to the minimum inhibitory concentration (MIC) (80 tested *T. dicentrarchi* isolates) (Nowlan et al., 2023). However, one single Chilean *T. dicentrarchi* isolate (of 55 tested) was shown to have a higher florfenicol MIC (8 μg/mL) compared to the wild types (4 μg/mL) (Irgang et al., 2021). Altogether, this infers that there may be a risk towards these tenacibaculosis associated bacteria developing antimicrobial resistance. This is even more daunting when considering that there are currently no preventative mouth rot treatments, such as a vaccine against the disease.

On a positive note, a review by Jonah et al. (2024) stated that antibiotic use in Canadian aquaculture has been cut in half from 2016 to 2021 (110.97 to 45.62 mg/kg), which shows a very

encouraging industry trend. However, the aquaculture antibiotic use graph from Jonah et al. (2024), shows that Chilian aquaculture uses about 400 mg/kg of antibiotics whereas Canadian, Scotland, and Norwegian aquaculture use less than 100 mg/kg. There are promising trends associated with a reduction in antibiotic use, although internationally there is still much work to be done. Overall, this antibiotic use change is a long-term goal of tenacibaculosis research, which will the sustainability of the industry.

1.3.3 *Tenacibaculum maritimum*

The salt-water, filamentous, and Gram-negative bacteria *Tenacibaculum maritimum* was first classified as [*Flexibacter maritimus*] by Wakabayashi et al. (1986) before reclassification into the *Tenacibaculum* genus by Suzuki et al. (2001). There were some early signs pointing towards the involvement of *T. maritimum* in BC Atlantic salmon infections regarding the presence of the filamentous bacteria on lesions (Ostland et al., 1999). However, *T. maritimum* was only confirmed to have a significant role upon experimental induction of tenacibaculosis in Atlantic salmon by a bath infection model (Frisch et al., 2018a; Frisch et al., 2018b). Although, current literature suggests a capacity of other *Tenacibaculum* spp. to be responsible for tenacibaculosis infections, resulting in added complexity around this disease (Nowlan et al., 2021c). Strong evidence of *T. maritimum* involvement was found by Wynne et al. (2020), that identified the microbial profiles of Atlantic salmon mouth rot lesions and determined *T. maritimum* to be the dominant bacterial species in the lesion of infected fish.

1.3.3.1 *Type IX secretion system*

A probable key facilitator for the virulence, gliding motility, and biofilm formation of *T. maritimum* is the assembly of a T9SS encoded in its core genome. The genome annotation by Pérez-Pascual et al. (2017) identified its existence and the secretome analysis by Escribano et al. (2023) confirmed its presence on the outer bacterial membrane. Unfortunately, the T9SS is one of the more recently discovered bacterial secretion systems resulting in a limited pool of knowledge on its mechanism and function. Further, the majority and origin of T9SS research has been done on the human pathogen, *Porphyromonas gingivalis* (Nakayama, 2015; Sato et al. 2010). The closest research to the *Tenacibaculum* genus is on the fish pathogens *Flavobacterium johnsoniae* (Kharade and McBride, 2015) and *Flavobacterium psychrophilum* (Barbier et al., 2020). The T9SS is broadly present across the Bacteroidetes clade and is formerly known as the Por secretion system (Paillat et al., 2023). Early studies associated this secretion system with bacterial motility, the secretion of proteins, and pathogenesis (Sato et al., 2010).

The T9SS has now been confirmed to have implications in several different bacterial functions, first and foremost being the gliding motility of the bacterium through the cell surface adhesions, SprB (Nelson et al., 2008) or RemA (Shrivastava et al., 2012). This movement mechanism uses the proton motive force to rotate and move the bacteria (Nakane et al., 2013), but it requires critical cytoplasmic membrane proteins for gliding motility function (GldK, GldL, GldM, and SprA) (Shrivastava et al., 2013). Secondly, the T9SS is involved in the secretion of various proteins across the outer membrane of the bacteria, such as soluble virulence factors (such as gingipains) (Benedyk et al., 2019) and proteins destined to be anchored to the outer bacterial membrane (Seers et al., 2003). Further, all the secreted proteins have conserved C-terminal domains (CTD) to allow for trafficking across the outer membrane by the T9SS (Veith et al. 2013;

Kulkarni et al., 2017). Proteins also contain a conserved N-terminal signal domain for trafficking across the cytoplasmic bacterial membrane using the Sec translocation protein (Xie et al., 2022), although this is not T9SS specific since the Sec protein is rather universally present for protein exportation (Tsirigotaki et al., 2017). Salmonid infection trial evidence, using T9SS component knockouts, revealed that the secretion system is an integral part of the pathogenesis of *Flavobacteriaceae* (Barbier et al., 2020; Li et al., 2017). Lastly, the T9SS is also involved in biofilm formation, likely due to it being essential for protein secretion and cell motility (Eckroat et al., 2021). All of this is relevant since the *T. maritimum* genome encodes the Gld, Spr, and Por T9SS proteins critical to the noted pathogen properties of gliding motility, protein secretion, and biofilm formation (Pérez-Pascual et al., 2017).

1.3.3.2 *Potential virulence factors*

Several potential virulence factors were identified in the *T. maritimum* NCIMB 2154 (type strain) genome annotation by Pérez-Pascual et al. (2017) including: chondroitin AC lyase, sphingomyelinase, ceramidase, sialidase, hemolysins, and other peptidases. This encompasses a wide list of potential soluble factors that may be used by the bacterium for involvement in host cell lysis, tissue degradation, and other pathogenesis mechanisms (Pérez-Pascual et al., 2017). These potential virulence factors were not just seen in the *T. maritimum* NCIMB 2154 strain, but also from Canadian *T. maritimum* isolates as well (Nowlan et al., 2023). In the study by Nowlan et al. (2023), the presence of an M12B Zinc metalloprotease with a T9SS CTD was identified in the core genome. Metalloproteases with a Zinc subunit can be utilized by opportunistic pathogens due to their versatility in cleaving host proteins (Miyoshi and Shinoda, 2000). Therefore, *T. maritimum* does have a repertoire of potential virulence factors that it could use against salmonid

host cells. Although, the influence and impact on pathogenesis from these *T. maritimum* produced products is unknown.

1.3.4 *Environmental conditions*

The temperature, salinity (ppt), and oxygen content of the Ocean varies depending on the water depth, the season, or the year (Alexander et al., 2001; Stramma and Schmidtko, 2021). Surprisingly, these differences in the environment may have an impact on disease through its influence on the hosts or on the pathogenic bacteria, which is discussed in detail in the following sections. The only exception is oxygen content, which is less of a worry regarding its impact on disease. Although, some literature points to the effect of hypoxic conditions inducing stress in fish, which could result in negative effects on the host immune system (Niklasson et al., 2011; Kvamme et al., 2013).

1.3.4.1 *Salinity*

The impact of salinity on the immune system of teleosts is rather understudied when compared to the impact of temperature. A review by Makrinos and Bowden (2016) highlighted some studies that saw changes to phagocytosis, proliferation of immune cells, and an inhibition of the adaptive immune system when salinity was adjusted. However, there was no clear association that heightened salinity was better or worse for the immune system of teleosts, only that changes to salinity could cause stress and additional energy expenditure depending on the species (Makrinos and Bowden, 2016). The prominent diseases often differ greatly in effectiveness between salinity levels, with the common theme being that the pathogens do worse outside of their

natural range. For example, the treatment for Atlantic salmon infected with amoebic gill disease is actually a freshwater bath (Parsons et al., 2001). It has also been shown that preventing freshwater *Flavobacterium columnare* salmonid infection can be done by adding salt to the water, inturn reducing the total number of viable bacteria and its growth rate (Suomalainen et al., 2005; Soltani et al., 1996). The differences between diseases in fresh (low salinity) and salt (high salinity) water are likely because they are caused by distinct pathogens and less so to do with the hosts immune responses.

An anonymous source in Ostland et al. (1999) states that a potential disease requirement for mouth rot is a salinity above 30‰ (or 30 ppt). This assessment is echoed by Soltani et al. (1996), which showed that in Atlantic salmon *T. maritimum* infection mortalities in 33–35‰ salinity water was much higher than the same experiment in 15‰ salinity water, despite a greater initial bacterial load. In Canadian waters near BC the mean water salinity is 23.44 ppt but the range goes from 0 ppt to 33 ppt (Thakur et al., 2018). Therefore, Canadian waters do range past the supposed 30‰ lower salinity limit for mouth rot disease, which may help to explain the timing for disease outbreaks.

Interestingly, recent literature suggests that bacteria may be able to use quorum sensing to detect salinity changes and alter gene expression. A study by Bjelland et al. (2012), proposed that the gene *litR* in *Aliivibrio salmonicida* is involved in regulating virulence factors and biofilm formation to enhance the bacterial pathogenesis depending on the salinity. The capacity for any *Flavobacteriaceae* to alter its pathogenicity depending on environmental conditions is not yet known but should be investigated.

1.3.4.2 *Temperature*

Temperature can affect hosts and pathogens differently depending on unique adaptations to or detriments of the water condition. For example, there may be an impairing effect that lower water temperatures have on the salmonid immune system (Páez et al., 2021; Köllner and Kotterba, 2002). However, this is likely circumstantial, depending on the species of fish, degree of temperature change, the pathogen, and host response pathway (see review by Abram et al., 2017). An example of higher disease at lower temperatures would be the outbreaks of cold-water vibriosis in Norway, which occurred when water temperatures were below 10°C despite the causative agent *Aliivibrio salmonicida* (previously *Vibrio salmonicida*) having a higher optimal growth temperature (Enger et al., 1991). Further, this same trend was shown with *Flavobacterium psychrophilum* as well, regarding the highest bacterial cold water disease mortality in the 12–15°C range (Holt et al., 1989). Temperature has been shown to have effects on the gene expression of virulence factors in numerous aquatic bacterial pathogens, using litR gene (identified in *Aliivibrio*) and the two-component system (identified in the *Flavobacteriaceae* family, containing *F. psychrophilum*) (see review by Guijarro et al., 2015). It is difficult to determine whether increases to the virulence of the bacterial pathogen is more impactful than the depression of the host immune system at lower temperatures unless each factor is independently studied.

Outbreaks of Tenacibaculosis infections (involving *T. maritimum*) have been documented to occur at aquaculture sites with lower water temperatures in both Canada (9–14°C) (Frisch et al., 2018c) and Norway (12°C) (Småge et al., 2016b). However, outbreaks in other regions of the world document *T. maritimum* associated tenacibaculosis infections at higher temperatures, such as in Chile (>16°C) (Valdes et al., 2021) and New Zealand (>16°C) (Kumanan et al., 2024b). When the literature on tenacibaculosis is expanded to include infection trials, then the range of

water temperature is even wider. For example, *T. maritimum* associated tenacibaculosis infections in Senegalese sole (*Solea senegalensis*) only occurred at 26°C and not at 16°C (Mabrok et al., 2016). Likewise, in a study by Yamamoto et al. (2010), infection of Japanese Flounder (*Paralichthys olivaceus*) with *T. maritimum* only occurred from 17–26°C and not above or below this temperature range. Therefore, given the articles above, there is no consensus in the literature regarding the effect that temperature may have on the tenacibaculosis disease and its associated bacteria.

1.3.5 *Other bacteria of interest*

The current understanding of tenacibaculosis is of a dysbiosis event potentially caused by multiple bacteria. This is supported with the isolation of different tenacibaculosis associated bacteria from the ulcers of infected Atlantic salmon, such as *T. dicentrarchi* (Klakegg et al., 2019; Avendaño-Herrera et al., 2016), *T. maritimum* (Apablaza et al., 2017; Frisch et al., 2018c), *T. finnmarkense* (Spilsberg et al., 2022; Småge et al., 2016a), and *T. ovolyticum* (Nowlan et al., 2022). The ability for some of these bacteria (*T. dicentrarchi*, *T. maritimum*, and *T. finnmarkense*) to induce tenacibaculosis infections has also been demonstrated with bath emersion trials (Nowlan et al., 2021a). The genetic composition of these bacteria can be quite variable between the species, which may mean that different virulence factors or a limited pool of shared factors may be responsible for pathogenesis. All of these bacteria use a T9SS, which is believed to have a potential role in virulence (as stated in an earlier section). However, the mechanism for how each bacteria induces tenacibaculosis and if there are any fundamental differences is not yet known.

Co-infections with other bacteria may also contribute to the pathogenicity of mouth rot infections. Evidence towards co-infections in ulcerative diseases can be found in winter ulcer disease (caused by *Moritella viscosa*) often being associated with *Aliivibrio wodanis* at the site of the infection (Benediktsdóttir et al., 2000). The *A. wodanis* bacterium was believed to contribute to the infection through the production of cytotoxic extracellular products, but in actuality, the co-infection trial mortality rates show that the interaction is much more complicated (Karlsen et al., 2014). Interestingly, the infection mortality rates were reduced when Atlantic salmon had exposure to *A. wodanis* before *M. viscosa* (Karlsen et al., 2014). The authors of this study believed that there may be competition between the two bacteria, leading to the *M. viscosa*-only infection being more lethal (Karlsen et al., 2014). This was shown in a co-growth experiment, where *A. wodanis* outcompeted *M. viscosa* at an even ratio of starting bacteria (Hjerde et al., 2015). Interestingly, *A. wodanis* has also been isolated from mouth rot lesions. Therefore, it is possible and likely that multiple bacteria are contributing to the ulcers on mouth rot and tenacibaculosis infections. For example, the number/quantity of Vibrio species on Atlantic salmon skin have been shown to significantly increase during a mouth rot infection (Wynne et al., 2020). Further, the repeated isolation of multiple other *Tenacibaculosis* species from mouth rot infections, and the infection trial evidence (Nowlan et al., 2021a) implies involvement in infection. The cytotoxicity of the soluble extracellular products from these *Tenacibaculum* species are currently not known, despite its potential participation in tenacibaculosis disease.

1.3.5.1 *Tenacibaculum ovolyticum*

Tenacibaculum ovolyticum formerly [*Flexibacter ovolyticus*] (Suzuki et al., 2001) was first isolated from halibut eggs and larvae as a suspected pathogen (Hansen et al., 1992). Some studies

(Nowlan et al. (2023) and Habib et al. (2014)) have grouped *T. ovolyticum* into the *Tenacibaculum* genome with bacteria such as *T. finnmarkense*, *Tenacibaculum soleae* (Pineiro-Vidal et al., 2008), *Tenacibaculum piscium*, and *T. dicentrarchi*. Interestingly, all of the closely related bacteria listed are potential aquatic pathogens (see above references). The complete genome of *T. ovolyticum* was sequenced by Teramoto et al. (2016), which revealed a T9SS and potential virulence factors. *T. ovolyticum* has also been isolated from mouth rot infections, but not in every infection as it does not appear to be a pathogenesis driver (Nowlan et al., 2022). Although, the authors also assessed that more outbreaks should be tested to determine if *T. ovolyticum* is involved in mouth rot infections, or if only under the right circumstances. Unfortunately, *T. ovolyticum* has not been much of a focus for the international community, resulting in a lack of sequencing and experimental data of the bacteria. However, Nowlan et al. (2023) showed that there are iron acquisition siderophores and potential virulence factors encoded in the genome. Therefore, there is a gap in the *in vitro* experimental data on the virulence potential towards salmonid cells regarding this bacterium.

1.3.5.2 *Tenacibaculum dicentrarchi*

Tenacibaculum dicentrarchi was first suggested to be a pathogen when it was isolated and identified from a diseased European sea bass (*Dicentrarchus labrax*) by Piñeiro-Vidal et al. (2012). It has since been found on tenacibaculosis infected Atlantic salmon from Chile (Avendaño-Herrera et al., 2016), Norway (Klakegg et al., 2019), and Canada (Nowlan et al., 2021b). Infection trials by Nowlan et al. (2021a) have also demonstrated that this bacterium can induce tenacibaculosis disease in Atlantic salmon. Complete genome sequences have revealed numerous potential virulence factors and a T9SS, providing a foundation for pathogenesis (Grothusen et al., 2016).

Although the same virulence factors identified in *T. maritimum* are not identified in the *T. dicentrarchi* genome, suggesting a distinct mechanism for virulence (Bridel et al., 2018). The extracellular products secreted by *T. dicentrarchi* have not been examined on a functional level, which leaves a gap in the understanding of this pathogen.

1.3.5.3 *Cellulophaga baltica*

Cellulophaga baltica was isolated from seawater (just outside of the Baltic Sea) and was associated with a marine benthic macroalga, *Fucus serratus* (Johansen et al., 1999). *C. baltica* had catalase activity and diverse carbohydrate metabolism, making it more fit the characteristics of a symbiotic microbe to the algae (Johansen et al., 1999). It was also phylogenetically placed in the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, which also contains the *Tenacibaculum* genus. The virulence potential from this bacterium is unknown, but it has been isolated from a mouth rot infection (this study) and should be investigated.

1.4 Summary

Atlantic salmon aquaculture is a promising field of food production, and industry disease management. However, there are still problems with mouth rot disease that affects the industry's bottom line, and public perception. Despite the wealth of literature on the etiological agent of mouth rot disease, *T. maritimum*, much has yet to be done for functional analysis of this bacterium. The mechanism for pathogenesis is still unknown, as well as its virulence under different environmental conditions. Various genome annotation work has proposed different potential virulence factors, but minimal has been done on determining the factors responsible. Furthermore, there are various related fish pathogens (such as *T. dicentrarchi* and *T. ovolyticum*) that could

contribute to mouth rot disease but have little to no functional assessment of their pathogenic mechanisms. Overall, the literature has some striking gaps in the understanding of the various *Tenacibaculum* pathogens, that could be resolved with some *in vitro* experimentation on disease associated isolates.

Chapter 2: Materials and methods

2.1 Bacteria

2.1.1 *Isolation methods*

The environmentally isolated bacteria (*T. maritimum*, *T. dicentrarchi*, *T. ovolyticum*, and *C. baltica*) were obtained from a lesion on a deceased Atlantic salmon with mouth rot symptomatology. These bacteria were isolated by industry partners at Grieg Seafood Ltd. from associated net pen sites in Vancouver Island, British Columbia, Canada. The bacteria were purified and had the species identified first by the Grieg Fish Health team before being shipped to the Barreda lab where further confirmation and sequence type (ST) identification was done. All isolated bacteria were grown on Flexibacter marine media (FMM) supplemented with kanamycin (**Table 2.0**). Freezing conditions for all bacteria consisted of 15% glycerol (Sigma CAT: G8773-500) with storage in -80°C. Numerous aliquots were made to prevent freeze/thaw cycles. Standard broth culture growth was performed in a shacking incubator (Innova 4000 New Brunswick Scientific) set to 220 rpms with 50 mL of culture in a 125 mL Erlenmeyer flask.

2.1.2 *Determination of CFU/mL and MPN/mL*

All bacteria, except *T. maritimum*, were enumerated after incubation using a standard plate count method to determine CFU/mL. Optical density (OD) of the cultures were taken on a spectrophotometer (ThermoScientific Genesys 30). Serial dilutions of the bacteria were performed and then 5 µL from each dilution was dropped onto a different section of the agar plate. The plates were incubated for 1–3 days. This resulted in a small number of countable colonies where the 5 µL was dropped. This is referred to as the spot-plate bacterial enumeration method (Gaudy Jr. et al., 1963). The counted colonies (CC) on the plate, the serial dilution factor (DF), and the volume

added to the agar plate (0.005 mL) were used to get the CFU/mL of the culture. This was done using the equation below. Three replicates of CFU/mL were averaged to enumerate the bacteria.

$$\frac{CC * DF}{0.005} = CFU/mL$$

The most probable number (MPN) enumeration method was used to determine the number of live bacteria for *T. maritimum* due to the distinct lack of countable colonies the bacteria forms on FMM agar plates. The MPN method was conducted according to Frisch et al. (2018a), with a minor change to 5 replicates per dilution instead of 8. Round bottom 96 well plates (Corning™ CAT: 07-200-95) were inoculated with the diluted culture at 100 µL/well and were incubated at 18°C on a cold shaking incubator (Innova 4300 New Brunswick Scientific) set to 80 rpm. Counts of wells positive for growth were made 5 days after the start of incubation, in which case the table and dilution values were used to determine the MPN/mL of the culture (Blodgett, 2010). The enumeration with the MPN method is to be considered equivalent to the CFU determination (Gronewold & Wolpert, 2008). All determined bacterial enumeration values at every experimental inoculate and endpoint are documented with all 3 replicates, mean, and standard deviation in **Table 2.1**.

2.1.3 *Identification and sequencing*

The isolated bacteria were identified on a species level using 16s ribosomal DNA, with universal 27F and 1492R primers from Nowlan et al. (2023). All genomic DNA was collected with the PureLink Genomic DNA Mini Kit (Invitrogen Cat: K1820-00) according to manufacturer instructions (Gram-negative bacterial lysate protocol). Further, ST identification was done with

the annealed housekeeping (HK) gene primers (atpA, dnaK, gyrB, glyA, infB, rlmN, and tgt) from Olsen et al. (2017) and a PCR cycle amplification cycle performed according to the original protocol from Habib et al. (2014) in a Nexus Gradient Thermocycler (Eppendorf Cat: 05414456). All amplification products were purified using the QIAquick PCR Purification Kit (Qiagen Cat: 28104), before using forward and reverse Sanger sequencing at the MBSU at the University of Alberta. The MEGAX alignment tool aligned the sequences to existing HK sequences taken from GenBank to identify exact nucleotide differences between all *Tenacibaculum* ST. Novel nucleotide differences in housekeeping gene sequences were identified and submitted to NCBI, accompanying a new strain name for the *Tenacibaculum* isolate. All primers used in this work, including sequences (5' to 3') and references, are accessible in **Table 2.3**.

2.1.3.1 *Tenacibaculum maritimum TmarCan2*

T. maritimum was grown in FMM with kanamycin and at a preferred bacterial growth temperature of 18°C. The *T. maritimum* isolate was determined to have a 16S ribosomal RNA sequence similarity of 99.62% to the TmarCan2 isolate described in Frisch et al. (2018b). Further, the *T. maritimum* isolate was determined to have the same ST as TmarCan2 from Frisch et al. (2018b), with 100% similarity to all seven HK genes that were aligned.

2.1.3.2 *Tenacibaculum dicentrarchi VI23*

T. dicentrarchi was grown in FMM with kanamycin and at a preferred bacterial growth temperature of 18℃. The *T. dicentrarchi* VI23 isolate was determined to have a 16S ribosomal RNA sequence similarity of 99.85% to the strain *T. dicentrarchi* QCR46. *T. dicentrarchi* VI23 had nucleotide differences in the HK genes dnaK and gyrB. Whereas the 100% sequence similarity to

the other HK genes were spread across multiple different *T. dicentrarchi* strains. These partial codon sequences for distinct HK genes are available on GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/).

2.1.3.3 *Tenacibaculum ovolyticum WC22*

T. ovolyticum was grown in FMM with kanamycin and at a preferred bacterial growth temperature of 18°C. The *T. ovolyticum* WC22 isolate was determined to have a 16S ribosomal RNA sequence similarity of 100% to several *T. ovolyticum* strains. *T. ovolyticum* WC22 had nucleotide differences in the HK genes glyA, gyrB, and rlmN. These partial codon sequences for distinct HK genes are available on GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/).

2.1.3.4 *Cellulophaga baltica*

C. baltica was grown in FMM with kanamycin at a temperature of 23-24°C. The *C. baltica* isolate was determined to have a 16S ribosomal RNA sequence similarity of 99.93% to several *C. baltica* strains. Sequencing of HK genes was not performed as determination of the *C. baltica* strain would be inconsequential to the aims of the project.

2.1.3.5 *Escherichia coli k12*

The *E. coli* was gifted to us by Dr. Tracy Raivio for use as a negative control for secreted bacterial products. *E. coli* was grown in LB broth at 23-24°C. Sequencing of the 16S ribosomal RNA gene and HK alleles were not performed due to the *E. coli* strain already being known and was not an isolate from the environment.

2.1.4 *Collection of secreted bacterial products*

Bacteria were grown up to a specific concentration (CFU/mL) or for an allotted amount of time depending on the experiment. Isolation of supernatants (containing the soluble extracellular bacterial products) was done by centrifugation at 3000xg for 15 min (x2) at 23-24°C. Following the removal of bacteria, the supernatant was filter sterilized with a 0.22 μm low protein binding syringe filter (Millex PVDF, Millipore Sigma Cat: SLGVR33RS). Filtered supernatants were spread on agar and allowed to incubate for 5 days to confirm a lack of live bacteria in the soluble extracellular products (ECP). The supernatants were frozen at -80°C in 1 mL aliquots to preserve secretion contents and prevent loss of function due to freeze/thaw cycles.

2.2 Fish cell lines

2.2.1 *Cell culture maintenance*

Fish cell lines were continuously cultured in T75 cm^2 closed capped corning flasks (Corning Cat: 430720U). Cell culture media consisted of Leibovitz's L-15 Medium (Gibco Cat: 41300, phenol red) supplemented with 10% heat-inactivated Fetal bovine serum (FBS) (Gibco Cat: 12483) and 1% Pen-Strep (Gibco Cat: 15140). All standard cell line growth and treatment conditions were in a room temperature incubator (23-24°C) under atmospheric conditions. Cells were typically grown to 90% confluency before use in experiments. Cells were frozen with 5% Dimethyl sulfoxide (DMSO) (Sigma Aldrich Cat: D2650) in liquid nitrogen for long term storage. Cells were harvested using Trypsin 0.05% Ethylenediaminetetraacetic acid (EDTA) (Gibco Cat: 25300, phenol red) and were counted with a hemocytometer for consistent cell density in all of our experiments.

2.2.2 *ASimf20*

The Atlantic salmon intestinal myeloid fibroblast cell line (ASimf20) was graciously gifted to us by Dr. Brian Dixon and came from Dr. Niels Bols in origin (Kawano et al., 2010). These adherent cells were grown under the above stated conditions with passage numbers typically around 7 days when passed 3:1 from full confluency using Trypsin 0.05% EDTA. No changes in cellular morphology or the cellular responses to bacterial products were seen over the course of ASimf20 cell line use. An example image of the fibroblastic morphology is in **Fig 2.1 A**. The ASimf20 cell line was used as a model to represent Atlantic salmon cells from mucosal epithelial tissue. These cell lines, despite being isolated from the gut, are very critical to be examined since the skin epithelial layer is the first place the bacterium establishes itself in natural infections. To our knowledge, there are no such salmonid epithelial skin cell lines, so the gut was chosen. Rainbow trout and Atlantic salmon epithelial gill cell lines were considered but ultimately turned down for a cell line that is more robust for examining cell death. An example of the tissue similarities would be the skin and gut mucosal epithelial tissues of fish responding to immune challenge in a conserved manner (Xu et al., 2013). The viability of these Atlantic salmon cells in response to the co-incubation of secreted products from the *Tenacibaculum* genera is of interest to the industry, which was why this cell line was chosen.

2.2.3 *RTgutGC*

The rainbow trout intestinal epithelial cell line (RTgutGC or short form RTgut) was graciously gifted to us by Dr. Brian Dixon and came from Dr. Niels Bols in origin (Kawano et al., 2011). These cells were grown in the above stated conditions with passage numbers typically around 10 days when passed 3:1 from full confluency. No changes in cellular morphology or the

cellular responses to bacterial products were seen over the course of RTgutGC cell line use in this study. An example image of the round epithelial cell morphology is in **Fig 2.1 B**. The RTgutGC cell line was chosen to represent a mucosal epithelial cell from a related salmonid species. The potential for different salmonid species to have varied cellular responses to the co-incubation of *T. maritimum* ECP is of interest to the aquaculture industry, which was why this cell line was chosen.

2.2.4 *SHK-1*

The salmonid head kidney cell line (SHK-1 or short form SHK) was graciously gifted to us by Dr. Brian Dixon and came from Norway in origin (Dannevig et al., 1997; Dannevig et al., 1995). These cells were grown in the above stated conditions and were typically passaged every 5 days. No changes in cellular morphology or changes in responses to bacterial products were observed over the course of the study. An example image of cell morphology is in **Fig 2.1 C**. The SHK-1 cell line is a macrophage enriched leukocyte culture from Atlantic salmon allowing for the assessment of ECP on cells of the immune system. Further, it is important to examine more than just epithelial cells from Atlantic salmon, and to assess ECP cytotoxicity in other salmonid cell types.

2.3 Cell assays

2.3.1 *Annexin V/PI cell death differentiation*

The flow cytometry-based annexin V/propidium iodide cell staining protocol was used to assess the induction of apoptotic and necrotic cell death. Experimental cell culture densities consisted of seeding 2.5 x 10^6 salmonid cells in a corning T25 cm^2 flask, followed by adherence overnight at RT. ASimf20 cells underwent a 24 h treatment with 25% (v/v) of either FMM, *E. coli*

supernatant (OD_{600} = 0.5, 3.33 x 10^8 CFU/mL), or *T. maritimum* supernatant (OD_{600} = 0.5, 7.93 x 10^8 MPN/mL) (**Table 2.1**). Controls for apoptosis consisted of cells treated with cycloheximide (Alfa Aesar Cat: J66901) diluted to 10 μg/mL in cell culture media for a treatment lasting 24 h, whereas necrosis was induced with a 56℃ water bath for 1 min after cell harvest. Cells underwent a 15 min incubation in 2 mL of L15 at 4℃ to allow for the reconstitution of the cell membrane after harvesting via trypsinization. ASimf20 treated cells then underwent the annexin V/propidium iodide staining procedure according to Rieger et al. (2011), using annexin V fluorescein isothiocyanate (annexin V FITC, Invitrogen Cat: A13199) as the marker for apoptosis and propidium iodide (PI, Sigma Aldrich Cat: P4864-10mL) as the marker for necrosis. Modifications to the procedure included adjustments to the centrifugation steps to 330xg for 8 min and for longer incubation periods L-15 (without 10% FBS or 1% Pen strep) was used in the place of 1x PBS (**Table 2.2**) to minimize the death of viable cells during the assay. Cells and events were counted using the ImagestreamX Mark II flow cytometer (Amnis) with 10'000 events collected at 43x magnification. The RTgut cells underwent the same experimental procedure as the ASimf20 cells with the substitution of annexin V Pacific Blue (annexin V PB, Invitrogen Cat: A35122) to mark apoptosis instead of annexinV FITC. This was done to negate autofluorescence in the 488 channel from the RTgut cells (Note: annexin V FITC and annexin V PB measure the exact same marker of apoptosis but have different Ex/Em values). For each cell line analyzed, the experiment was independently replicated four times. A graphical depiction of the treatment of salmonid cells was included (**Fig 2.2**), from the time of ECP isolation to the annexin V/PI assessment endpoint. Gating strategies for collected cells, including verification of positive controls, are shown in **Fig 2.3**.

2.3.2 *Alamar blue & CFDA cell viability measurement*

Two fluorescent dyes, Alamar blue (AB, Invitrogen Cat: DAL1025) and 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM, Invitrogen Cat: C1354), were used as general cellular viability markers. Cells were seeded at a density of 1.0 x 10^4 cells/well in a 96-well tissue culture plate (ThermoScientific, Cat: 130188) and adhered overnight at 23–24°C. Cell monolayers were then treated in six-fold with either bacterial supernatants or media controls (diluted to either 25% or 50% (v/v) of the treatment in cell culture media). Following a 24 h treatment incubation at 23–24°C, each well was washed once with PBS before addition of AB and CFDA-AM as described previously by Semple et al. (2022). After 1 h of incubation at 23–24°C, the 96-well plate was read at an excitation of 560 nm and an emission of 590 nm for AB as well as an excitation of 480 nm and an emission of 520 nm for CFDA-AM. The plate reads were completed using a Synergy H1 plate reader (BioTek Instruments). For each cell line analyzed, three independent experimental replicates were performed, which contained six technical replicates per 96 well plate. The AB/CFDA protocol was visually illustrated in **Fig 2.4**.

2.4 Environmental conditions impact *T. maritimum*

2.4.1 *Effects of salinity on growth and secretion*

Ocean salinity was tracked by Grieg Seafoods Ltd. at 4 different West Coast Canadian sites to obtain an accurate range of ocean salinity for replicating environmental conditions *in vitro*. This range was determined to be between 25–33 ppt when the data from 5m and 10m depths are examined wholistically (**Fig. 2.5**). The salinity of FMM was adjusted in 3 conditions to cover this relevant range (Low: 25 ppt, Med: 29 ppt, High: 33 ppt). *T. maritimum* was grown at 18°C in each respective salinity conditions. After 48 hours, the MPN/mL of the culture was determined (**Table**

2.1), and the soluble ECP were harvested as described earlier. ASimf20 cells were treated with 25% (v/v) ECP for 24 h and underwent the AB/CFDA assay as previously described. The experiment was independently replicated three times.

The supernatant from these conditions was normalized to account for differences in the bacterial numbers. ECP from the various salinities were diluted to the lowest measured MPN/mL value, which happened to be the lowest salinity (25 ppt). ASimf20 cells were treated with the diluted supernatant at 25% (v/v) for 24 h and underwent the AB/CFDA assay as previously described. The experiment was independently replicated three times.

2.4.2 *Effects of temperature on growth and secretion*

The influence of temperature on the secretion of *T. maritimum* bacterial products was examined with the use of refrigerated incubators set at 12°C, 18°C, and 24°C. *T. maritimum* grown at 23–24°C was used to inoculate experimental cultures at an OD_{600} of 0.2, 1.25 x 10^8 MPN/mL. After 48 h of shaking in the respective refrigerated incubator MPN/mL growth measurements were taken (**Table 2.1**) and bacterial supernatants were harvested as previously described. ASimf20 cells were treated with the various supernatants at 25% (v/v) for 24 h before the cells underwent a CFDA/AB assay, as previously described. The experiment was independently replicated three times.

The supernatants from various temperatures were diluted to the lowest MPN/mL value, which corresponds to the highest temperature (24°C). This results in a normalization to account for growth differences. ASimf20 cells were treated with 25% (v/v) diluted supernatant (normalized for bacterial growth) for 24 h and underwent the AB/CFDA assay as previously described. The experiment was independently replicated three times.

2.5 Biofilm development

2.5.1 *Crystal violet quantification assay*

A crystal violet (CV) assay was used to measure the capacity of *T. maritimum*, *T. ovolyticum*, and *T. dicentrarchi* to form biofilms. The procedure described below was based on an article by Niu & Gilbert (2004). Modifications were made to closely reflect the current study's bacterial growth conditions. These bacteria were grown to an OD_{600} of 0.2 before being transferred in 20 mL aliquots to 50 mL Erlenmeyer flasks. Biofilm measurement timepoints were 0, 24, and 48 h after the transfer to new flasks. Each timepoint had 1 mL of broth culture set aside for OD_{600} measurement before 1.25 mL of 0.3% CV (BD CAT: 212525) was added. After 15 min in the shaking incubator, the flasks were washed 7x with 30 mL of dH_2O before the final rinse with 25 mL of ethanol:acetone (80:20) to release the CV from the biofilm. The collected CV was measured at OD_{570} with a spectrophotometer (ThermoScientific Genesys 30). An abiotic control of FMM broth alone was also performed in the same manner as described above. The specific biofilm formation (SBF) for each bacterium was calculated as described previously in Niu & Gilbert (2004). The SBF value is just a calculation that refers to the amount of CV released by the biofilm (a larger biofilm releases more CV which results in a higher SBF; whereas no biofilm will release no CV and will have a low SBF). This number is obtained for the purpose of quantitatively comparing the capacity to form biofilms between the *Tenacibaculum* species. Images were taken at the 48 h timepoint to visually illustrate the biofilm differences between the three *Tenacibaculum* species. The CV assay was independently replicated three times.

2.6 Protein

2.6.1 *Denaturation of secreted products*

Secreted soluble products were obtained from the *T. maritimum* bacterial culture of an OD_{600} of 0.9 and an enumeration of 1.13 x 10^9 MPN/mL (**Table 2.1**). The secreted products were submerged in 100°C water for 10 minutes to functionally denature all proteins in the solution. ASimf20 cells were treated with 25% (v/v) boiled and non-boiled supernatants for 24 h before the cells underwent a CFDA/AB assay, as previously described. The experiment was independently replicated three times.

2.6.2 *Protease inhibitor cocktail and EDTA*

Soluble ECP from *T. maritimum* ECP (0.5 OD, 18°C **Table 2.1**) were treated with varying doses of Halt™ Protease Inhibitor Cocktail, EDTA-free (100X) or 0.5M EDTA (100X) (Thermofisher Cat: 87786). A list of all protease inhibitors, inhibitor types, and concentrations are obtained from the product user guide, and is made available in **Table 2.4**. The acting concentrations for P.I. and EDTA were scaled down to 0.01X, 0.1X, 1X, 2X, and 3X for determination of the dose-response and the optimal concentration associated with minimal cytotoxicity but the most inhibition. An example dilution for a 1X EDTA treatment would be adding 2 µL of 0.5M EDTA (100X) to 200 µL of *T. maritimum* supernatant. After a 5 min incubation, 600 µL of cL15 was added and SHK cells were treated with the 25% supernatant mixture with 1X EDTA. Incubation occurred for 24 h before the general cellular viability was measured with the CFDA/AB assay, as previously described. The experiment was independently replicated three times.

After the *T. maritimum* P.I. and EDTA experiment, *Tenacibaculum* spp. ECP were also treated with P.I. or EDTA at the 2X concentration. These treated and non-treated ECP were incubated with SHK cells before the general cellular viability was measured with the CFDA/AB assay, as previously described. The experiment was independently replicated three times.

2.6.3 *SDS-PAGE gel prep*

Preparation of the SDS-PAGE protein gels were created in accordance with Barreda lab protocols (**Table 2.5**). An 8% polyacrylamide gel and 4% stacking gel was used in all 1 mm 10 well experimental gels. The 5 µL of protein ladder used per gel was an unstained broad range ladder (Thermofisher Cat: 26630). Bacterial ECP were heat-treated at 100°C for 10 min with 4X Laemmli loading dye (Bio-Rad Cat: 161-0747) and 2-Mercaptoethanol (BME) (10X) (EMD Millipore Cat: 6010-100mL) (note that the resulting concentration was 1X). After, 35 µL of the denatured protein was loaded into the well. The protein gel was run for 30 min at 90 V to stack the protein, followed by 40 min at 170 V to create protein band separation.

2.6.4 *SYPRO ruby protein gel stain*

The total protein in the gel was stained with SYPRO Ruby (Invitrogen Cat: S12000) according to manufacturer instructions and visualized on a ChemiDoc fluorescent gel imager (Bio-Rad Cat: 12003153) at the 460 nm excitation setting. An unstained protein ladder was used to aid in determining the protein band size for all supernatant fractions. According to the product information, SYPRO Ruby is sensitive down to 1 ng of protein as a lower detection limit (lower than Coomassie) with much less background staining compared to silver staining. Further, it can

stain most classes of protein, including glycoproteins. Altogether, this makes it a good stain for examining ECP protein gel bands.

2.7 Analysis

Imagestream flow cytometry data was analyzed with the Ideas V6.2 (Amnis, EMD Millipore) software. Statistics were performed on GraphPad Prism V10. For statistics involving more than two data comparisons, a one-way analysis of variance (ANOVA) was used. A post-hoc Tukey's test was used for pair-wise comparison analysis to all data sets. A two-way ANOVA was used for assessing the effects of EDTA and Protease inhibitors (P.I.), as well as differences between the negative control and bacterial supernatants under these treatments. A Šídák's multiple comparisons test was used for the pair-wise comparison analysis across all data sets.

2.8 Figures and tables

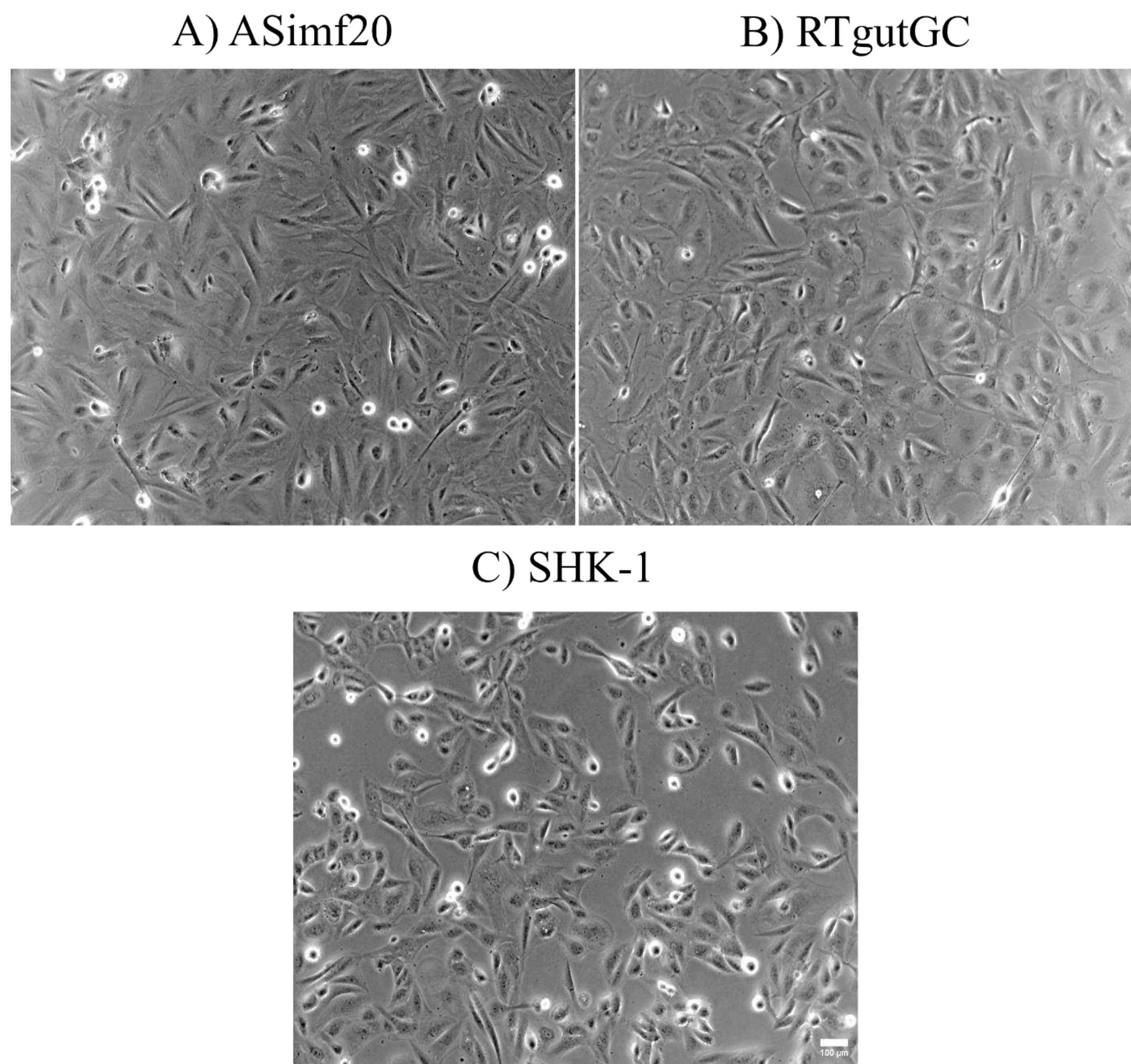


Fig 2.1 Cell line images of ASimf20 (A), RTgutGC (B), and SHK-1 (C). The scale bar is in bottom right corner and is set to 100 µm. Images taken on 43x magnification. All cell lines were seeded to 4 x 10^6 cells/well in a 12 well plate 24 hours before images were taken.

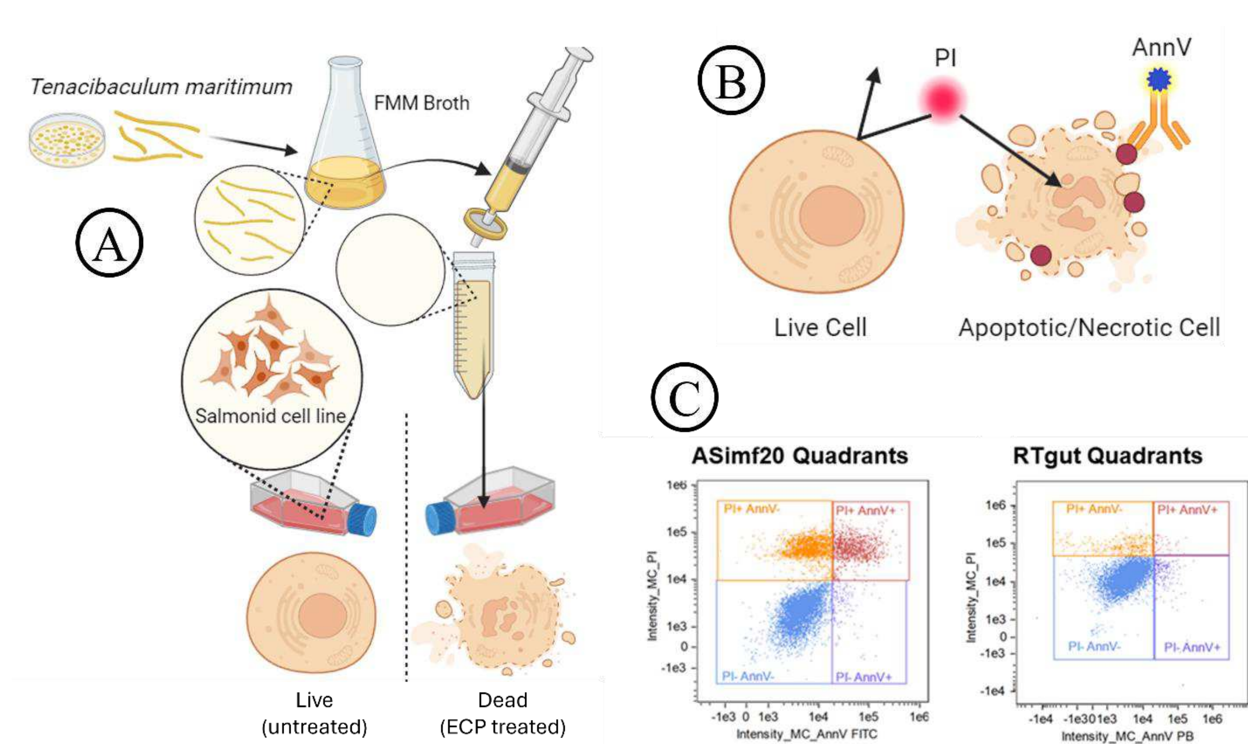


Fig 2.2 *T. maritimum* extracellular products (ECP) isolation and treatment of salmonid cells, using annexin V (Ann V) and propidium iodide (PI) to differentiate and then quantify the cell death. The first section (A) illustrates the collection of *T. maritimum* ECP from Flexibacter marine media (FMM) broth and then the treatment of salmonid cell line cultures with ECP, resulting in cell death. The second section (B) illustrates the cell death markers for necrosis (PI enters nucleus after necrotic membrane permeabilization) and apoptosis (AnnV binds to phosphatidyl serine displayed on the membranes of apoptotic cells). The third section (C) is the gates for the different cell death mechanisms after the cells (untreated ASimf20 and RTgut cells) were quantified by the Imagestream flow cytometer. More details on these cell death quadrants are available on **Fig 2.3**. The majority of this protocol graphic was made in Bio Render.

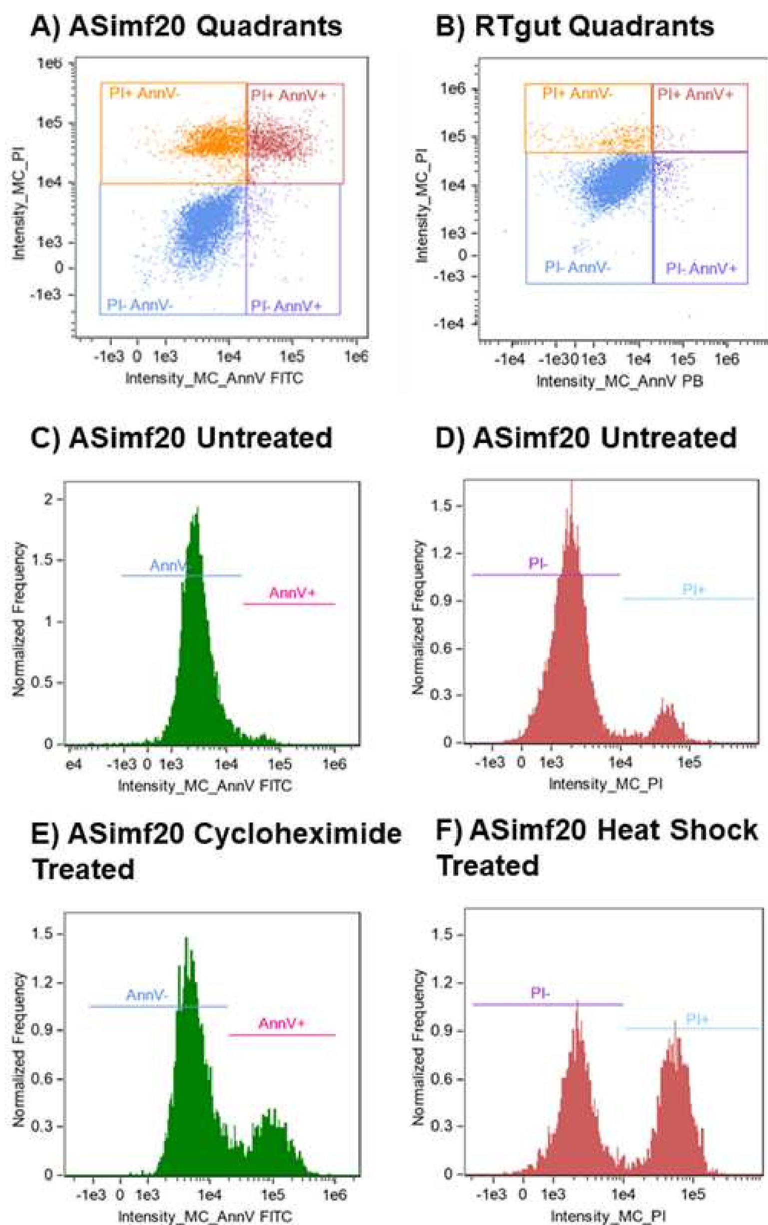


Fig 2.3 Gating strategies for assessing cell death pathways in ASimf20 and RTgut cells. The cell death quadrants are visualized for ASimf20 cells (A) and RTgut cells (B) from untreated cells. Untreated ASimf20 cells (C and D) are compared to the induced cell population for apoptosis (E) and necrosis (F) for positive gate verification. These positive gates allowed for the construction of the complete quadrants for assessing the quantity of each cell death mechanism. As a reminder, AnnV+ is the positive apoptosis marker, whereas PI+ is the positive necrosis marker.

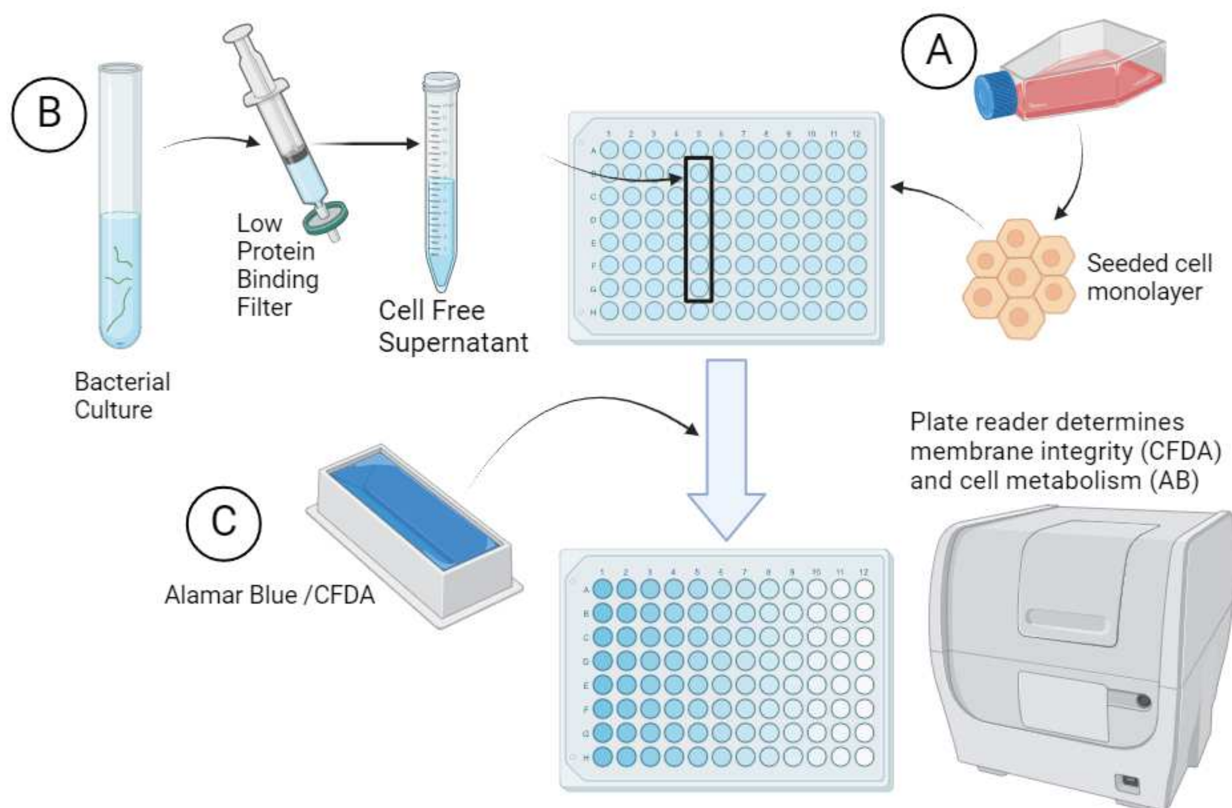


Fig 2.4 The general Alamar blue (AB) and 5-Carboxyfluorescein Diacetate (CFDA) protocol for assessing cellular viability of the adherent salmonid cell lines. The first step (A) encompasses seeding the plate with the salmonid cells at a concentration of 1×10^4 cells per well. After a 24 h incubation, the second step (B) is the treatment of these salmonid cells with the bacteria free supernatant. After a 24 h incubation, the third step (C) is the incubation of AB and CFDA with the treated cells and subsequent measurement of fluorescent intensity after 1 h. This step-by-step graphic was made with Bio Render.

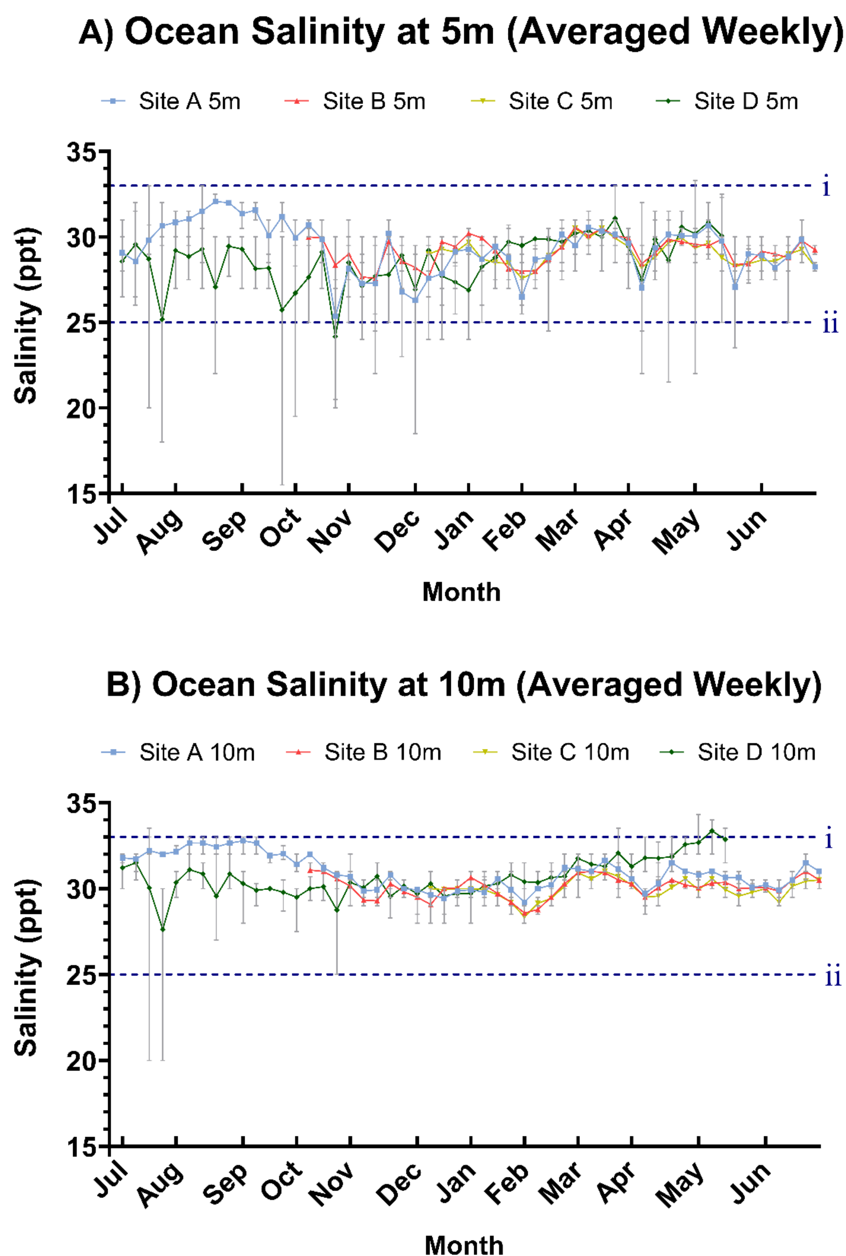


Fig 2.5 Measurements of ocean salinity from depths of 5 m and 10 m were taken daily from four separate sites and over the course of a year. The data points represent the weekly averages, whereas the error bars represent the min and max values within that week. Upper and lower salinity observations were marked with i and ii lines (which correspond to 33 ppt and 25 ppt, respectively). These were considered our high and low environmental restrictions for the *in vitro* experiment.

Table 2.0 Composition of FMM salt-water bacterial growth media, including catalog numbers. Agar was added if FMM agar plates were being made. The FMM solution was autoclaved and cooled before the addition of kanamycin under a sterile flame. FMM broth and plates were stored at 4°C until use.

	FMM (1 L)	Catalog number
Trypticase casein	5.00 g	Gibco #211921
Yeast extract	0.5 g	Fischer chemical #BP1422-500
Sodium acetate	0.01 g	Fischer #S210-500
Vibrant sea salt	31 g	
Kanamycin (add after autoclave)	1.0 mL (50 mg/mL aliquot)	Fischer #BP906-5
dH_2O	Fill to 1 L	
Agar (optional, for agar plates)	14 g	BD #214010

Table 2.1 Bacterial enumeration at each experimental inoculation and endpoint. Values calculated with the most probable number (MPN) method are denoted with an asterisk (*). All other values were done with a spot-count method (CFU/mL).

OD Endpoint Cytotoxicity (24°C)	Rep 1	Rep 2	Rep 3	Average	SD
0.5 OD TM*	$1.10x10^9$	$4.90x10^8$	$7.90x10^8$	$7.93x10^8$	$3.05x10^8$
0.9 OD TM*	$1.30x10^9$	$1.30x10^9$	$7.90x10^8$	$1.13x10^9$	$2.94x10^8$
0.5 OD EC	$5.00x10^8$	$1.00x10^8$	$4.00x10^8$	$3.33x10^8$	$2.08x10^8$
0.9 OD AS	$8.00x10^8$	$1.20x10^9$	$1.40x10^9$	$1.13x10^9$	$3.06x10^8$
0.5 OD TD	$3.00x10^8$	$1.00x10^8$	$1.00x10^8$	$1.67x10^8$	$1.15x10^8$
0.5 OD TO	$3.00x10^8$	$2.00x10^8$	$3.00x10^8$	$2.67x10^8$	$5.77x10^7$
Salinity Endpoints	Rep 1	Rep 2	Rep 3	Average	SD
0.2 OD TM (inoculate)*	$3.50x10^5$	$2.40x10^5$	$2.40x10^5$	$2.77x10^5$	$6.35x10^4$
25 ppt TM (48 h later)*	$3.50x10^7$	$5.40x10^7$	$9.20x10^7$	$6.03x10^7$	$2.90x10^7$
29 ppt TM (48 h later)*	$5.40x10^8$	$2.20x10^8$	$9.20x10^8$	$5.60x10^8$	$3.50x10^8$
33 ppt TM (48 h later)*	$1.60x10^9$	$9.20x10^8$	$5.40x10^8$	$1.02x10^9$	$5.37x10^8$
Temperature Endpoints	Rep 1	Rep 2	Rep 3	Average	SD
0.2 OD TM (inoculate)*	$5.40x10^7$	$2.40x10^5$	$3.50x10^5$	$1.82x10^7$	$3.10x10^7$
12°C TM (48 h later)*	$1.60x10^9$	$1.60x10^9$	$2.40x10^9$	$1.87x10^9$	$4.62x10^8$
18°C TM (48 h later)*	$5.40x10^9$	$2.40x10^9$	$9.20x10^9$	$5.67x10^9$	$3.41x10^9$
24°C TM (48 h later)*	$3.50x10^8$	$1.70x10^8$	$1.70x10^8$	$2.30x10^8$	$1.04x10^8$

Table 2.2 Composition of 10X PBS-/- stock concentration. Includes ingredients and catalog numbers for making the buffer. Obtained from Barreda Lab protocols. Dilute 10X PBS -/- one in ten (1/10) in dH_20 to get 1X PBS-/- working concentration. Buffers were stored at 4°C.

	10X PBS (1 L)	**Catalog number**
KCL	2.0 g	Sigma-Aldrich #P3911-500G
KH_2PO_4	2.0 g	Fisher Scientific #P285-500G
NaCl	80.0 g	Fischer Scientific #S271-3
$NaHPO_4$	11.44 g	Sigma-Aldrich #S9763-500G
dH_2O	Fill to 1 L	

Table 2.3 Primers used in bacterial identification, as specified in the text. For 16S rDNA sequencing whole bacterial DNA was amplified and then sequenced using the same 16S rDNA F or 16S rDNA R primer. For multilocus sequence typing (MLST), bacterial DNA was amplified using the 7 primers listed (atpA, dnaK, glyA, gyrB, infB, rlmN, and tgt) in both the forward (F) and reverse (R) directions. The amplification products were sequenced using the respective Seq F or reverse primer (M13).

Primer names	Sequence 5' to 3'	Reference
16S rDNA F (27F)	AGAGTTTGATCATGGCTCAG	Nowlan et al., 2023
16S rDNA R (1492R)	GGTTACCTTGTTACGACTT	Nowlan et al., 2023
Seq F (M13)	CAGGAAACAGCTATGACC	Habib et al., 2014
Seq R (M13)	TGTAAAACGACGGCCAGT	Habib et al., 2014
atpA 567 F	CAGGAAACAGCTATGACCATTGGWGAYCGTCAAACWGG	Olsen et al., 2017
atpA 567 R	TGTAAAACGACGGCCAGTCCAAAYTTAGCRAAHGCTTC	Olsen et al., 2017
dnaK 573 F	CAGGAAACAGCTATGACCGGWACYACNAAYTCDTGTGT	Olsen et al., 2017
dnaK 573 R	TGTAAAACGACGGCCAGTTCWATCTTMGCTTTYTCAGC	Olsen et al., 2017
glyA 558 F	CAGGAAACAGCTATGACCCAYTTAACWCAYGGWTCDCC	Olsen et al., 2017
glyA 558 R	TGTAAAACGACGGCCAGTACCATRTTTTTRTTTACHGT	Olsen et al., 2017
gyrB 597 F	CAGGAAACAGCTATGACCAGTATYCARGCRCTRGAAGG	Olsen et al., 2017
gyrB 597 R	TGTAAAACGACGGCCAGTGTWCCTCCTTCRTGYGTRTT	Olsen et al., 2017
infB 564 F	CAGGAAACAGCTATGACCATGCCDCAAACWAAAGARGC	Olsen et al., 2017
infB 564 R	TGTAAAACGACGGCCAGTGTAATHGCTCCAACYCCTTT	Olsen et al., 2017
rlmN 549 F	CAGGAAACAGCTATGACCGCKTGTGTDTCDAGYCARGT	Olsen et al., 2017
rlmN 549 R	TGTAAAACGACGGCCAGTCCRCADGCDGCATCWATRTC	Olsen et al., 2017
tgt 486 F	CAGGAAACAGCTATGACCGAAACWCCWATWTTYATGCC	Olsen et al., 2017
tgt 486 R	TGTAAAACGACGGCCAGTTAYAWYTCTTCNGCWGGTTC	Olsen et al., 2017

Table 2.4 Protease inhibitor cocktail (P.I.) components, types, and concentrations from the Halt™ Protease Inhibitor Cocktail (100X). Information and table are obtained from the product user guide (https://www.thermofisher.com/order/catalog/product/87786). The cocktail was stored at 4°C until use.

Protease Inhibitor Cocktail Component	Inhibitor Type	Concentration (1X)
AEBSF	Serine proteases	1mM
Aprotinin	Serine proteases	800nM
Bestatin	Amino-proteases	50μM
E64	Cysteine proteases	15μM
Leupeptin	Serine and cysteine proteases	20μM
Pepstatin A	Aspartic acid proteases	10μM

Table 2.5 Gel electrophoresis ingredients and respective catalog numbers from the Barreda Lab protocols. 10% Ammonium Persulfate (APS) was stored in aliquots at -20°C, whereas other solutions were stored at 4°C. Both gel solutions were made on the same day as use, with 10% APS and N,N,N′,N′-Tetramethylethylenediamine (TEMED) being added last before polymerization. The solutions were enough to make two 1 mm gels at one time.

	8% Separating gel	4% Stacking gel	Catalog number
1.5 M Tris pH 8.8 for 8% 0.5 M Tris pH 6.8 for 4%	2.50 mL	1.25 mL	BioShop #TRS001.1
dH_2O	5.55 mL	3.30 mL	
10% SDS	100 μL	50 μL	Sigma #L3771-500G
40% Acrylamide	1.95 mL	490 μL	Bio-Rad #161-0144
10% APS	50 μL	25 μL	Bio-Rad #161-0700
TEMED	15 μL	10 μL	Sigma #T9281-50mL

Chapter 3: Characterization of *T. maritimum* extracellular secreted products (ECP)

*Parts of this chapter were previously published under the title “The use of salmonid epithelial cells to characterize the toxicity of *Tenacibaculum maritimum* soluble extracellular products” in the Journal of Applied Microbiology.

3.1 Introduction

There are several significant gaps in the literature regarding the unknown mechanism of *Tenacibaculum maritimum* pathogen virulence. One such unknown is the minimal inflammation and immune response seen at the site of mouth rot infections despite mats of filamentous bacteria (Frisch et al., 2018a). Further, the bacteria likely are systemic (Frisch et al., 2018b), resulting in the transport of the bacteria and its secreted products throughout the salmonid. Although there is minimal internal tissue damage associated with these findings (Frisch et al., 2018b). This interesting disease symptomatology, and the undetermined mechanisms behind it, results in a variety of questions surrounding the suspected pathogen *T. maritimum*. Most notably, “How?”

Previous genomics research on *T. maritimum* has identified a type IX secretion (T9SS) system as well as potential virulence factors in the core genome (Pérez-Pascual et al., 2017). Other studies by Barbier et al. (2020), Eckroat et al. (2021), and Li et al. (2017) have demonstrated (in *Flavobacteriaceae*) that a functioning T9SS is necessary for virulence, motility, biofilm formation, and the secretion of proteins. Numerous T9SS components and potential virulence factors have been identified and confirmed with mass spectrometry to be present in *T. maritimum* extracellular products (ECP) (Escribano et al., 2023). This infers a working T9SS in *T. maritimum* which would allow for the extracellular secretion of potential virulence factors of currently unknown nature and mechanisms. These ECPs could have cytotoxic effects and may contribute to pathogenesis, making

them of interest to study. Furthermore, there are also questions regarding any differential ECP effects between some of the disease targeted species, specifically within salmonids (Atlantic salmon and rainbow trout).

Environmental conditions, such as salinity and temperature, are thought to have a role in Atlantic salmon mouth rot infections. Higher incidence of tenacibaculosis disease has previously been associated with higher ocean salinities (Avendaño-Herrera et al., 2006a; Kent & Poppe, 2002). Although, the link of disease to environmental coniditions is not fully understood. Temperature has a much more interesting and conflicting relationship. Globaly, most of the tenacibaculosis concerns regard higher water temperatures increasing the incidence and severity of disease (as in reviews by Avendaño-Herrera et al., (2006a) and Mabrok et al., (2023)). However, mouth rot in British Columbia, Canada has more commonly aflicted fish in lower water temperatures (~8–14°C) (Frisch et al., 2018c). Ironically, these water temperatures are not stated within the ideal growth temperature range for *T. maritimum* in the above reviews. It is also currently unclear whether the mouth rot disease precence at lower temperatures may be due to temp-dependent impairments of the salmonid immune system (Köllner & Kotterba, 2002), or to do with the virulence of the opportunistic bacteria. One of these options is easier to study *in vitro*. Therefore, the influence of these environmental conditions on the physiology of the bacteria must be examined to determine why the mouth rot disease occurs more frequently at lower temperatures.

Due to the currently unknown nature and undetermined components of the soluble ECP secreted by *T. maritimum*, it is difficult to pinpoint the virulence factor(s) responsible. Despite annotations of *T. maritimum* (Pérez-Pascual et al., 2017; Nowlan et al., 2023) and its secreted products (Escribano et al., 2023), there are still a large portion of identified genes or proteins that have unknown functions. Basic tests (such as Azocoll hydrolysis) of the proteolytic activity of *T.*

maritmum ECP have already been preformed (Escribano et al., 2023), but nothing to our knowledge on what inactivates ECP cytotoxicity. Determining the nature of the cytotoxic ECP through component inactivation may help narrow down what the product of interest is and any strategies that can be used to deal with these products.

The main goals of this chapter were to collect and demonstrate *T. maritimum* ECP cytotoxicity and: 1) uncover the mechanism of cell death in *T. maritimum* ECP treated salmonid epithelial cells, 2) examine the influences of temperature and salinity on *T. maritimum in vitro* growth and ECP secretion/cytotoxicity, and 3) to better elucidate the characteristics of the soluble product(s) responsible for *T. maritimum* ECP cytotoxicity. Herin, this research adds further insight into the mechanisms associated with *T. maritimum* soluble extracellular products and its potential contributions to mouth rot disease. Furthermore, this work will allow for better pointed future research on these secreted products and their potential impacts on tissue degradation and disease severity.

3.2 Results

3.2.1 *Apoptosis-mediated cellular death of ASimf20 cells treated with T. maritimum ECPs*

As the first step to functionally characterizing the toxicity of *T. maritimum* ECPs, the cellular mechanism and extent of cytotoxicity was determined. The relevant (as previously stated in chapter 2) Atlantic salmon cell line from intestinal epithelial tissue cell line (ASimf20) was used as a model. The treatment of 25% bacterial supernatant and 75% cell culture media was determined to be the optimal conditions for elucidating toxicity; this was due to the reduction in cell viability associated with 50% FMM treatment control (Michnik et al., 2024). The mechanism of cellular

death was determined using the annexin V (AnnV) and propidium iodine (PI) assay. AnnV FITC binds phosphatidyl serine on the outside membrane of apoptotic cells and emits fluorescence in the yellow green photo range (488 nm), whereas PI (emits in the red photo range (561 nm)) stains the nucleus of cells with porous outer and nuclear membranes (indicative of necrosis). The four cell treatment outputs (viable cells, early apoptosis, late apoptosis/necrosis, and necrosis) are illustrated in **Fig 3.1 A**. The baseline for ASimf20 cellular viability was determined to be roughly 90% for the untreated cells (control) (**Fig 3.1 B**). ASimf20 cells treated with either the FMM (bacterial culture media), or *E. coli* supernatant did not show a reduction in cellular viability compared to the control (**Fig 3.1 B**). On the contrary, ASimf20 cells treated with *T. maritimum* supernatant showed a marked reduction in cellular viability compared to all other treatments (**Fig 3.1 B**). The predominant cell death mechanism of ASimf20 cells treated with *T. maritimum* ECPs was apoptosis (combined fractions of early and late apoptosis) and not necrosis (**Fig 3.1 C**). Expectedly, the FMM and *E. coli* ECP treatments showed no increase in either apoptosis or necrosis (**Fig 3.1 C**).

3.2.2 *Apoptosis-mediated cellular death of RTgutGC cells treated with T. maritimum ECPs*

To examine differences between salmonid species, rainbow trout epithelial cells originating from the intestines (RTgut) were used. The annexin V/PI assay (as previously mentioned) was used to determine the cell death mechanism. However, AnnV FITC was substituted with annexin V Pacific Blue (AnnV PB) due to the autofluorescence of RTgut cells in the 488 nm emission spectra. AnnV PB has the same cellular interaction as AnnV FITC (binds to phosphatidyl serine on cell membrane) except for an emission peak of 405 nm instead of 488 nm. The four cell treatment outputs (viable cells, early apoptosis, late apoptosis/necrosis, and necrosis) are illustrated in **Fig 3.2 A**. The baseline for cellular viability for RTgut was determined to be

roughly 90% for the untreated cells (**Fig 3.2 B**). RTgut cells treated with either the FMM, or *E. coli* supernatant did not show a reduction in cellular viability compared to the control (**Fig 3.2 B**). Unexpectedly, the cellular viability of the *T. maritimum* ECP treated cells also was not significantly different from the controls (**Fig 3.2 B**). The lack of a marked difference was most likely due to the variation within the *T. maritimum* ECP treatment since there was a significant amount of apoptosis cell death in the same treated group compared to the control (**Fig 3.2 C**). However, this difference was not seen between *T. maritimum* and *E. coli* ECP (**Fig 3.2 C**).

3.2.3 *The effects of salinity on T. maritimum growth and ECP cytotoxicity*

In vitro growth of *T. maritimum* at salinities of 25, 27, and 33 ppt were used as a comparison between low, medium, and high environmental conditions, respectively. Supernatants taken from these 48 h cultures grown at 18°C were all cytotoxic compared to the FMM control (**Fig 3.3 A and B**). The lack of a cytotoxicity difference between salinity groups is unexpected but is most likely due to the resolution of the assay. *T. maritimum* growth determined by the MPN method indicates that there was significantly better growth in the high salinity condition compared to the low salinity condition (**Fig 3.3 C**). This growth was then normalized to dilute ECPs to the same number of live *T. maritimum* in the lowest condition (6.03×10^7 MPN/mL in the 25 ppt condition) (**Fig 3.3 D**). The dilutions were 1 in 9 for the 29 ppt ECP and 1 in 16 for the 33 ppt ECP (**Fig 3.3 D**). The normalized ECP treatments showed no difference between groups, however they were all still cytotoxic compared to the FMM control (**Fig 3.3 E and F**). This could suggest a similar ECP toxicity per secreting bacteria, but the resolution of the assay is still too low to confirm. However, despite the significant dilution to the 33 ppt and 29 ppt supernatants, these ECPs still cause significant cytotoxicity. These results could indicate secreted product toxicity in dilute quantities when the right environmental conditions are present. It should be noted that the

low salinity condition did not turn off the production of cytotoxic ECP, despite the reduction in growth.

3.2.4 *The effects of temperature on T. maritimum growth and ECP cytotoxicity*

T. maritimum was grown *in vitro* at 12°C, 18°C, and 24°C in FMM+Kan to compare secreted ECPs at environmentally relevant conditions. It should be noted that 8°C and 4°C should have also been done to capture the complete environmental temperature spectrum in West Coast British Columbia, Canada. However, the limitation was the refrigerated shaking incubator used could not reach temperatures this low to grow the bacteria. *T. maritimum* ECP from the 12°C and 18°C growth condition demonstrated cytotoxicity compared to the FMM condition (**Fig 3.4 A and B**). ECP from the 24°C growth condition was cytotoxic compared to the FMM condition with the CFDA measurement but not the AB measurement of cellular viability (**Fig 3.4 A and B**). The growth conditions of the bacteria were not significantly different between the three temperature conditions (12, 18, and 24°C) (**Fig 3.4 C**). The ECP from all the *T. maritimum* growth conditions were normalized to the lowest bacterial concentration (6.83×10^8 MPN/mL in the 24°C condition) (**Fig 3.4 D**). The dilutions were 1 in 3 for 18°C ECP and 1 in 9 for 12°C ECP (**Fig 3.4 D**). The diluted 12°C and 18°C supernatants were still cytotoxic in ASimf20 cells; although, its cytotoxicity was in a temperature dependent manner as the 18°C diluted ECP was now in between the diluted 12°C and 24°C ECP cytotoxicity (**Fig 3.4 E and F)**. This is further demonstrated by *T. maritimum* ECP from the 12°C condition having induced more cytotoxicity per live bacterium than the 24°C ECP (**Fig 3.4 E and F**). This difference could be due to an elevation of general (or specific) secreted products at 12°C or an increased stability of the secreted toxic product at this low temperature.

3.2.5 *ECP toxicity post heat-treatment*

Further elucidation as to the proteinaceous nature of the *T. maritimum* extracellular product(s) was done by heating the supernatant to 100°C for 10 min, allowing it to cool, and then measuring its cytotoxicity towards ASimf20 cells. The functional denaturation of proteins due to the high temperature treatment was shown to have a complete knock down effect regarding the cytotoxicity of the supernatant (**Fig 3.5**). The heat-treatment of ECP resulted in a similar overall cell viability to the FMM control, further supporting the loss of function.

3.2.6 *Protease inhibitor cocktail and EDTA effects on T. maritimum ECP cytotoxicity*

The commercial protease inhibitor cocktail (P.I.) was added to *T. maritimum* ECP with the intention of knocking down the cytotoxicity of the unknown virulence factor(s). Unexpectedly, the cytotoxicity of *T. maritimum* ECP remained high in both cell viability measurements, despite the co-incubation of P.I. up to a 3X concentration (**Fig 3.6**). It should be noted that there was also a slight decrease in the cellular viability of the FMM media control treatment with 3X P.I., which was possibly due to the protease inhibitors being dissolved in DMSO (0.75% DMSO at 3X P.I., >0.5% DMSO is slightly cytotoxic depending on the cell line (Sangweni et al., 2021)) (**Fig 3.6**). Therefore, future experiments focused on using the 2X P.I. concentration to minimize effects on the CFDA measurement with the DMSO solvent. According to the manufacturer's instructions, the 2X P.I. treatment should have enough inhibitors to deactivate the targeted proteases that may be responsible for the ECP cytotoxicity.

The commercial EDTA (0.5 mM at 100X; 0.015 mM at 3X) was added to *T. maritimum* ECP to remove metal ions from proteins through chelation. There was a significant rise in the cellular viability of *T. maritimum* ECP treated cells attributed to a dose dependent increase in EDTA treatment (**Fig 3.7**). At the higher EDTA concentrations (2X and 3X), EDTA treated *T. maritimum* ECP had no difference in SHK cellular viability compared to EDTA (2X and 3X) treated FMM bacterial media (negative control) (**Fig 3.7**). Further, both cellular viability measurements in SHK cells treated with *T. maritimum* ECP (EDTA free) and *T. maritimum* ECP with 2X EDTA were significantly different (AB: p=0.0243 and CFDA: p=0.0003) (**Fig 3.7 B**). Both of these differences provide evidence of a *T. maritimum* ECP cytotoxicity knockdown using a concentration of 0.010 mM EDTA (2X).

3.2.7 *T. maritimum ECP protein bands visualized*

The presence of two metalloproteases of interest was visually probed on an SDS-PAGE gel of the *T. maritimum* ECP (**Fig 3.8**). It should be noted that this is not diagnostic and is only a visualization to see if bands are present at the rough predicted sizes of these proteins. In the SYPRO Ruby stained gel, there are light bands at the rough sizes of the proteins of interest, indicating that they could be present in the ECP (**Fig 3.8**).

3.3 Discussion

3.3.1 *T. maritimum ECP induce apoptosis in salmonid cells*

It is shown in the above work that ECP isolated from *T. maritimum* induces apoptosis in mucosal epithelial cell lines from two salmonid species (specifically ASimf20 and RTgut).

Apoptosis is commonly regarded as part of the pro-resolution and anti-inflammation pathway (Birge & Ucker, 2008). Further, the cellular mechanism of apoptosis is conserved throughout vertebrates (including salmonids) (Verleih et al., 2019). Some bacteria are capable of hijacking the apoptosis pathway in host cells to promote infection (Monack & Falkow, 2000; Wanford et al., 2022). Further evidence that this is possible can be found in a closely related fish pathogen, *Flavobacterium psychrophilum*, which induces apoptosis in host salmonid cells through the action of cleaving caspases (Iturriaga et al., 2017). Supernatants (such as the ones examined in this study) from *Pseudomonas aeruginosa* have been shown to be capable of inducing apoptosis in host cells (Zhang et al., 2003). Therefore, the induction of apoptosis by *T. maritimum* supernatants may align with the current understanding of mouth rot disease regarding the lack of clinical inflammation at the site of infection (Frisch et al., 2018a; Nowlan et al., 2021a; Handlinger et al., 1997; Frelier et al., 1994). Although, it should be mentioned that the presence and extent of involvement of this *T. maritimum* ECP mechanism remains to be shown in live mouth rot infections.

3.3.2 *Generalized toxicity in T. maritimum ECP*

The cell lines (ASimf20 and RTgut) tested in the thesis work were isolated from different salmonid species (Atlantic salmon and rainbow trout, respectively) and showed little difference in the mechanism of cytotoxicity upon exposure to *T. maritimum* ECP, just sensitivity. Therefore, *T. maritimum* ECP toxicity may not be specific to one fish species, which would support the bacteria having a more generalized toxicity. This can further be supported by the range of tenacibaculosis-infected hosts summarized in a review by Mabrok et al. (2023). However, the definitive role of *T. maritimum* and whether apoptosis is induced in all susceptible genera and cell types of those genera is still unknown.

3.3.3 *The role of high salinity in T. maritimum pathogenesis in vitro*

The range of salinity (25–33 ppt) illustrated in the methods of this thesis is what some British Columbia aquaculture net pen sites experienced during over one year. This documented range is relevant since previous literature had suggested that tenacibaculosis is more commonly seen in high salinity ocean waters (Kent & Poppe, 2002; Avendaño-Herrera et al., 2006a). These disease observations are supported with the evidence of improved *in vitro* growth of *T. maritimum* in the high salinity condition (33 ppt). This data goes hand in hand with disease spread and infection potentially being reduced at lower salinities based on what is seen with bacterial growth in the low salinity condition (25 ppt). To bring in further support, a study by Drønen et al. (2022), suggested that differences in the microbiota of ulcerative skin lesions may be due to the differential preferences or selection pressures of/on bacteria at varying environmental conditions (i.e. salinity and temperature). Therefore, mouth rot disease, predominantly colonized by *T. maritimum* (Wynne et al., 2020) may be observed more frequently at higher salinities and lower temperatures due to it being favorable conditions for the bacteria.

There was not a marked difference in the cytotoxicity of the *T. maritimum* ECP in either the raw or the normalized supernatants from the three salinity conditions (25, 29, and 33 ppt). Whether salinity influences the quantity or pattern of soluble extracellular products secreted from the type IX secretion system is not currently known and was not clearly demonstrated by this work in the thesis. Most likely, the better growth from *T. maritimum* cultures in high salinity media (33 ppt) would be synonymous with improved pathogenesis but not more cytotoxic soluble ECP secretions.

3.3.4 *The role of low temperature in T. maritimum pathogenesis in vitro*

Measured ocean temperatures in British Columbia (BC), Canada have been shown to range between 2.60–21.80°C (Thakur et al., 2018), with documented mouth rot incidents involving an isolated *T. maritimum* occurring from temperature ranges of 8.7–14.7°C (Frisch et al., 2018c). It is therefore an interesting connection that at the lowest experimental incubation temperature (12°C, well within the range of documented mouth rot disease), there is also the highest degree of cytotoxicity from soluble *T. maritimum* ECP towards Atlantic salmon epithelial cells. However, this should not be surprising since low temperatures, as well as iron deprived conditions, have been shown to upregulate virulence factor secretion in other aquatic pathogens. For example, *Vibrio anguillarum* shows a change at the transcriptome level, as well as an increase in various hemolysins and proteolytic activity when grown at a lower incubation temperature (15°C) (Lages et al., 2019). This difference exists despite the more optimal growth condition for the bacteria being the higher temperature condition (25°C) (Lages et al., 2019). Low temperatures have also been shown to activate the two-component system (closely tied to the production and secretion of proteins of various functions) in the closely related fish pathogen, *Flavobacterium psychrophilum* (Hesami et al., 2011). Additionally, the impact of this may be directly seen regarding an increased secretion of extracellular metalloproteases from *F. psychrophilum* at 12°C (Secades et al., 2001), which is within the most lethal temperature range for bacterial cold-water disease (BCWD) (Holt et al., 1989). However, the mechanism between how lower temperatures cause an increase in *T. maritimum* ECP cytotoxicity is still unknown, but the fact that temperature does induce a strong change is an impactful conclusion. Especially since the highest cytotoxicity of *T. maritimum* ECP was at 12°C, well within the range of most frequently seen mouth rot disease (personal

communication, Patrick Whittaker). This also likely demonstrates that the secreted product causing virulence is a secondary metabolite, due to its interaction with the environment.

3.3.5 *A proteinaceous component involved in cytotoxicity*

The heating of non-thermoresistant protein-based virulence factors to induce a loss of function (Naik & Duncan, 1978; Woodburn et al., 1979) and protein structure changes (Regenthal et al., 2017) is a validated and well-established method used to inactivate protein toxins, including metalloproteinases (Alves et al., 2018). The current study shows that the *T. maritimum* ECP virulence factor(s) can be inactivated with only 10 minutes of heat-treating the soluble secreted products. Therefore, this experiment indicated that the cytotoxic product likely has a proteinaceous component necessary to the structure and function of the virulence factor.

To add further context to the experiment, an example of a heat-resistant virulence factor would be the endotoxin (LPS-based) from *Vibrio cholerae*, discussed in the review by Beutler and Rietschel (2003). For this endotoxin, heat-treatment does not inactivate its function and the treatment can instead be used as a way of isolating the endotoxin from the bacteria (Brock-Utne and Gaffin, 1989). Staphylococcal enterotoxins, produced by *Staphylococcus aureus*, are another type of heat-stable toxin, which has been shown to require one or more hours of incubation at 100°C to reduce its activity (Fung et al., 1973). These other products are in stark contrast to what is seen with *T. maritimum* ECP activity after a heat-treatment.

3.3.6 *EDTA knocks down T. maritimum ECP cytotoxicity*

In the current study, a cocktail of general protease inhibitors (EDTA free) was shown to have minimal effects on the cytotoxicity of *T. maritimum* ECP against salmonid cells. Whereas EDTA induced a loss of function in the cytotoxic effects of *T. maritimum* ECP. These results are consistent with the chelation of a metal ion from a metalloprotease resulting in an inactivation of the protein. For example, this EDTA interaction has been documented to occur in metalloproteinases from bacterial origin (*Pseudomonas fluorescens*) (Diermayr et al., 1987), to snake venom (Tanjoni et al., 2005), and even jellyfish toxin (Li et al., 2022).

The EDTA inactivation results from the current study may point to the use of a hypothetical M12B metalloprotease as a putative virulence factor, which was first identified in the *T. maritimum* genome by Nowlan et al. (2023). The genome of *Tenacibaculum maritimum* NCIMB 2154 (type-strain) encodes two probable M12B metalloprotease genes (MARIT_2045 and MARIT_2638), which have predicted metalloprotease functions based on homology. The type-strain also encodes a probable M43 family metalloprotease gene (MARIT_3130), which has been theorized to be involved in virulence through host tissue degradation in related *Flavobacteriaceae* species (Hesami et al., 2011). Furthermore, all of these genes listed above encode a Type IX secretion system C-terminal domain for secretion of the product into the extracellular space. There are visual indications of *T. maritimum* ECP proteins present near the predicted protein size range of the SDS-PAGE gel, although this is hardly diagnostic in confirming the presence of metalloproteases in the supernatant. However, in a secretome study by Escribano et al. (2023), they found that the M12B metalloprotease was present in the secreted soluble ECP products, which were virulent against cyprinid cells. It should also be noted that despite the *T. maritimum* Canadian isolate used in the

experiments not being sequenced, these metalloprotease genes appear to be conserved across all/most sequenced *T. maritimum* strains.

3.3.7 *Metalloproteinase implications*

Further information on the family of M12B metalloproteases is necessary to understand why the hypothetical *T. maritimum* protein is of such interest. The M12B metalloproteases (also known as reprolysin or adamalysin) are a structurally diverse peptidase family that is most well-known for its implications in snake venom (Takeda, 2016). This M12B metalloproteinase family has implications in host immune suppression (Lin et al., 2018), host cell adhesion/cytoskeleton disruption (Lin et al., 2018; Bustillo et al., 2017), and host cell apoptosis (Bustillo et al., 2017; Takeda et al., 2012). Another related bacterium, *F. psychrophilum*, and causative agent for bacterial cold-water disease (BCWD), secretes Fpp1 which is a M12B metalloprotease with implications in host tissue degradation and potentially virulence (Secades et al., 2001). Further, there is also an interesting semblance to *T. maritimum* ECP cytotoxicity, where *F. psychrophilum* Fpp1 secretion increases at lower incubation temperatures (Secades et al., 2001). These similarities are stressed since the same functional type IX secretion system, responsible for the movement of proteins over the outer membrane for *T. maritimum*, is also used by *F. psychrophilum* (Barbier et al., 2020). Another secreted ECP metalloprotease theorized to be involved in virulence is Fpp2, which contains a M43 family cytophagalysin region (Pérez-Pascual et al., 2011; Secades et al., 2003). However, it should be noted that in an fpp1-fpp2 *F. psychrophilum* knockout study by Pérez-Pascual et al. (2011), there was not a drop in the LD_{50} of the Fpp1$^-$ or Fpp2$^-$ mutant infected rainbow trout. Although, the article states that proteolytic activity in the Fpp1$^-$ mutant did not decrease, which could speak to the genetic redundancy of the encoded M12B proteases. Therefore,

the knockout study stresses that it does not rule out the involvement of metalloproteinases in virulence.

3.4 Figures

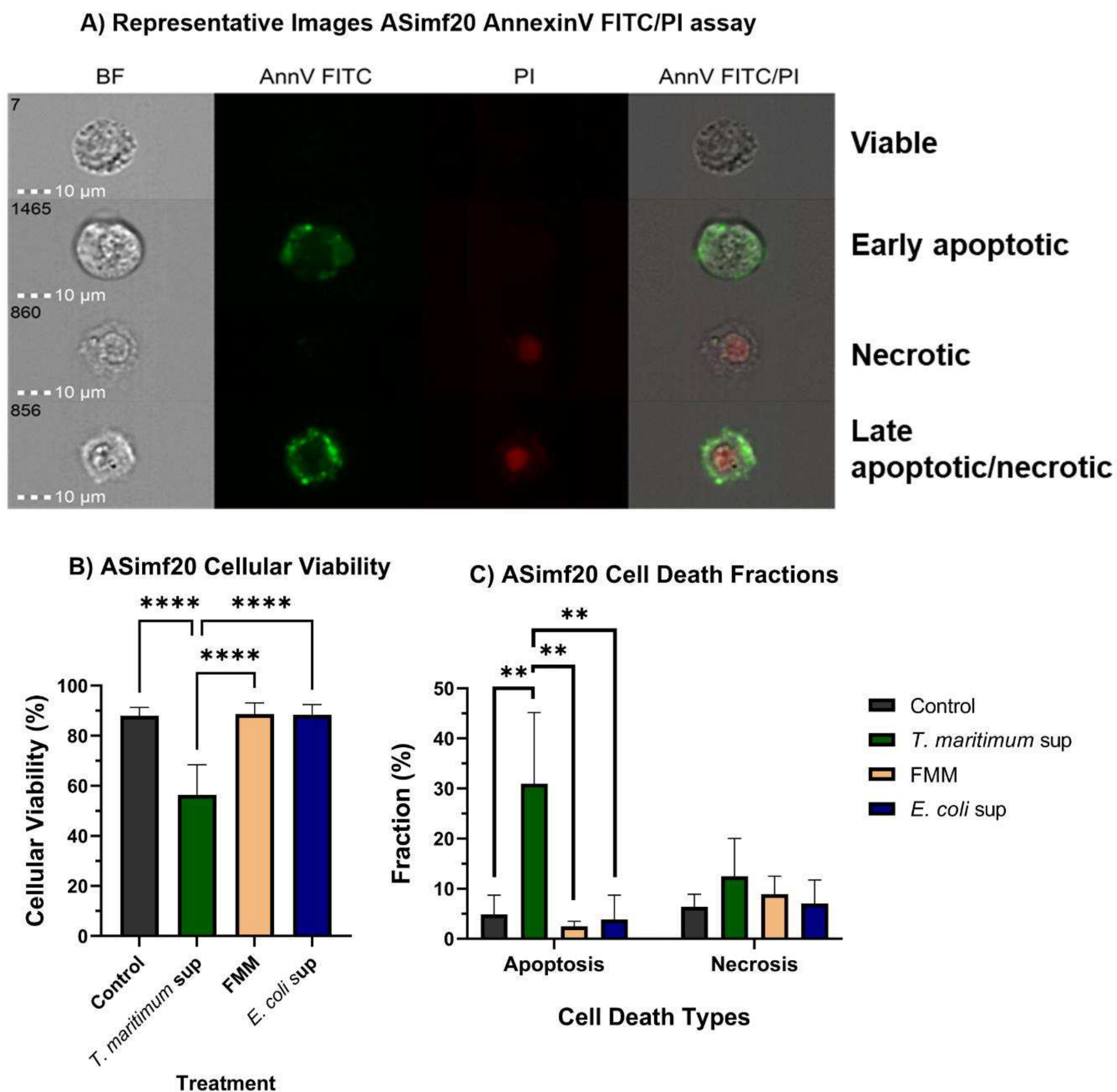


Fig 3.1 Mechanism of cell death in ASimf20 cells following a 24 h exposure to *T. maritimum* extracellular products (ECP). Representative images of cellular processes (viable, early apoptosis, necrosis, and late apoptosis/necrosis) for cells highlight the imaging flow cytometry outputs for quantifying cell death types (A). The lack of any fluorescence is associated with viable cells (B). The control bar is the baseline (untreated) salmonid cellular viability. The presence of annexin V (AnnV) and Propidium Iodide (PI) markers indicate the induction of apoptosis or necrosis, which

is quantified as fractions of the total cell population. All bacterial secreted products were harvested at OD_{600} of 0.5. For each data point, 10’000 events were collected. Error bars represent +SEM. Stars represent statistically significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) compared across treatments, n=4.

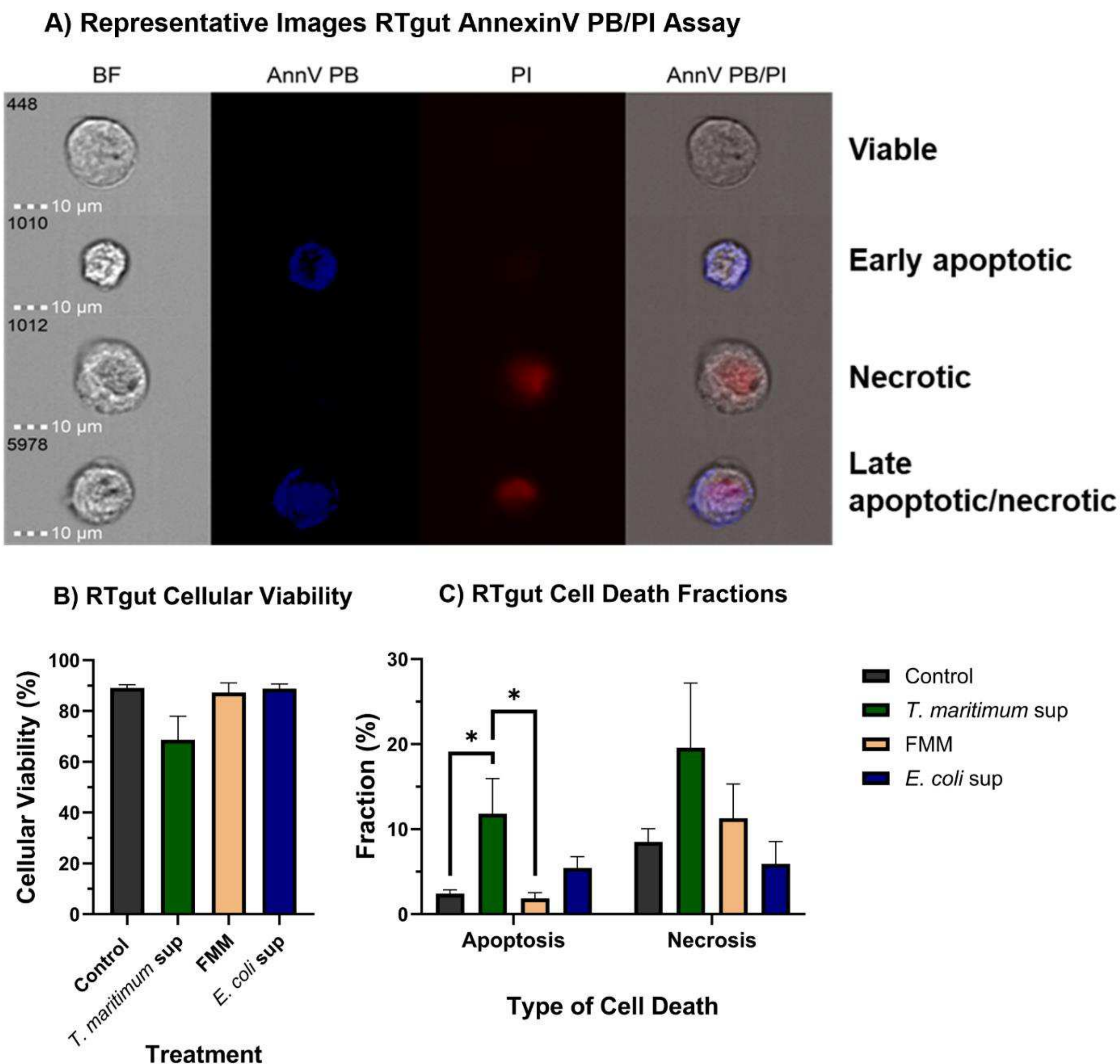


Fig 3.2 Mechanism of cell death in RTgut cells following a 24 h exposure to *T. maritimum* ECPs. Representative images of cellular processes (viable, early apoptosis, necrosis, and late apoptosis/necrosis) for cells highlight the imaging flow cytometry outputs for quantifying cell death types (A). The lack of any fluorescence is associated with viable cells (B). The control bar is the baseline (untreated) salmonid cellular viability. The presence of annexin V (AnnV) and Propidium Iodide (PI) markers indicate the induction of apoptosis or necrosis, which is quantified as fractions of the total cell population. All bacterial secreted products were harvested at OD_{600} of

0.5. For each data point, 10’000 events were collected. Error bars represent +SEM. Stars represent statistically significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) compared across treatments, n=4.

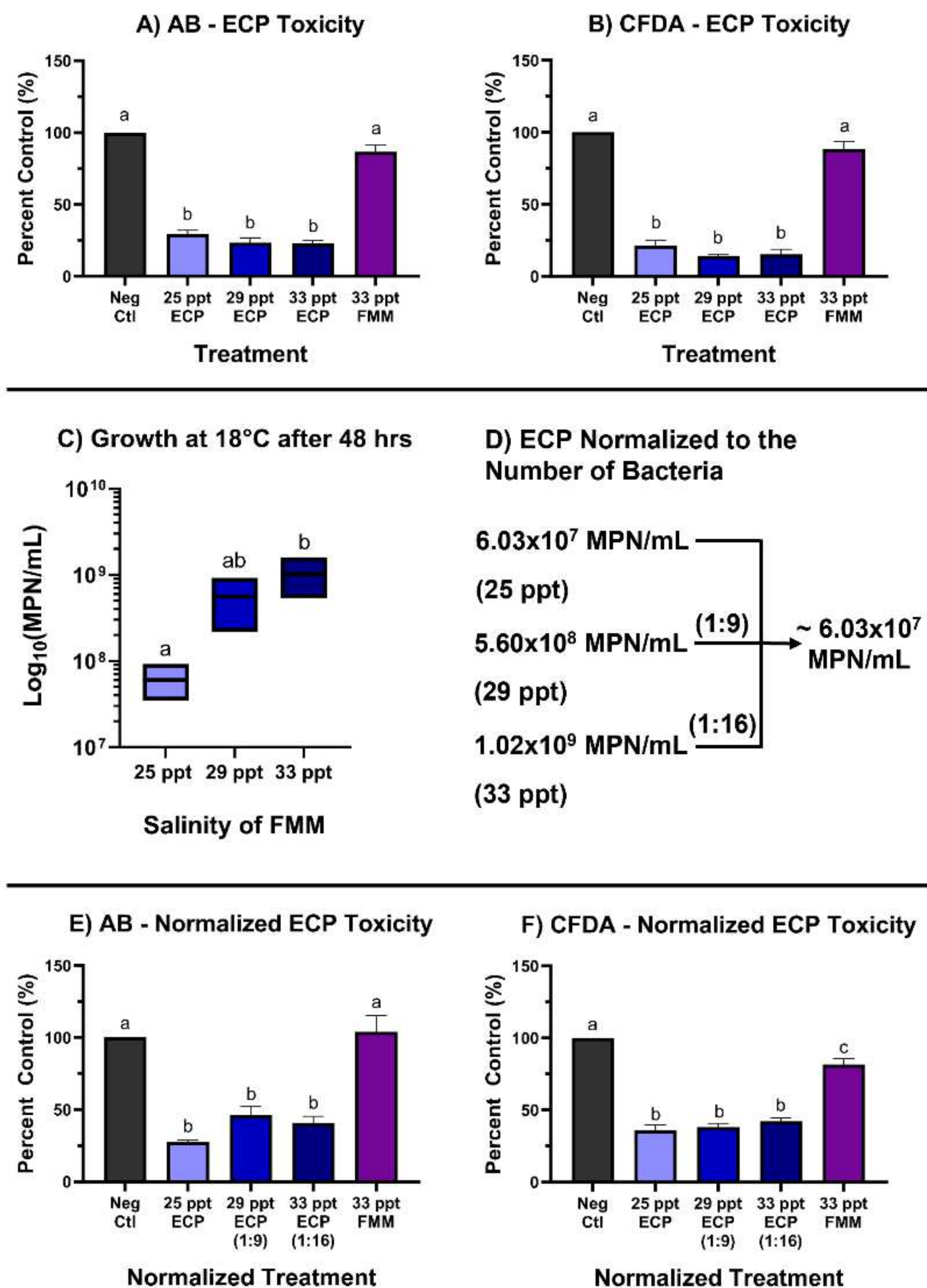


Fig 3.3 Effects of various broth salinities on *T. maritimum* growth and secreted extracellular product (ECP) toxicity. ASimf20 cells were treated with *T. maritimum* ECP from different salinity conditions for 24 h with Alamar blue (AB) (A) and 5-Carboxyfluorescein Diacetate (CFDA) (B) as the measurements of cellular viability. The negative control ("Neg Ctl") was the untreated

cellular baseline. The 33 ppt flexibacter marine media (FMM) was the bacterial media-only control. The live bacterial concentration (MPN/mL) of *T. maritimum* was determined after 48 h of growth and was plotted on a logarithmic scale (C). The upper and lower limits of the box plots detail the maximum and minimum measured MPN/mL, with the central line in the plot being the mean (C). Secreted ECPs were diluted to the lowest mean MPN/mL (6.03×10^7 MPN/mL, 25 ppt) with a dilution in FMM (33 ppt) (D). The dilutions normalized all ECPs to the smallest bacterial concentration as outlined in the flowchart (1:9 for 29 ppt; 1:16 for 33 ppt) (D). ASimf20 cells were treated with diluted ECPs for 24 h with AB (E) and CFDA (F) as the measurements of cellular viability. Different lower-case letters above the bars represent significant differences of at least $p<0.05$, n=3. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

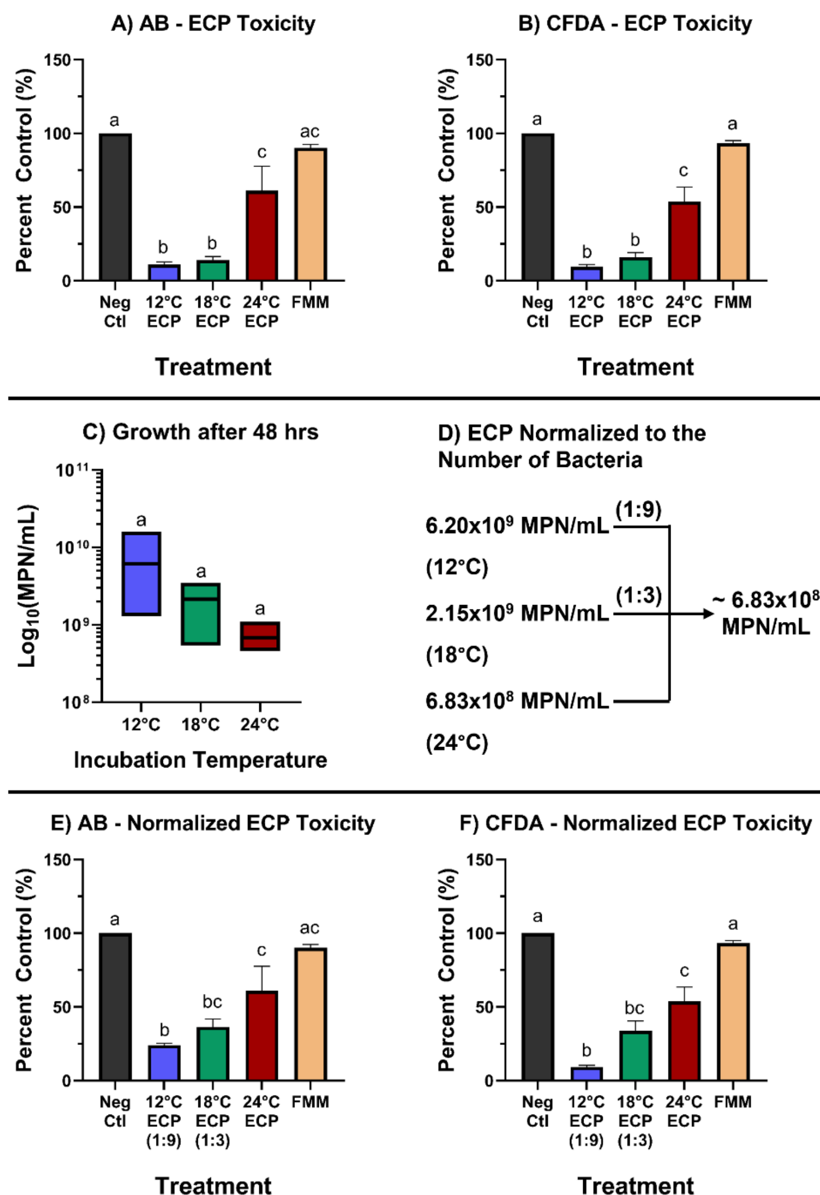


Fig 3.4 Effects of various incubation temperatures on *T. maritimum* growth and secreted extracellular product (ECP) toxicity. ASimf20 cells were treated with *T. maritimum* ECPs from different temperature conditions for 24 h with Alamar blue (AB) (A) and 5-Carboxyfluorescein Diacetate (CFDA) (B) as the measurements of cellular viability. The MPN/mL of *T. maritimum* was determined after 48 h of growth (immediately before ECP isolation) and was plotted on a logarithmic scale (C). The upper and lower limits of the box plots detail the maximum and

minimum measured MPN/mL, with the central line in the plot being the mean (C). Secreted ECPs were diluted to the lowest mean MPN/mL (6.83x108 MPN/mL, 24°C) with FMM (D). The dilutions normalized all ECPs to the smallest bacterial concentration, as outlined in the flowchart (1:3 for 18°C; 1:9 for 12°C) (D). ASimf20 cells were treated with diluted ECPs for 24 h with AB (E) and CFDA (F) as the measurements of cellular viability. Different lower-case letters above the bars represent significant differences of at least $p<0.05$, n=3. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

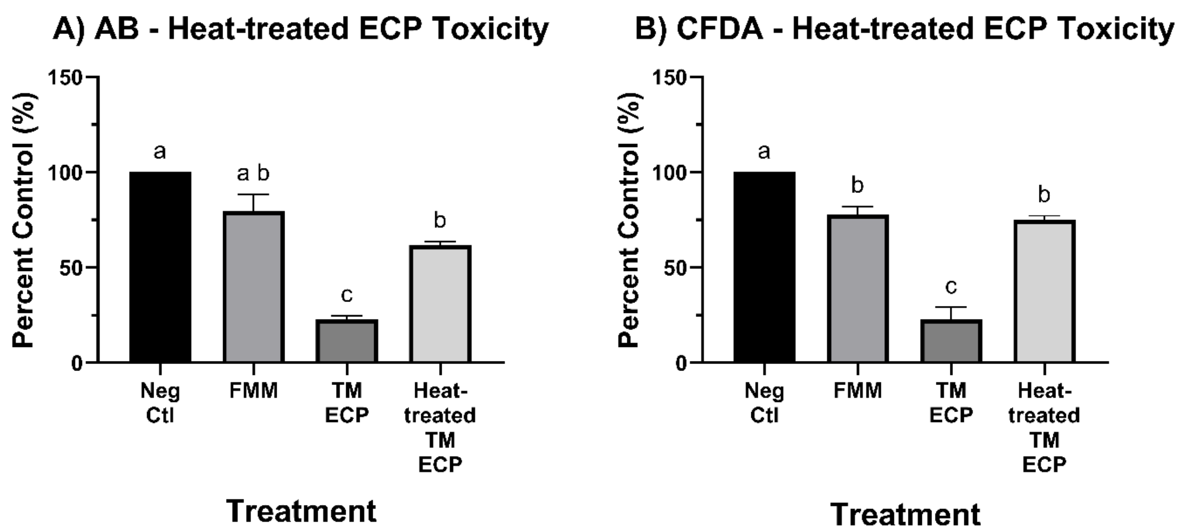


Fig 3.5 *T. maritimum* extracellular product (ECP) cytotoxicity effects on salmonid cells after heat-treatment. ASimf20 cells were treated (50% v/v) with *T. maritimum* ECPs before and after heating the supernatant to 100°C for 10 min. The negative control ("Neg Ctl") was the untreated cellular baseline and FMM was the bacterial media-only control. Alamar blue (AB) (A) and 5-Carboxyfluorescein Diacetate (CFDA) (B) were complimentary measurements for quantifying cellular viability. Different lower-case letters above the bars represent significant differences of at

least $p<0.05$, n=3. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

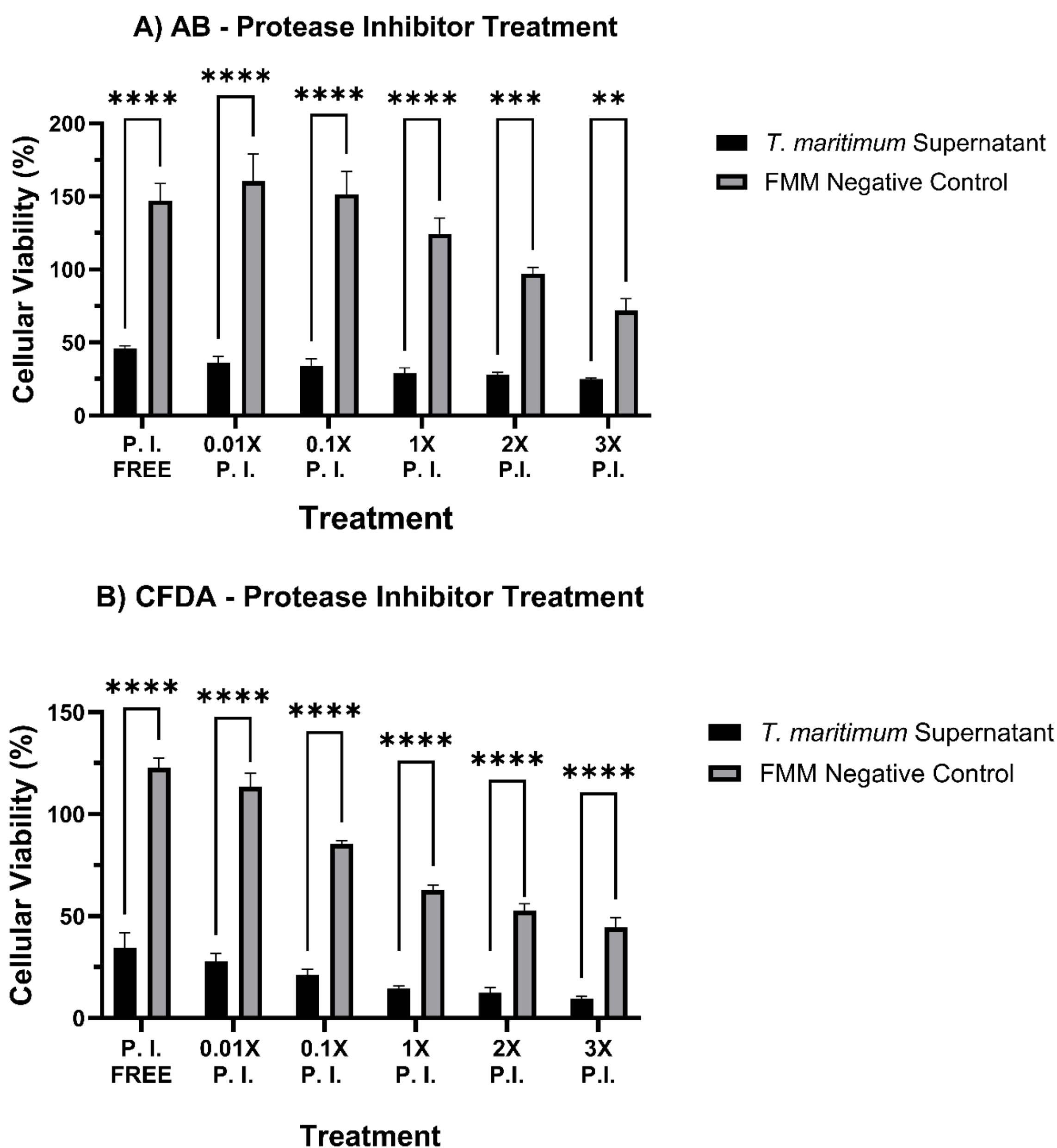


Fig 3.6 The cellular viability of SHK cells co-incubated with different doses (0.01X to 3X) of protease inhibitor cocktail (P.I.) and either *T. maritimum* supernatant or FMM bacterial media. SHK cells were treated with the mixtures for 24 hours before measurement of general cellular viability with Alamar blue (AB) (A) and 5-Carboxyfluorescein Diacetate (CFDA) (B). Cellular viability is the percentage of AB and CFDA fluorescence compared to untreated baseline cells

(100% cellular viability). Error bars represent +SEM. Stars represent statistically significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), n=3.

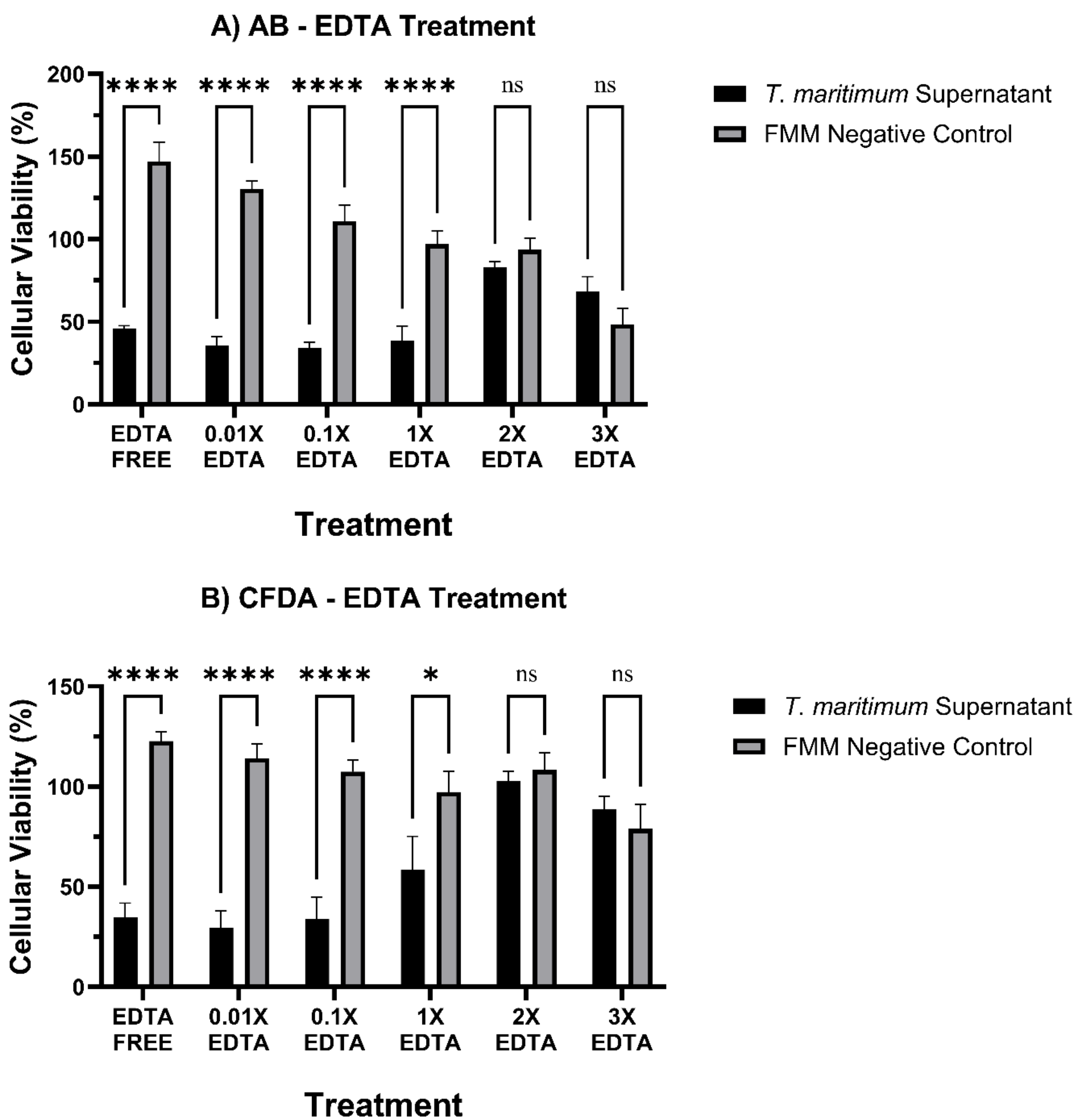


Fig 3.7 The cellular viability of SHK cells co-incubated with different doses (0.01X to 3X) of EDTA and either *T. maritimum* supernatant or FMM bacterial media. SHK cells were treated with the mixtures for 24 hours before measurement of general cellular viability with Alamar blue (AB) (A) and 5-Carboxyfluorescein Diacetate (CFDA) (B). Cellular viability is the percentage of AB and CFDA fluorescence compared to untreated baseline cells (100% cellular viability). Error

bars represent +SEM. Stars represent statistically significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), n=3.

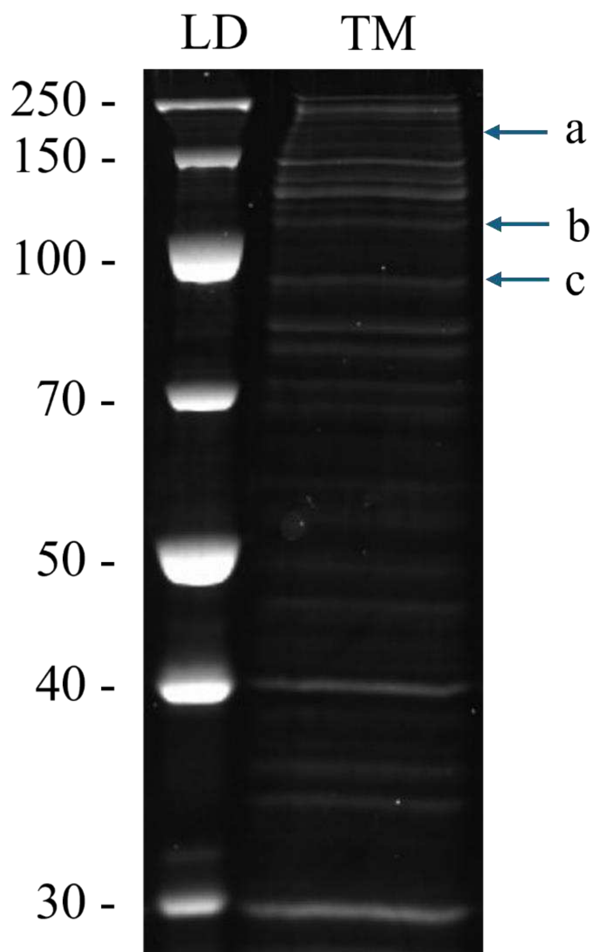


Fig 3.8 An SDS-PAGE gel with SYPRO Ruby in gel protein stain for the rough identification of band presence in the *T. maritimum* ECP (TM) at the predicted sizes for the M12B and M43 metalloproteases of interest. Predicted M12B metalloproteases "MARIT_2045" with a size of 179 kDa (a), "MARIT_2638" with a size of 107 kDa (b). The predicted M43 family metalloprotease "MARIT_3130" with a size of 95.6 kDa (c). The arrows are strictly indicating an estimation of where these predicted proteins may be on the SDS-PAGE gel, relative to the protein ladder.

Chapter 4: Functional comparison of various *Tenacibaculum* species

4.1 Introduction

There is still a significant mystery around the true cause of tenacibaculosis and if *T. maritimum* is a sole, primary, or just a secondary opportunistic pathogen. Current literature suggests that even in *T. maritimum* dominated Atlantic salmon (*Salmo salar*) mouth rot lesions, an increased presence of *Tenacibaculum* spp. and *Vibrio* spp. is correlated with the infections (Wynne et al., 2020). This could be due to bacteria (outside of *T. maritimum*) contributing to a dysbiosis, host cell death, and the host colonization, which may lead to the mortality of the infected fish. An example of this would be in Salmonid rickettsial septicemia (SRS) (predominately caused by *Piscirickettsia salmonis*), where *Aliivibrio wodanis* and *Tenacibaculum dicentrarchi* are identified on the SRS Atlantic salmon ulcers and could potentially contribute to pathogenesis (Godoy et al., 2024). This is also true for winter-ulcer disease, which is associated with *Moritella viscosa* and *A. wodanis* infections/coinfections (Benediktsdóttir et al., 2000); although, the contribution from each bacterium is not fully characterized yet (Karlsen et al., 2014). Further, Amoebic gill disease (AGD) infection of Atlantic salmon is also strongly associated with the presence of *T. dicentrarchi* (Slinger et al., 2020). These other ulcerative diseases in fish provide context into what may be happening in tenacibaculosis infections, which is why all mouth rot associated bacteria should be investigated.

Various *Tenacibaculum* species have been isolated and identified in tenacibaculosis lesions on infected Atlantic salmon, such as *T. dicentrarchi* (Avendaño-Herrera et al., 2016), *Tenacibaculum finnmarkense* (Småge et al., 2016a; Spilsberg et al., 2022), and *Tenacibaculum ovolyticum* (Nowlan et al., 2022; Avendaño-Herrera et al., 2022). Further, there is a significant amount of bacterial diversity in Norwegian Atlantic salmon ulcers, signifying differential impacts

that the various *Tenacibaculum* species may have on ulcerative fish disease, despite the common tenacibaculosis label (Olsen et al., 2011; Olsen et al., 2017). Multiple *Tenacibaculum* species have also been shown to be capable of causing Atlantic salmon tenacibaculosis in a bath infection model (Canadian isolates of *T. maritimum*, *T. dicentrarchi* and *T. finnmarkense*) (Nowlan et al., 2021a); although, their disease mechanisms remain unknown. According to a QPCR study by Nowlan et al. (2022), *T. ovolyticum* does not appear to be a driver of mouth rot infection (despite isolation from Atlantic salmon lesions). However, the authors do state that these results could be different in other outbreaks (different locations, environmental conditions, or other unknown reasons), meaning that the bacteria could still contribute to disease in the right circumstances. Overall, these studies detail the potential for differential involvement of a number of *Tenacibaculum* species isolated from the ulcers of Atlantic salmon. The current study examined two *Tenacibaculum* species (*T. ovolyticum* and *T. dicentrarchi*) and another microbe (*Cellulophaga baltica*), all of which were isolated from mouth rot infected Atlantic salmon in British Columbia, Canada.

The genome composition and characteristics associated with these bacteria may give insights into the potential mechanisms of action by which the bacteria could influence ulcerative fish disease progression. This is highlighted in studies by Bridel et al. (2018) and Grothusen et al. (2016) that have identified potential virulence factors, including peptidases and metalloproteinases containing T9SS CTD, in the *T. dicentrarchi* genome (although they are distinct from potential virulence factors encoded by *T. maritimum*). This is regarding the main proposed virulence factors encoded by *T. maritimum* (Pérez-Pascual et al., 2017), and these protein classes are not present in the examined *T. dicentrarchi* isolates (Nowlan et al., 2023). In a full genome annotation of *T. ovolyticum*, potential virulence factors were identified, including metallopeptidases and hemolysins (Teramoto et al., 2016). Interestingly, Nowlan et al. (2023), suggests that due to the

genome similarities (16S rDNA, MLST) between *T. dicentrarchi* and *T. ovolyticum*, it is possible they have similar or shared pathogenic factors in the core genome. The algae-associated marine microbe, *C. baltica*, is likely not pathogenic and is more of a symbiont (Johansen et al., 1999), but it is in the *Flavobacteriaceae* family just like the *Tenacibaculum* genus and should be investigated as a potential contributor to pathogenesis.

Function assessment of the extracellular products from *T. maritimum* is in the literature (Escribano et al., 2023) and has been expanded upon in **Chapter 3**. However, the cytotoxic assessment of *T. dicentrarchi* extracellular products (ECP) had not yet been done, outside of outer membrane vesicles (which are not examined here) (Echeverría-Bugueño and Avendaño-Herrera, 2024). The ECP cytotoxicity of *T. ovolyticum* and *C. baltica* is also unknown. Therefore, if it is thought that secreted products from *T. maritimum* may have a role in the pathogenesis of mouth rot, then the assessment of secreted products from other disease associated bacteria could bring to light other facets of this disease. Furthermore, characteristics, such as the ability for *T. dicentrarchi* to form biofilms (Levipan et al., 2019b) like that of *T. maritimum* (Levipan et al., 2019a), may be involved in facilitating infection. The capacity for *T. ovolyticum* and *C. baltica* to form biofilms is unknown, as well as the direct comparison between *Tenacibaculum* species, making it of interest to examine.

4.2 Results

4.2.1 *Bacterial morphology*

Agar plate images were taken of the three *Tenacibaculum* species and *Cellulophaga baltica* grown on FMM+Kan agar at 15°C (**Fig 4.1**). Clear differences in the general growth of the bacteria

are seen, such as the rough edges and no clear colony formation of *T. maritimum* (making it uncountable), and the round countable colonies from *T. ovolyticum*, *T. dicentrarchi*, and *C. baltica* (**Fig 4.1**). The four bacterial species consist of different shades of yellow, with *T. maritimum* being the palest (**Fig. 4.1 A**). All bacterial isolates were fixed with 95% methanol to a glass slide for Gram-staining and morphological assessment. All bacteria were determined to be Gram-negative, indicated by the pink/reddish color (**Fig 4.2**). Morphological assessment of the bacterial shapes showed filamentous rods, with varying sizes, shapes, and structures between and within all examined *Tenacibaculum* species, whereas *C. baltica* were small rod-shaped bacteria (**Fig 4.2**). Something to note is that *T. maritimum* broth cultures contain large aggregates and fewer flocculent bacteria when visually compared to the other species (**Fig 4.2 A**). Evident of this is the deep red color of the aggregate after safranin staining, indicating a large number of bacteria and the difficultly of washing the stain (**Fig 4.2 A**). Interestingly, *T. dicentrarchi* produces long string-like filamentous structures that are not seen in any other *Tenacibaculum* spp. (**Fig 4.2 B**).

4.2.2 *Biofilm development*

Specific biofilm formation (SBF) is a well-tested measurement of biofilm development in broth cultures using crystal violet (CV) to stain biofilms and acetone to release the CV for quantification (measurable at OD_{570nm}). The more biofilm formed by the bacteria results in higher CV released and a higher SBF value for that timepoint. Starting at 0 h, all bacterium (*T. maritimum*, *T. dicentrarchi*, *T. ovolyticum*, and *C. baltica*) SBF values were not significantly different from each other (**Fig 4.3 A**). At the 24 h timepoint, *T. maritimum* had an SBF of 0.8 (indicating biofilm) whereas *T. dicentrarchi*, *T.* ovolyticum, and *C. baltica* had SBF values of ~ 0 (indicating no biofilm) (**Fig 4.3 A**). This SBF difference was exhibited in the 48 h timepoint as well (**Fig 4.3 A**).

Overall, this data suggests that *T. maritimum* has a stronger capacity to form biofilms to glass than either *T. dicentrarchi*, *T. ovolyticum*, or *C. baltica* Canadian isolates at 22–23°C. This experiment was further complemented by pictures of the flasks and bacterial cultures at 48 h (**Fig 4.3 B**). The images show bacteria adhering to the flask in the *T. maritimum* culture in contrast to a lack of any visual biofilm in *T. dicentrarchi*, *T. ovolyticum*, and *C. baltica* (**Fig 4.3 B**).

4.2.3 *Cytotoxicity of Tenacibaculum spp. ECP*

Soluble extracellular products (ECP) from the *Tenacibaculum* species of interest and other microbiota (*C. baltica*) were harvested at 0.5 OD_{600} (**Table 2.0**) to assess cytotoxicity against salmonid cells. Predictably, *C. baltica* ECP treatment was not significantly different from the negative control, indicating that it does not have any cytotoxic effects against the SHK cells (**Fig 3.4**). Whereas ECP from *T. dicentrarchi* and *T. ovolyticum* were more cytotoxic to SHK cells than *T. maritimum* ECP despite having a lower measured CFU/mL, and *T. maritimum* being grown at a lower temperature (18°C) (**Fig 3.4**). Overall, it shows that the secretion of cytotoxic ECP may be more broadly present in the *Tenacibaculum* genus. Further, there could be pathogenesis contributions from these additional *Tenacibaculum* spp. in the form of cytotoxic ECP production; although this contribution needs to be demonstrated *in vivo*.

4.2.4 *Effect of heat-treatment on Tenacibaculum spp. ECP*

To assess the proteinaceous nature of the ECP from various *Tenacibaculum* species, a heat treatment of 100°C for 10 min was performed. It should be noted that although this experiment was done with *T. maritimum* ECP in **Fig 3.5**, this was repeated in chapter 4 with a different cell

line (SHK instead of ASimf20) (**Fig 4.5**), which may explain any subtle differences. Although the results of the experiment in chapter 3 were conclusively the same for *T. maritimum* as is shown here. Note that the *Tenacibaculum* species and *C. baltica* baseline ECP cytotoxicity against SHK cells was shown in **Fig 4.4**. The heat-treatment resulted in the complete knockdown of SHK cytotoxicity in both *T. dicentrarchi* and *T. ovolyticum* ECP, mirroring what is seen with heat-treated *T. maritimum* ECP (**Fig 4.5**). The cytotoxicity of *C. baltica* ECP was also not significantly different from the control (Neg Ctl) after heat-treatment (**Fig 4.5**). Therefore, the soluble extracellular products from *T. dicentrarchi* and *T. ovolyticum* may have proteinaceous components responsible for the cytotoxicity towards salmonid cells. This is the same as what is seen in *T. maritimum* ECP.

4.2.5 *Effect of protease inhibitor cocktails and EDTA on Tenacibaculum spp. ECP*

The *T. dicentrarchi* ECP and *T. ovolyticum* ECP were diluted to a more comparable cytotoxicity to that of *T. maritimum* ECP. It should be noted that in **Fig 4.6** and **Fig 4.7**, the *T. maritimum* cytotoxicity data was from **Fig 3.6** and **Fig 3.7** and is only displayed to allow for the comparison between all *Tenacibaculum* species. The 2X concentration for P.I. and EDTA was chosen as for this experiment since it provided the most inactivation of cytotoxicity in *T. maritimum* ECP, with the least control cytotoxicity, which is demonstrated in **chapter 3**. The *T. dicentrarchi* supernatant was diluted 1:6 (33 µL of ECP, 167 µL of FMM) whereas *T. ovolyticum* supernatant was diluted 7:10 (140 µL of ECP, 60 µL of FMM), resulting in a mean cytotoxicity that was generally not significantly different between the *Tenacibaculum* species. From there, a protease inhibitor (P.I.) cocktail was added to the ECP before cell treatment to knockdown the cytotoxic effects of any serine proteases, amine proteases, etc. (See complete table of inhibitor

types, **Table 2.0**). All *Tenacibaculum* spp. ECP remained cytotoxic to the SHK cells, with no knockdown of cytotoxicity due to 2X P.I. observed (**Fig 4.6**). However, this could be due to the protease inhibitor treatment not being effective at inhibiting the bacterial product induced cytotoxicity since no reductions were seen in any bacterial ECPs.

When EDTA was added to the diluted *Tenacibaculum* spp. ECP, there was an inhibition in the cytotoxic effects of ECP from some bacteria but not in all measurements (**Fig 4.7**). The *T. dicentrarchi* ECP and 2X EDTA did not see a significant knockdown in cytotoxicity in the AB measurement but did however see one in the CFDA measurement (**Fig 4.7**). Although, it should be noted that it was not a complete knockdown of cytotoxicity since the 2X EDTA negative control (L15 media only) and *T. dicentrarchi* ECP with 2X EDTA were still significantly different (**Fig 4.7**). The *T. ovolyticum* ECP saw no cytotoxicity knockdowns in either measurement of cellular viability despite the 2X EDTA treatment that worked on both *T. maritimum* and *T. dicentrarchi* ECP (**Fig 4.7**). These results indicate that there are likely differences in the cytotoxic mechanism from all three of the related *Tenacibaculum* species ECP.

4.2.6 *Protein gel of Tenacibaculum spp. ECP*

The SDS-PAGE gel of all the different *Tenacibaculum* spp. supernatants displayed different protein band profiles between all groups, with very few shared protein bands between all three (**Fig 4.8**). It is noteworthy to mention that the protein in the wells was not equalized between the bacteria since the FMM media also contains small peptides (<6 kDa) which makes it difficult to accurately quantify the ECP protein present (despite concentrating peptides >9 kDa). It is still relevant to identify large differences in the secreted ECP protein band sizes and the overall

signature, likely indicating many different products being secreted by all of the *Tenacibaculum* species.

4.3 Discussion

4.3.1 *Differential growth of Tenacibaculum spp.*

There are some notable visual differences between the *Tenacibaculum* species in terms of their growth on plates, in broth, and ability to form biofilms. This is despite *T. maritimum*, *T, ovolyticum*, and *T. dicentrarchi* all having a T9SS encoded in their genomes, which is associated with the use of gliding motility. There are different levels to gliding motility, which may produce the rough, spread-out edges of colonies such as with *T. maritimum* or more round countable colonies such as in the other bacteria. This phenomenon is detailed in a study by Kientz et al. (2012), which displayed the differing extent of gliding motility and iridescence within the *Bacteroidetes* clade. Differences between *T. maritimum* and the other *Flavobacteriaceae* grown were evident by the colony morphology on agar plates. Interestingly, in the current study *C. baltica* (ST: unknown) was shown to have minimal gliding motility and iridescence when compared to the *C. baltica* CIP106307 described in an article by Kientz et al. (2012). However, this difference could be due to gliding motility being growth media (nutrient) dependent and/or a difference between *C. baltica* strains.

4.3.2 *Biofilm forming capacity of Tenacibaculum spp.*

The movement of the bacteria by gliding motility is not indicative of an ability to form biofilms, as seen in a study by Sorongon et al. (1991). However, the ability of aquatic pathogens

to form biofilms and expand them with the use of gliding motility may improve the virulence of the bacteria (Sato et al., 2021). This is why it is essential to characterize each of these capabilities for every mouth rot associated species and strain. In the current study, *T. dicentrarchi* was not able to adhere to the glass Erlenmeyer flask and readily form a biofilm. This is distinct from conclusions made by Levipan et al. (2019b), which saw *T. dicentrarchi* form biofilms in polystyrene 96-well plates. The differences could be due to the growth temperature, the *T. dicentrarchi* strain, the media composition, the speed of shaking, and/or the material of culture flask used (polystyrene vs glass). For a reference regarding *Tenacibaculum* strain-to-strain differences, Rahman et al. (2014) documented a lack of biofilm forming abilities and reduced virulence in a Japanese *T. maritimum* strain. Therefore, there could be a number of reasons responsible for deviations from the previous *T. dicentrarchi* biofilm literature. Although, the simple answer may be that *T. dicentrarchi* does not form biofilms as readily as *T. maritimum* in unfavorable biofilm conditions. The biofilm forming capabilities of *T. maritimum* were consistent with previous literature, despite the differences in study conditions mentioned earlier (Levipan et al., 2019a). Something novel is the lack of biofilm forming capabilities of *T. ovolyticum* and *C. baltica* at 22–23°C. Overall, this recent data suggests that *T. maritimum* may be a stronger biofilm producer than the *T. dicentrarchi* or *T. ovolyticum* Canadian isolates. The production of biofilms could be a mechanism for stronger virulence and persistence compared to that of non-biofilm producing *Tenacibaculum* species and may be one reason why *T. maritimum* is more dominant in Canadian waters. This is in contrast to *T. dicentrarchi* and *T. finnmarkense* being dominant in Norway or Chilean waters (Lagadec et al., 2021) and the differences in biofilm production (between *T. dicentrarchi* strains) could explain why. Although a comparison using the same study conditions and different geographical strains should be performed to ensure true strain to strain differences as well as remove experimental

effects. The study conditions attempted to mimic environmental conditions of ocean waves with the shaking of the flasks, but it is reasonable to think that stationary growth may give a better opportunity for the other non-biofilm forming species to form biofilms, which may be a limitation of the current study.

4.3.3 *Comparative ECP cytotoxicity from various Flavobacteriaceae*

In a study by Bridel et al. (2020), they analyzed the genomes of twenty-five *T. maritimum* strains and found that the core genomes of every strain contained the virulence factors and predicted toxins identified by Pérez-Pascual et al. (2017). Further, Habib et al. (2014) identified that there is very little divergence in the core genome reference sequences used in MLST. Overall, this shows remarkable conservation in the protein coding genes, similar genome sizes, and average nucleotide identities between the examined *T. maritimum* strains (Bridel et al., 2020). This results in a lot more confidence when attempting to identify the responsible cytotoxic product(s) in ECP from a non-sequenced *T. maritimum* strain. In **Chapter 3**, the *T. maritimum* ECP mechanism for salmonid cell cytotoxicity was measured, but it was unknown as to how this compared to other related (and potentially pathogenic) *Tenacibaculum* species. Interestingly, the cytotoxicity of *T. maritimum* ECP were significantly lower than that of *T. dicentrarchi* and *T. ovolyticum*, despite having a higher CFU/mL at the time of supernatant isolation. This points towards different ECP cytotoxic mechanisms between *T. maritimum* and the other *Tenacibaculum* species.

The other *Tenacibaculum* species examined in this study, *T. dicentrarchi* and *T. ovolyticum,* do not have the same level of genome sequencing and comparative examination that *T. maritimum* does. A study by Satyam et al. (2023), compared genome sequences from a number of *Tenacibaculum* spp. (including *T. dicentrarchi* but not *T. ovolyticum*) and found that only a handful of proposed virulence genes were present across all species. Although, this examination

of virulence factors was neither exhaustive, nor the main focus of their study. A comparison study between *T. dicentrarchi* and *T. finnmarkense* by Bridel et al. (2018), suggested that the lack of any species-specific virulence factor(s) likely indicates shared factor(s) between these two closely related *Tenacibaculum* species. A study of Canadian isolates by Nowlan et al. (2023), supports this observation with a large number of 95% and 80% identity shared genes between *T. dicentrarchi* and *T. finnmarkense*. Where *T. ovolyticum* fits into this relationship, and if it contains any proposed "shared virulence factor(s)" is unknown. However, Nowlan et al. (2023) identified a number of different virulence factors that may be present in the *T. ovolyticum* strain, that weren't present in *T. dicentrarchi* and *T. finnmarkense* genomes (and vice versa). Further, Nowlan et al. (2023) examined the virulence factors identified in the *T. maritimum* genome by Pérez-Pascual et al. (2017) and determined that they were not broadly present in *T. dicentrarchi*, *T. finnmarkense*, or *T. ovolyticum*. Although, *T. ovolyticum* did have some shared virulence factor categories (Sphingomyelinase and Sialoglycan Degradation) with *T. maritimum* that *T. dicentrarchi* and *T. finnmarkense* did not (Nowlan et al., 2023). Overall, these genetics studies support what is seen regarding differential virulence mechanisms involving secreted ECP, but there are still likely conserved factors. The extent of involvement of each side (species conserved or unique), and if it is different between strains is unknown.

4.3.4 *Comparison of the Tenacibaculum spp. cytotoxic mechanism of secreted ECP*

The heat-treatment of all bacterial supernatants completely knocked down the cytotoxicity in the 24 h exposure trials against SHK-1 cells. *T. dicentrarchi* and *T. ovolyticum* showed drastic reductions in cytotoxicity due to the proteinase denaturation effect of the heat-treatment, indicating some proteinaceous component to the secreted product(s) of interest. This was the same as the

effect of the heat-treatment on *T. maritimum*, as described in **Chapter 3.3.5**. However, differences between the knockdown of cytotoxicity can be found in the addition of EDTA and protease inhibitors (P.I.) to the ECP from various *Tenacibaculum* species, when diluted to a similar cytotoxicity baseline. For example, *T. dicentrarchi* ECP cytotoxicity did not change upon addition of P.I. and was only slightly reduced from EDTA treatment (in only one measurement). Likewise, *T. ovolyticum* ECP cytotoxicity was not impaired by P.I. or EDTA addition. All of the cytotoxicity levels from *Tenacibaculum* species ECP were slightly distinct when the inhibitors were added. This could point towards different cytotoxic mechanisms/secreted extracellular product(s) regarding the *Tenacibaculum* species identified here. As noted by Olsen et al. (2017), *T. ovolyticum*, *T. dicentrarchi*, and *T. finnmarkense* (all known to be isolated from tenacibaculosis ulcers) are grouped together in the same clade, which could indicate a much closer pathogenesis relationship between these *Tenacibaculum* species, compared to *T. maritimum*. Further, the lack of identified virulence genes shared between these *Tenacibaculum* species and *T. maritimum* promotes this idea of distinct pathogen ancestors (Olsen et al., 2017; Nowlan et al. 2023). Although, the extent of similarities and dissimilarities between the pathogens is still unknown.

4.3.5 *Potential for ECP metalloprotease*

All three *Tenacibaculum* species genomes contain metalloproteases, albeit from different protease families and not all containing a T9SS CTD. Since the work in this study focused on the supernatant, only metalloproteases with a T9SS CTD were examined. In the type-strain of *T. dicentrarchi* TD3509 (Piñeiro-Vidal et al., 2012), a functionally uncharacterized metalloprotease with a T9SS CTD [SOS52666] was found in the genome annotation by Bridel et al. (2018). This predicted protein of unknown function was shown to have a M36 family region (endopeptidase in

pathogenic fungi), which has the potential to cleave elastin (Markaryan et al., 1994) and keratin (Mercer & Stewart, 2019). It has also been shown to be involved in the pathogenesis of fungi (Wang et al., 2022). For reference, salmonid mucus contains keratin with anti-microbial properties (Molle et al., 2008), which is relevant since *T. dicentrarchi* can grow in fish mucus (Echeverría-Bugueño et al., 2023). This metalloprotease may aid in the colonization of the fish, but this has yet to be shown with any knockout studies.

In a genome annotation of the Chilean strain *T. dicentrarchi* TDCHD05 by Bridel et al. (2018), the bacterium was shown to encode the previously discussed uncharacterized metalloprotease (M36 family, or fungalysins) [SOU87347] and another uncharacterized metalloprotease (M43 family, or cytophagalysin) [SOU85710]. Cytophagalysin has been found in the secreted ECP of other pathogenic members of *Flavobacteriaceae*. The ECP (containing cytophagalysin) from *F. psychrophilum* have been shown to be capable of causing necrosis in rainbow trout muscles; further, this activity was inhibited with 1,10-phenanthroline (Zn2+ metalloprotease inhibitor) (Ostland et al., 2000). Although full genome sequencing by Nowlan et al. (2023) on a Canadian strain, *T. dicentrarchi* 18-3141, elucidated that the isolate was missing both of the uncharacterized metalloproteinases present in the Chilean strain and the type-strain. Additionally, there were no metalloproteases in the *T. dicentrarchi* 18-3141 genome that contained the T9SS CTD. It should be noted that the live pathogenesis differences between these *T. dicentrarchi* strains (genome containing metalloproteases vs not containing) is unknown. In the case of the *T. dicentrarchi* VI22 ST used in this experiment, it is not sequenced so the presence of any metalloproteinases with T9SS CTD in the genome is unknown. However, due to the partial reduction in cytotoxicity after EDTA treatment of the *T. dicentrarchi* ECP there may be a metalloprotease present that contributes to cytotoxicity (however this is only seen in one of the

two cellular viability measurements). It should also be noted that *T. dicentrarchi* ECP were harvested after 23–24°C growth incubation. This may be relevant since metalloprotease secretion from the T9SS may increase at lower growth temperatures (Secades et al., 2001).

Interestingly, *T. ovolyticum* and *T. dicentrarchi* have a lot of shared metalloproteases in their genomes. For example, a 449-nucleotide gene for RIP metalloprotease ResP [WBX76499] (*T. ovolyticum*) and [WBX68669] (*T. dicentrarchi*). However, no shared metalloproteases with suspected virulence or even a T9SS CTD. It may be reasonable to state that the Canadian *T. ovolyticum* WC23 isolate does not use a soluble ECP metalloprotease in the mechanism of secreted extracellular product cytotoxicity. This was demonstrated when there was no cytotoxicity knockdown after the treatment of *T. ovolyticum* ECP with EDTA. Therefore, there appears to be different mechanisms for secreted product(s) inducing salmonid cytotoxicity between the various *Tenacibaculum* species, and potentially even ECP differences between strains. Although this last point is not verified and should be considered in future experiments.

4.3.6 *Other cytotoxic pathways for T. dicentrarchi*

There are a number of well-known toxin pathways and peptidases encoded in some tenacibaculosis isolated *T. dicentrarchi* and *T. ovolyticum* strains. Thus far, potential virulence factors within the *T. dicentrarchi* annotations have included a serine endopeptidase, siderophores for iron acquisition, bacterial collagenases, and Fic proteins (Saldarriaga-Córdoba et al., 2021; Bridel et al., 2018). The above potential virulence factors that contain a T9SS CTD would be limited to bacterial collagenases [SOU86340] (*T. dicentrarchi* TDCHD05). Interestingly, Bridel et al (2018) pointed out that the *T. dicentrarchi* bacterial collagenase is highly similar to that of

Clostridium perfringens, which is involved in tissue necrosis (Matsushita et al., 1994). The Canadian isolate study by Nowlan et al. (2023), only showed this bacterial collagenase in the genomes of both *T. dicentrarchi* strains, only one of the two *T. finnmarkense* strains, and it was not present in the *T. ovolyticum* strain examined. It also appears that the majority of *T. dicentrarchi* strains encoded proteins with a T9SS CTD are uncharacterized and have unknown functions. It is possible that some of these uncharacterized proteins are secreted into the bacterial extracellular space and may be responsible for the virulence against salmonid cells, but there is currently no way of knowing their degree of involvement.

An article by Saldarriaga-Córdoba et al. (2021) details that some (but not all) Chilean *T. dicentrarchi* strains have a type II toxin-antitoxin system (*yefM-yeoB* locus), a type VI secretion system (T6SS), and/or the HigB-HigA toxin-antitoxin system. The T6SS encoded in *T. dicentrarchi* TDCHD05, containing the VgrG gene, may contribute to ECP cytotoxicity, but it wasn't identified in other *T. dicentrarchi* strains (Saldarriaga-Córdoba et al., 2021). Based on the study by Saldarriaga-Córdoba et al. (2021) there seems to be a core genome conservation of encoded virulence factors between *T. dicentrarchi* strains, such as peptidases and collagenases, but not in the toxin-antitoxin systems. This could be due to the variable genome of *T. dicentrarchi* (2.7-2.9 MB), resulting in a pool of genes with regional differences, due to the presence of genomic islands, but similar core genes (Nowlan et al., 2023). This is substantiated by Lagadec et al. (2021), with the suggestion of highly diversity, virulent strains, with relatively broad host pathogenicity in *Tenacibaculum* species. It should be noted that even a non-hemolytic strain (often associated with reduced pathogenesis) of *T. dicentrarchi* ST-160 was still shown to be pathogenic and associated with tenacibaculosis (Lagadec et al., 2021). Therefore, due to the inability to identify a non-pathogenic strain of *T. dicentrarchi*, ECP cytotoxicity (if involved in host pathogenesis) is likely

based in the conserved core genome and not genomic islands. Further, the presence of these additional virulence factors has yet to be demonstrated as helpful for bacterial infection and should be considered in the future. Ultimately, the main focus should likely stay within the core bacterial genome as this has the most broad and relevant application. Overall, there are not any clear candidates as virulence factors for salmonid cell cytotoxicity with *T. dicentrarchi* ECP, which is in contrast to what is seen with *T. maritimum*.

4.3.7 *Potential components responsible for T. ovolyticum ECP cytotoxicity*

T. ovolyticum genetics are not as well characterized as *T. maritimum* or *T. dicentrarchi*. Although, a number of different section system components have been found in the sequenced genomes of some *T. ovolyticum* strains. A study by Avendaño-Herrera et al. (2022), found genes for the type IV secretion system (T4SS), T6SS, and T1SS secretion systems in the genome of *T. ovolyticum* To-7Br, on top of the T9SS widely seen across the *Tenacibaculum* genera. A study by Nowlan et al. (2023), identified components of the T4SS, T6SS, T3SS, and T2SS, and T9SS in a Canadian *T. ovolyticum* isolate 20-4135-2. Both of these isolates were from farm salmonids with tenacibaculosis infections. However, a genome sequence of *T. ovolyticum* da5A-8 by Teramoto et al. (2016), found that the genome of that *T. ovolyticum* isolate only contained a T9SS. Although it should be noted that this bacterium was isolated from seawater and not from an infected host. Naturally, the question should arise as to if these additional secretion systems are necessary for bacterial virulence and pathogenicity. However, Teramoto et al. (2016) noted that shared potential virulence genes were found in this isolate as well as the fish pathogen *T. dicentrarchi* AY7486TD (now *T. finnmarkense* AY7486TD) (Grothusen et al. 2016). To our knowledge, there is no clear

virulence factor responsible for any ECP cytotoxicity, presently identified in the genome of *T. ovolyticum*. Therefore, examination should continue into how this cytotoxicity is induced.

4.3.8 ***Summary and future of Tenacibaculum spp. comparison***

It is possible that reported regional differences associated with tenacibaculosis could be due to the array of bacterial species involved, the strain-associated differences within species, or other unknown factors. This study highlights potential extracellular product differences between the strains of *Tenacibaculum* species, which may be responsible for regional differences in tenacibaculosis disease presentation, and the predominant associated bacterial species in the region. Although, this is just an interpretation of what could be happening. There could be other such reasons, such as different colonization capacities, biofilm formation, etc. A concrete experiment would involve comparing the cytotoxicity of ECP from various *T. dicentrarchi*/*T. ovolyticum* strains and determining if there is a relationship to infection salmonid trials, as well as the strain genetics. This could tie all of the genomics and functional work together, in order to identify the product of interest. These virulent product(s) from *Tenacibaculum* species could not be identified in the current study, but it did provide evidence that ECP cytotoxicity may be involved in bacterial pathogenesis, not just from *T. maritimum* in mouth rot infections. Further, cytotoxicity from these *Tenacibaculum* species may be associated with distinct product(s) and mechanisms. This was supported by visualizing the *Tenacibaculum* spp. secreted products on a gel to see obvious differences in proteins being secreted, as well as EDTA knocking down the ECP cytotoxicity to varying degrees, depending on the bacterial species.

4.4 Figures

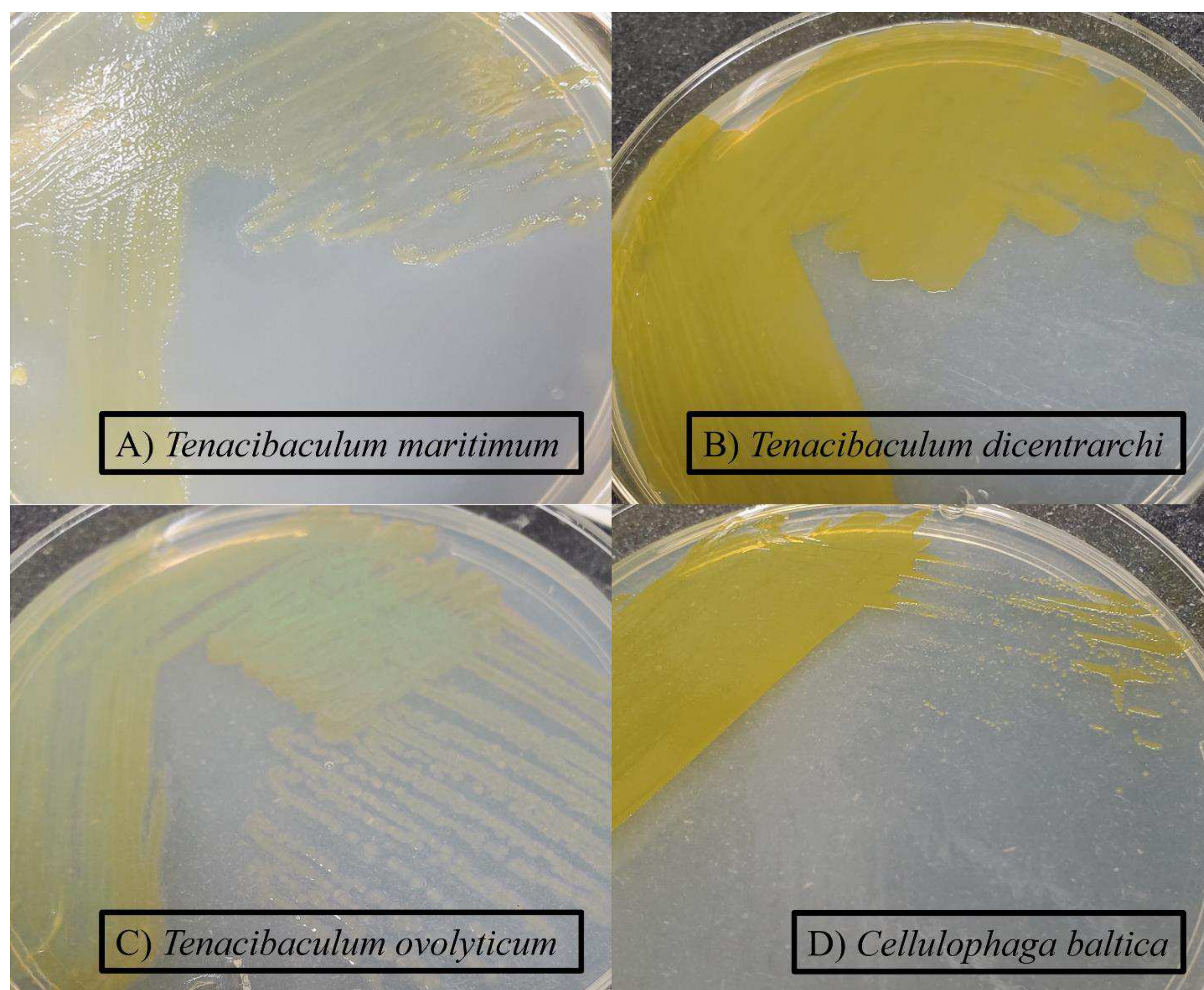


Fig 4.1 Images of bacterial plates, illustrating agar growth differences on Flexibacter marine media (FMM). *Tenacibaculum maritimum* (A), *Tenacibaculum dicentrarchi* (B), *Tenacibaculum ovolyticum* (C), *Cellulophaga baltica* (D). Images were taken after 72 h of 15°C growth on FMM+Kan Agar, from a -80°C 15% glycerol stock.

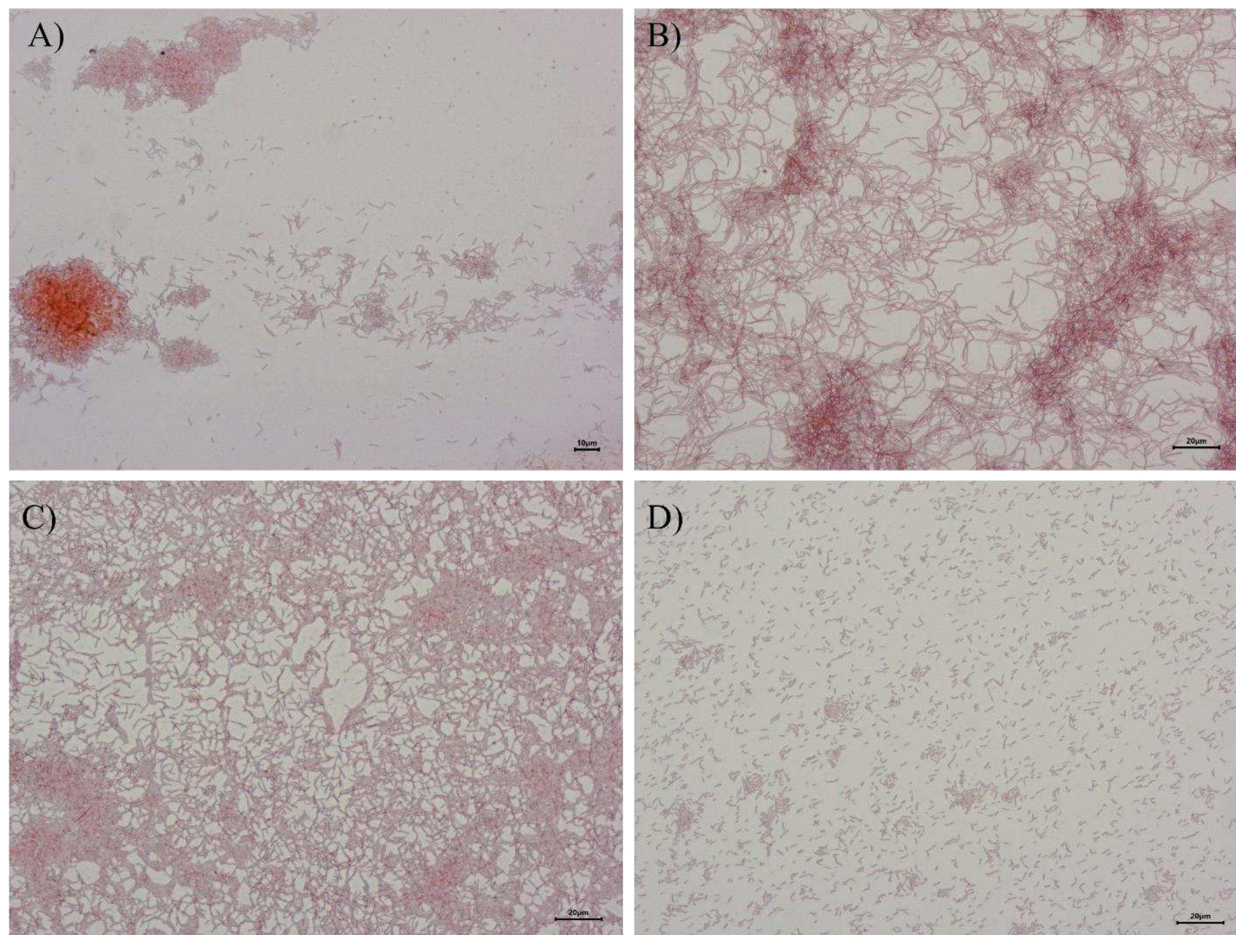
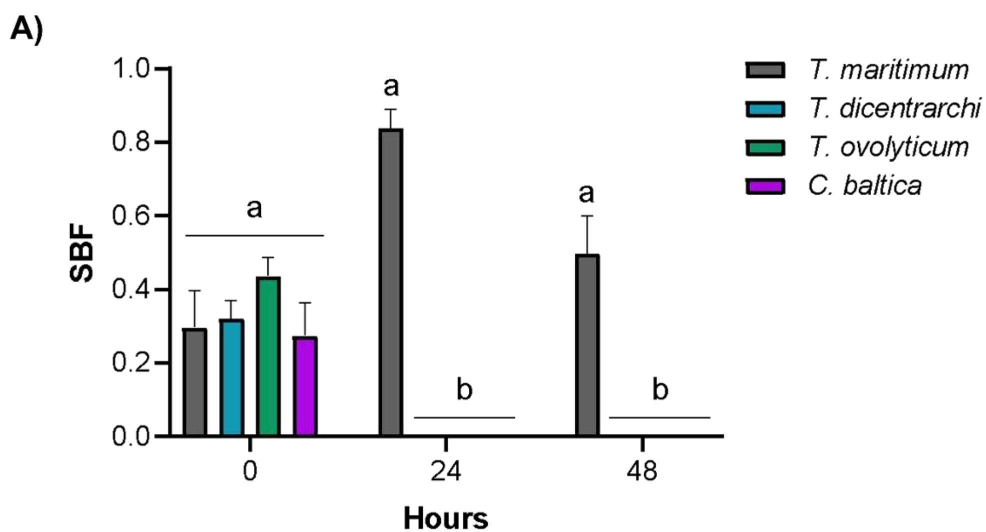


Fig 4.2 Microscopy images of the Gram-stained bacteria of interest. *Tenacibaculum maritimum* (A), *Tenacibaculum dicentrarchi* (B), *Tenacibaculum ovolyticum* (C), and *Cellulophaga baltica* (D). Images taken at 40x magnification with either a 10 μm or 20 μm ruler in the bottom right. Pink/reddish color indicates Gram-negative bacteria.



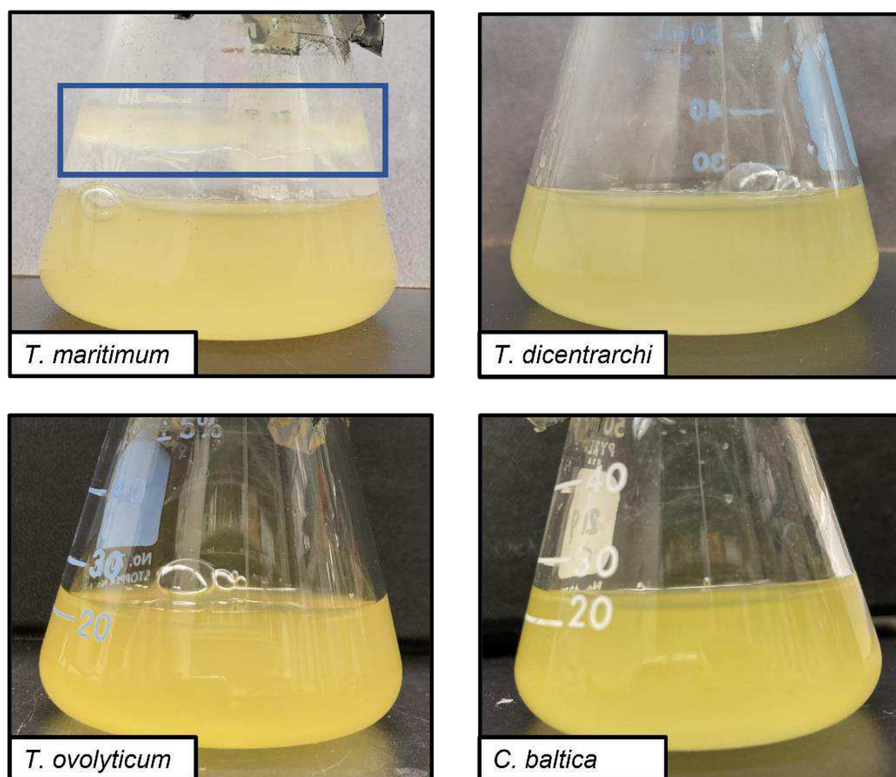


Fig 4.3 Biofilm quantification of various *Flavobacteriaceae* species with crystal violet (CV). Liquid broth cultures were seeded at similar cell density starting points and specific biofilm

formation (SBF) values were determined at 0, 24 and 48 h of 22–23°C shaking incubator growth (A). Different lower-case letters above the bars represent significant differences of at least $p<0.05$, $n=3$. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM. Representative pictures were taken at the 48 h timepoint to visually illustrate the difference in bacterial adherence to the Erlenmeyer flask (B).

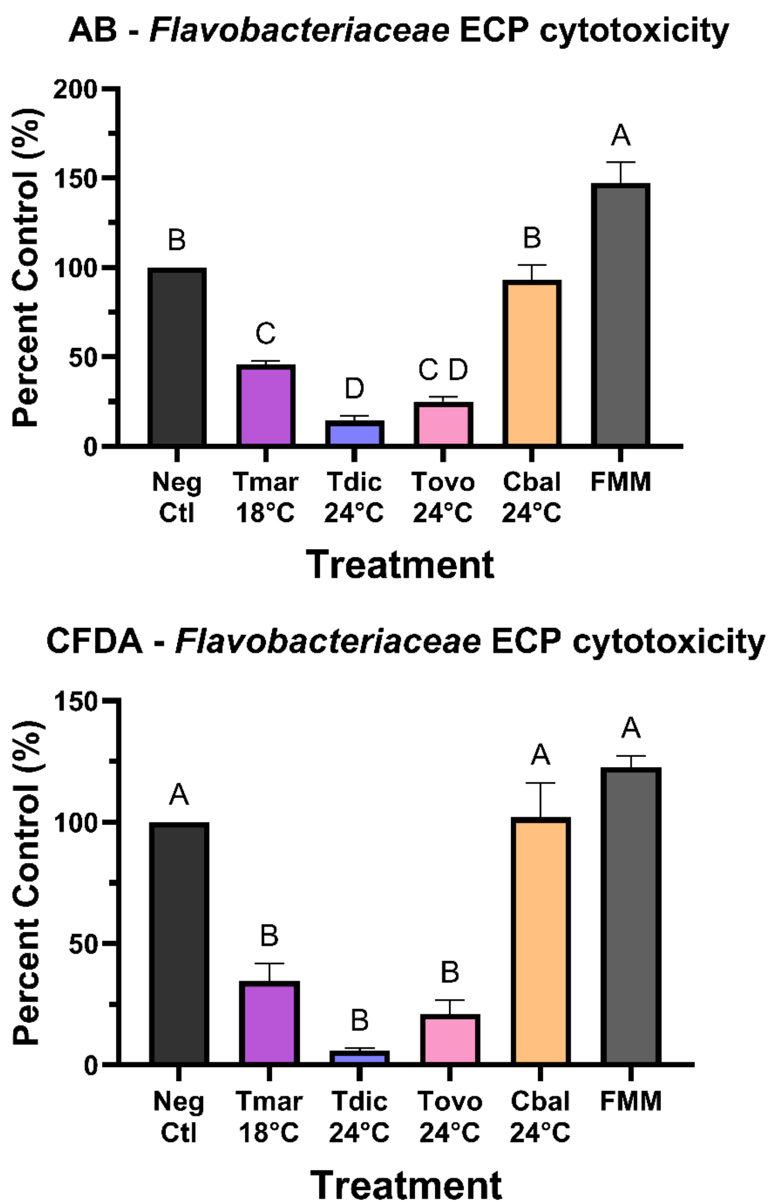


Fig 4.4 Cytotoxicity of various *Flavobacteriaceae* species secreted soluble extracellular products (ECP) against SHK-1 cells. The specific species examined were *T. maritimum* (Tmar), *T. dicentrarchi* (Tdic), *T. ovolyticum* (Tovo), and *C. baltica* (Cbal). Bacterial growth temperatures are listed below the bacterial species. SHK-1 cells were treated with the extracellular products (ECP) for 25 h before measuring metabolism with Alamar blue (AB) (A) and membrane

integrity with 5-Carboxyfluorescein Diacetate (CFDA) (B). The cellular viability for each treatment is relative to the percent control (%) of untreated cells (Neg Ctl). Different lower-case letters above the bars represent significant differences of at least $p<0.05$, $n=3$. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

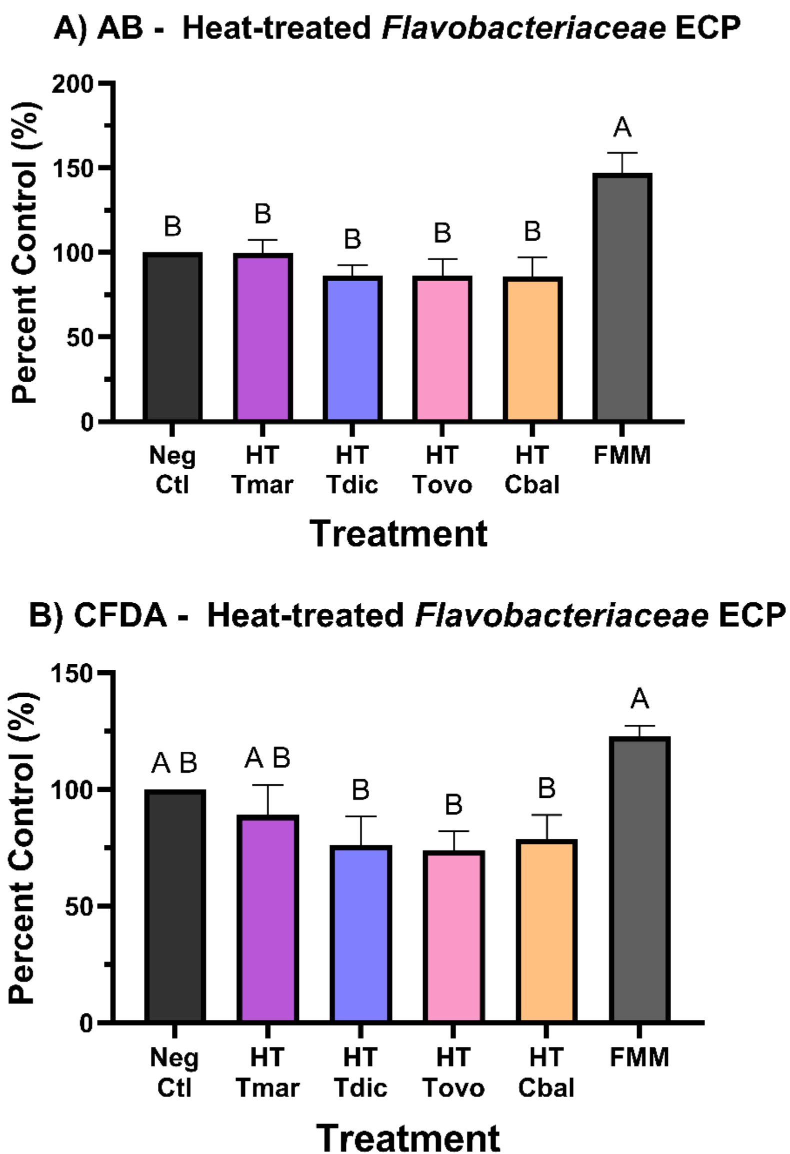


Fig 4.5 The cytotoxicity of soluble extracellular products (ECP) from various *Flavobacteriaceae* species after ECP heat-treatment (HT) of 100°C for 10 min. The specific species examined were *T. maritimum* (Tmar), *T. dicentrarchi* (Tdic), *T. ovolyticum* (Tovo), and *C. baltica* (Cbal). SHK-1 cells were treated with HT ECPs for 24 h before measuring metabolism with Alamar blue (AB) (A) and membrane integrity with 5-Carboxyfluorescein Diacetate (CFDA) (B). The cellular viability for each treatment is relative to the percent control (%) of untreated cells (Neg Ctl).

Different lower-case letters above the bars represent significant differences of at least $p<0.05$, $n=3$. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

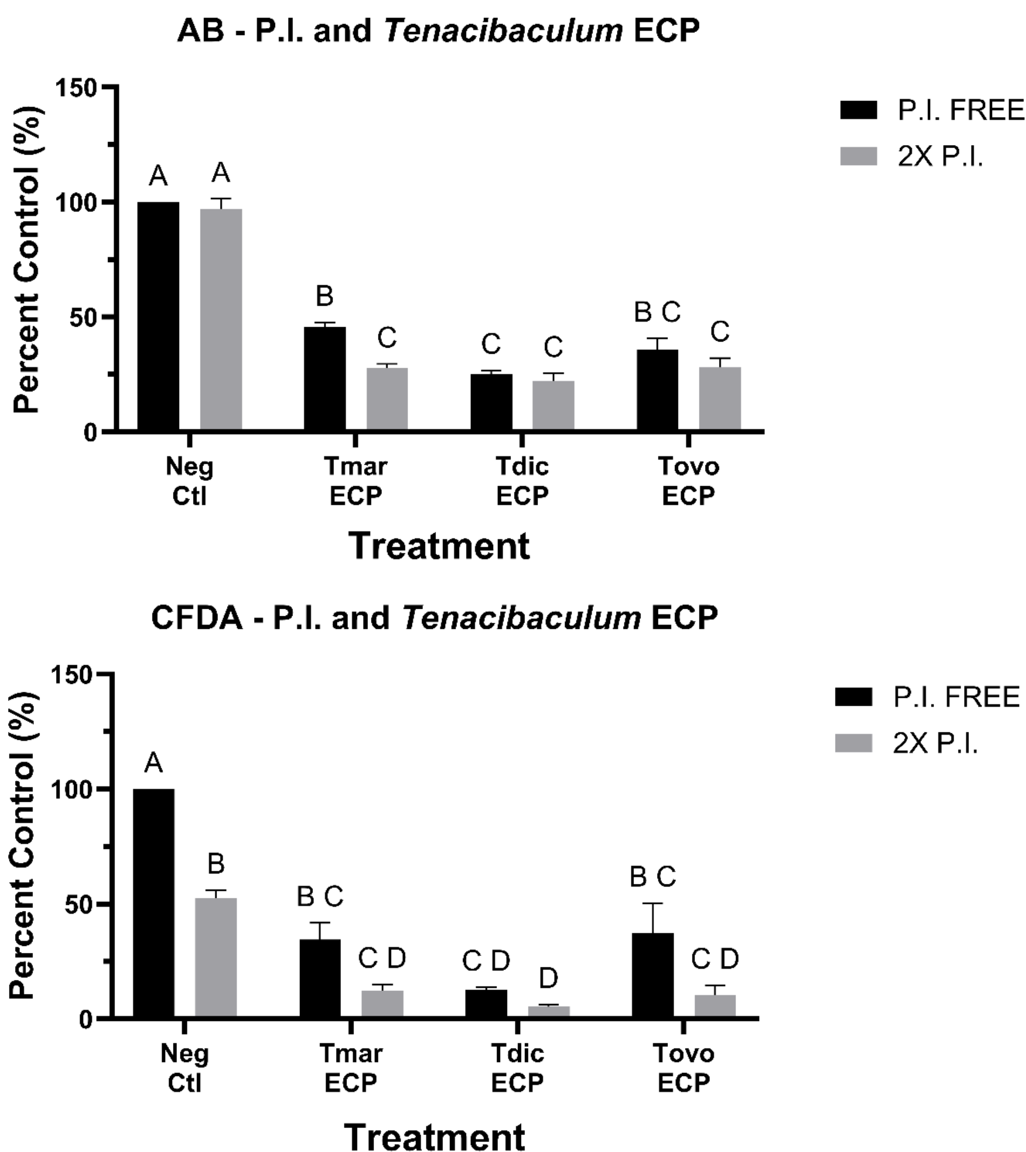


Fig 4.6 Protease inhibitor cocktail (P.I.) treatment of various mouth rot associated *Flavobacteriaceae* extracellular products (ECP). The specific species examined were *T. maritimum* (Tmar), *T. dicentrarchi* (Tdic), *T. ovolyticum* (Tovo), and *C. baltica* (Cbal). SHK-1 cells were treated with ECP under P.I. free or 2X P.I. conditions for 24 h before measuring metabolism with Alamar blue (AB) (A) and membrane integrity with 5-Carboxyfluorescein Diacetate (CFDA) (B). The cellular viability for each treatment is relative to the percent control

(%) of untreated cells (Neg Ctl). Different lower-case letters above the bars represent significant differences of at least $p<0.05$, $n=3$. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

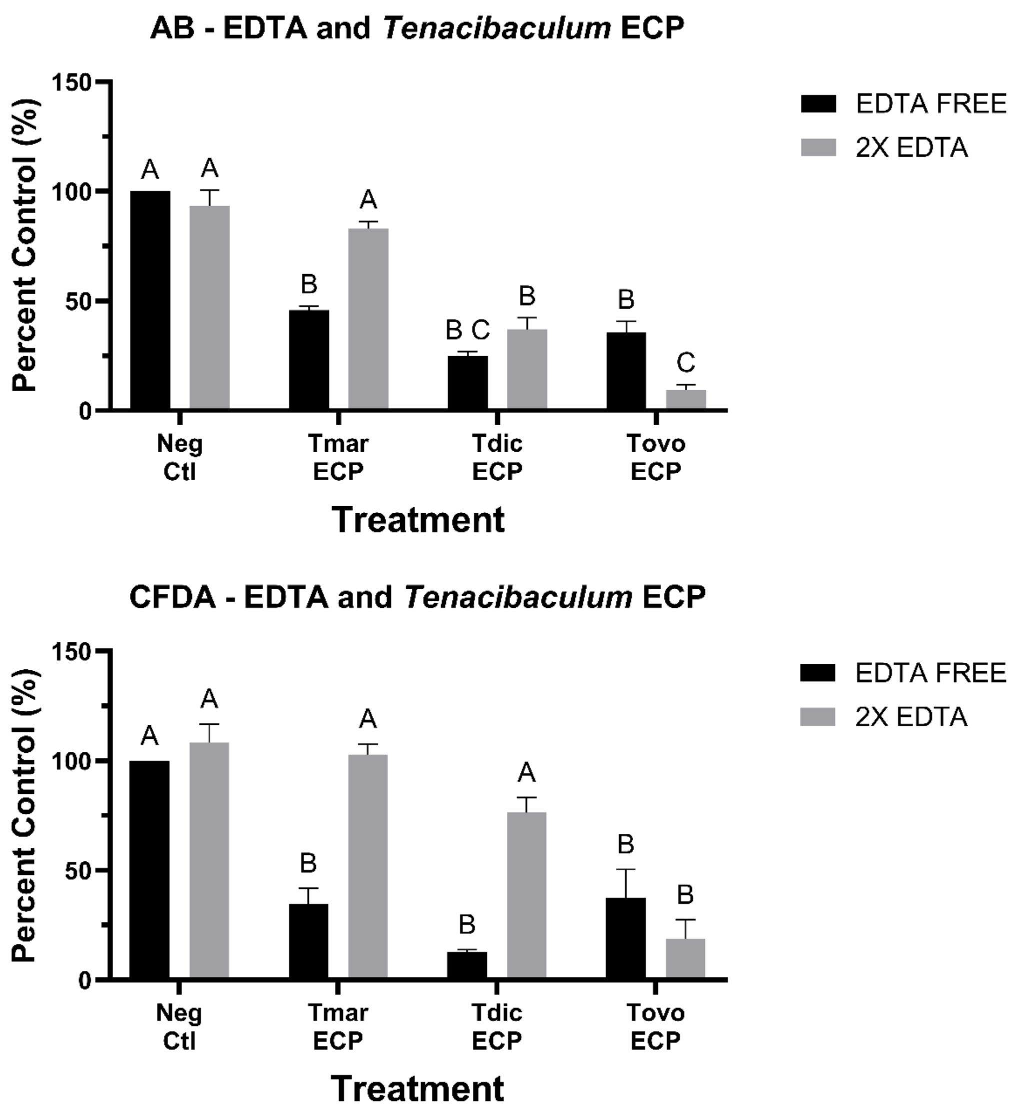


Fig 4.7 Ethylenediaminetetraacetic acid (EDTA) treatment of various mouth rot associated *Flavobacteriaceae* extracellular products (ECP). The specific species examined were *T. maritimum* (Tmar), *T. dicentrarchi* (Tdic), *T. ovolyticum* (Tovo), and *C. baltica* (Cbal). SHK-1 cells were treated with ECP under EDTA free or 2X EDTA conditions for 24 h before measuring metabolism with Alamar blue (AB) (A) and membrane integrity with 5-Carboxyfluorescein Diacetate (CFDA) (B). The cellular viability for each treatment is relative to the percent control

(%) of untreated cells (Neg Ctl). Different lower-case letters above the bars represent significant differences of at least $p<0.05$, n=3. Lower case letters that are the same indicate no significant difference between treatments. Error bars represent +SEM.

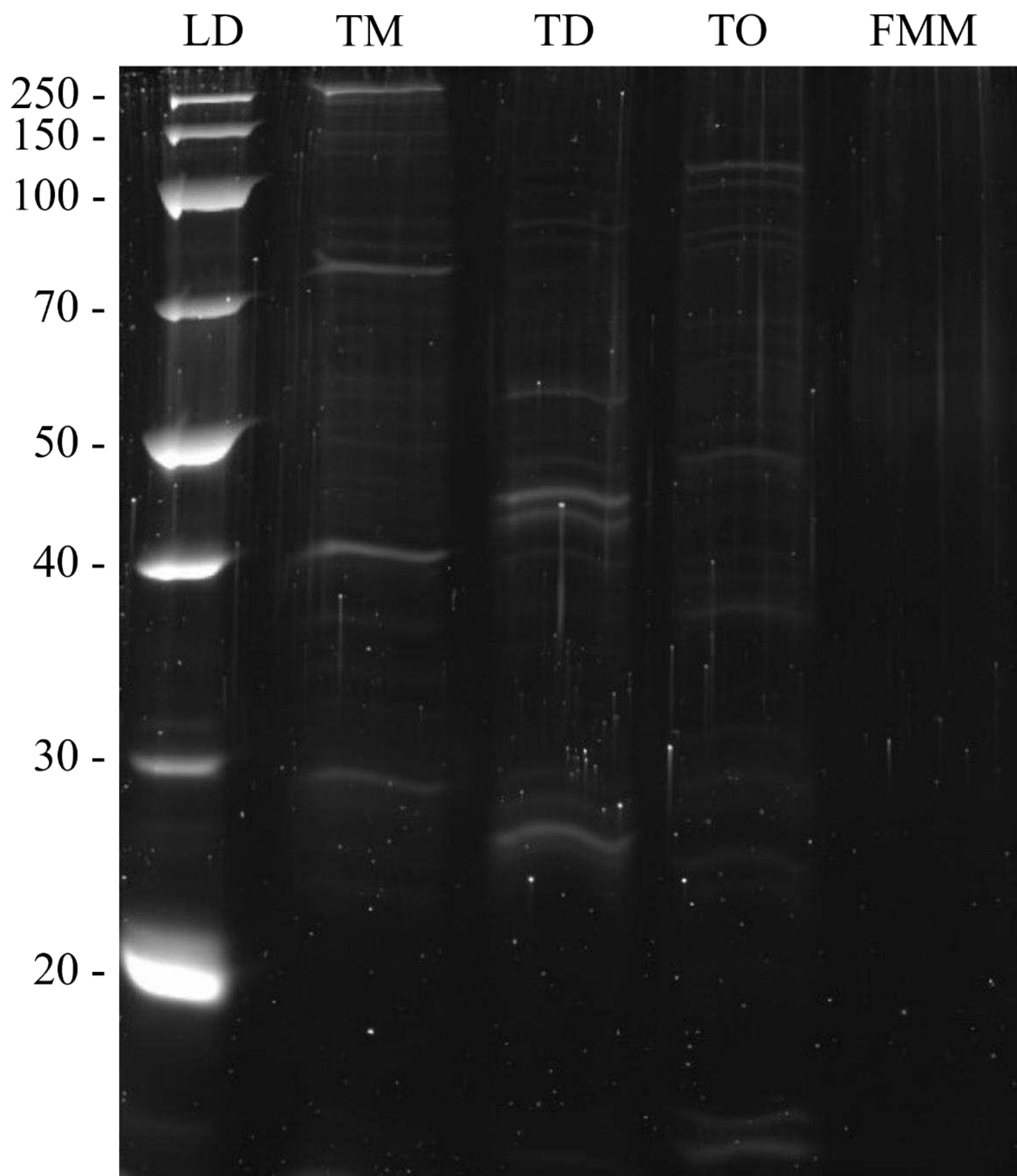


Fig 4.8 Supernatants of *T. maritimum* (TM), *T. dicentrarchi* (TD), and *T. ovolyticum* (TO) on an SDS-PAGE gel and visualized with SYPRO Ruby protein gel stain.

Chapter 5: Discussion

5.1 Introduction

5.1.1 *Relevance and Importance of Tenacibaculum research*

As discussed in **chapter 1**, tenacibaculosis is a prominent disease worldwide, responsible for a large portion of aquaculture antibiotic use and industry profit loss. Mouth rot (regional presentation of tenacibaculosis) is defined by a lack of knowledge around how its bacterial pathogen, *T. maritimum*, causes Atlantic salmon mortality. Consequently, more research was needed (and still needs) to be done on how the bacteria interacts with host cells and if there is a potential toxin secreted from the bacteria that may contribute to fish mortality. The work in this study has the potential to increase the understanding of which factors promote mouth rot disease, as well as discover potential weak points that can be targeted by the industry. The overall long-term goal is to aid in the eradication of this disease from aquaculture to allow for a more sustainable industry, with a lower perceived risk to the wild salmon populations. Given the international impact of other *Tenacibaculum* species, and their potential contributions to mouth rot, it was also critical to analyze the potential for toxin secretion from these related pathogens. Further, there is even less research done on these bacteria than *T. maritimum*, which makes this study important on an international level.

5.1.2 *The value of in vitro cell lines for understanding ECP cytotoxicity*

Cell lines are useful tools that can help enhance pathogen understanding on a fundamental level, due to their ease of access, reproducibility, and cost-effectiveness (Goswami et al., 2022). Two of the cell lines used in this study originated from salmonid mucosal epithelial tissue,

ASimf20 and RTgutGC (See **chapter 2** for more details on the cell lines). This is relevant since mouth rot infection manifests symptomatology afflicting the skin mucosal epithelium tissues (such as the mouth, gills, and skin) (Frisch et al., 2018a), which made these cell lines appropriate models for measuring the effect of soluble *T. maritimum* ECP on salmonids. *Tenacibaculum* species have also been detected and isolated from internal organs of the fish (including the kidney) (Nowlan et al., 2021a; Yardimci and Timur, 2016; Frisch et al., 2018b; Klakegg et al., 2019), which makes the SHK cell line important for examining the effect that the soluble *Tenacibaculum* ECP have on kidney origin cells. Interestingly, more traditional ECP characterizations have measured the activity (sialidase, sphingomyelinase, etc.) of the secreted bacterial products from *T. maritimum* (Pérez-Pascual et al., 2017), or have injected the supernatant into fish to see its effect (Van Gelderen et al., 2009; Escribano et al., 2023). These methods have limitations regarding the quantification of cell death caused by these secreted products and ultimately the virulence factor's mechanism of action on cells. With the use of relevant cell lines (ASimf20, RTgutGC, and SHK-1), these tools can allow for the assessment and comparison of the cytotoxicity from various proposed bacterial pathogens. Fish are expensive, are of ethics concerns, and require aquatics facilities compared to incubating cells in a flask, which is why cell lines are useful in the context of testing toxicity (Schirmer, 2006). Though this is mainly referring to toxicology, it can pertain to toxicity from secreted bacterial products as well. Further, cytotoxicity rather than activity may be better for identifying the main product of interest in supernatant. This would be through the use of EDTA or protease inhibitors, where activity of a protease might be inhibited but it may not have an impact on salmonid cell death.

5.1.3 *Application and justification of cell death assays*

The first (and most detailed) cell death assay performed was the annexin V/PI assay that sorted and quantified cells into various categories of cell death stages. Fortunately, our lab had access to an Imagestream flow cytometer and previously did some innovative methods work regarding this assay (Rieger et al., 2011). The Imagestream flow cytometer allowed for visualization of positive and negative cell death populations at a single cell level; this added more confidence to the assay and allowed for the assessment of mechanism regarding *T. maritimum* ECP induction of host cell death. Some downsides associated with this assay was the cost (reagents and price to run hourly), the time associated with acquiring data (time to run and perform assay steps, equaling roughly 6 hours every time), and the large number of cells required for a single datapoint (10,000 cells counted). Another assay was selected for a more rapid assessment of general cell death from ECP exposure, which was the AB (Rampersad, 2012) and CFDA (Kamiloglu et al., 2020) assay. This assay used two measurements for quantifying general cell death in an exposure population to give greater depth than one measurement alone (Schreer et al., 2005). This assay was less expensive, much less time consuming (1.5 hours total), and used just over half the number of cells per data point (6,000). Considering that fish cells can take a long time to grow, this assay saved time, money, and allowed for the assessment of additional inhibition trials (EDTA, Heat-treatment, and protease inhibitors) not in the original project aims. Overall, both assays were critical to the results of this study and should be considered for future experiments examining the cytotoxicity of bacterial soluble extracellular products on salmonid cells.

5.1.4 *Inhibitors of the secreted Tenacibaculum product(s) cytotoxic mechanism*

The inactivation of the cytotoxic bacterial products followed by testing the products on cells was difficult to troubleshoot. Firstly, the inactivation of protease activity is normally done by measuring the cleavage of a protein after adding ECPs and a single inactivating agent (Lee et al., 1999); however, in our case, a lack of protease activity does not necessarily mean loss of cytotoxicity. Therefore, since an already established measurement for cytotoxicity has been established in our study, it makes sense to apply the inactivation experiment to cells instead of protein only. Interestingly, the protease inhibitors did not reduce the ECP cytotoxicity in any of the trials. This could be due to a number of reasons, such as the concentration of DMSO (dissolved the protease inhibitors) causing cytotoxicity itself (Sangweni et al., 2021), the individual inhibitors not being in a high enough concentration inhibit the ECP, or that the inhibitors are ineffective at impairing the protease toxin activity (as discussed in the review by Supuran et al. (2002)). There may be a reason that, to our knowledge, no one has published their attempt to inactivate a bacterial toxin with a commercially available protease inhibitor cocktail. Altogether, the next step should be acquiring more effective protease inhibitors (such as various cysteine and serine protease inhibitors from Lee et al. (1999), as well as others) that may target the specific activity of the proteases in the *Tenacibaculum* ECP. This should hopefully inform researchers if there is a combination of products responsible for cytotoxicity (not just a metalloprotease for *T. maritimum*), as well as being able to better narrow down the cytotoxic secreted product(s) through matching the specific action of the individual inhibitors to the active sites of identified putative toxins in the genomes.

Broad ranging metal ion chelators have been used for impairing metalloprotease toxins before, such as botulinum and tetanus toxins in Simpson et al. (1993). In the current study EDTA

(a well-known chelator) was effective at eliminating cytotoxicity from *T. maritimum* secreted products, which happens to have some putative virulence factor metalloproteases encoded in the core genome (Nowlan et al., 2023). However, more specific metalloprotease inhibitors exist, such as o-Phenanthroline (1,10-Phenanthroline), which is essentially a Zinc (Zn^{2+}) metal chelator with a history of metalloprotease toxin inactivation use (Bjarnason et al., 1987). If the secreted virulence factor is a Zn^{2+} metalloprotease, then its action should be inhibited by o-Phenanthroline when combined. This would allow for higher confidence in the results that the secreted metalloprotease is the product of interest from *T. maritimum*. This is a logical continuation of the experiment and may provide clarity to the results regarding the partial inhibition of *T. dicentrarchi* ECP cytotoxicity with EDTA.

5.2 Original hypotheses

The original hypothesis of my MSc project was to 1) test if *T. maritimum* secretes a toxin(s) that causes salmonid cell death, 2) identify environmental factors that may increase virulence factor production to promote pathogenesis, and 3) test if *Tenacibaculum* species had a similar ECP mechanism of cell death induction and growth capacities, which would explain why tenacibaculosis is a wide-ranging disease with lots of associated bacterial pathogens. Although the first two hypotheses were accepted, the last hypothesis was rejected based on the results of the *in vitro* study.

5.3 Conclusions and Implications

It was determined through validated assays that *T. maritimum* secretes a toxin that induced apoptotic cellular death in host salmonid cells. The secretion of this unknown virulence factor(s) from *T. maritimum* can likely increase under cold incubator conditions (12°C vs 24°C) resulting in greater cytotoxicity of the ECP, however the increase was not confirmed. Further experimentation revealed that one contributor to *T. maritimum* secreted product cytotoxicity may be an extracellular metalloprotease, since this product would be inactivated with heat-treatment and EDTA (as seen in the current study). Interestingly, it was shown that the *Tenacibaculum* species examined in the study secreted different ECP (visualized on SDS-PAGE), and that the inactivation assays (EDTA and P.I.) suggest differential cytotoxic mechanisms. Therefore, *Tenacibaculum* species may not be as interchangeable regarding disease as once imagined; meaning that there may be more depth to the differences between tenacibaculosis presentations and the pathogenic microbes involved that has yet to be elucidated. The majority of the results in this study were supported by recent literature, including the rejected hypothesis.

Implications from these conclusions regard documenting the possible reasons as to why the environmental trends of mouth rot disease are observed. The identification of a primary *T. maritimum* product for virulence may allow for a targeted vaccine against the product, resulting in protection of these salmonids from the disease. Both of these implications are important to the aquaculture industry through their possible contribution to improved fish welfare and potential economic savings. To our knowledge there is also novelty in these thesis experiments, regarding the fish time biofilms from *Tenacibaculum* species have been directly compared, the first time *T. dicentrarchi* and *T. ovolyticum* soluble extracellular products have been examined for cytotoxicity

against salmonid cells, and the first time a protease inhibitor cocktail and EDTA have been used to attempt a knockdown of cytotoxicity from soluble extracellular products, not just activity.

5.4 Future directions

5.4.1 *Comparison of Tenacibaculum isolates internationally and locally*

There are some interesting differences in tenacibaculosis disease internationally and regionally between sites. Interestingly, tenacibaculosis disease manifests itself in different ways depending on the region. For example, mouth rot in North America is primarily associated with *T. maritimum* (Frisch et al., 2018c), whereas Atlantic salmon tenacibaculosis in Norway appears to be associated with *T. finnmarkense* (Spilsberg et al., 2022) or *T. dicentrarchi* (Klakegg et al., 2019). This is despite both locations using the same Norwegian origin of farmed fish (meaning it is likely not the host). Interestingly, Spilsberg et al. (2022) stated that the differences between *Tenacibaculum* caused outbreaks might be due to temperature, as *T. finnmarkense* outbreaks occurred from 4–6°C whereas *T. dicentrarchi* outbreaks occurred at 12°C and 18°C (per references cited earlier). *T. maritimum* has also been isolated from Norwegian aquaculture sites (Småge et al., 2016b), but does not appear to be the main tenacibaculosis threat for Atlantic salmon mortality (Olsen et al., 2017). It should be noted that there are also differences between Canadian *T. maritimum* isolates regarding their ability to induce disease mortality in infection trials (Frisch et al., 2018b). High *Tenacibaculum* strain diversity was reported in Norway by Lagadec et al. (2021), which mentioned that low hemolytic strains may be less pathogenic compared to highly hemolytic strains. Besides genome examination and basic phenotypic characteristics, no one outside of this MSc project (to our knowledge) had examined the functional differences between geographically distinct *Tenacibaculum* isolates. Therefore, there could be differences in the protein production of

these isolates, which may be directly involved in tenacibaculosis disease and the bacterium's pathogenicity towards Atlantic salmon. Answers to these questions could explain the mysterious differences regarding this disease.

A simple place to start would be repeating the annexin V/PI assay with the other *Tenacibaculum* bacteria (such as *T. dicentrarchi*, *T. ovolyticum*, and even adding in *T. finnmarkense*). This would allow for a mechanistic comparison between the cytotoxic pathway induced by the ECP of all three *Tenacibaculum* species. Another intriguing idea would be a competition assay between *Tenacibaculum* species. Seeing if under certain conditions, some species out compete with others or if the secreted products from certain bacteria inhibit the growth of others. This can be done in an experiment set up like the *Aliivibrio wodanis* versus *Moritella viscosa* co-cultivation assay in an article by Hjerde et al. (2015). The compared bacteria could be *T. maritimum*, *T. dicentrarchi*, and *T. finnmarkense* together in various combinations and under various conditions to see if there are different dominant bacteria in different conditions (nutrient rich/poor, growth temperatures, oxygen content, presence of salmonid serum). Overall, this could elucidate the connections between bacteria using a simple *in vitro* method, as well as the influence of environmental conditions on these other species.

5.4.2 *Improvements to understanding the effects of environmental conditions*

Alternatively, more examination could be done on what happens at these lower temperatures (elevated ECP cytotoxicity) and if the M12B metalloprotease, proposed in this thesis as the primary virulence factor, is truly increasing in concentration. If so, looking into the mechanism of increase and if this same low temperature increase occurs in Chilean *Tenacibaculum*

isolates (often associated with disease at higher temperatures) would be interesting to see. Specifically, identifying the virulence factor transcription machinery and if it is triggered differently in a geographically distinct isolate accustomed to higher temperatures. Some work on virulence factor transcription has already been done in a related *Flavobacteriaceae* described in an article by Guérin et al. (2021), where *F. psychrophilum* upregulates virulence genes in certain conditions, and down regulates them in others (e.g. downregulates fpp1-fpp2 in high salinity). However, this work has not been done on *T. maritimum* but could greatly increase the understanding of this pathogen's virulence strategies and the differences between geographically distinct isolates. Further, it might be a good idea to expand the examination of ECP cytotoxicity at various environmental conditions to the other *Tenacibaculum* species. This may be critical information for the greater aquaculture industry, regarding the prediction of when tenacibaculosis infections may occur and which *Tenacibaculum* species would be dominant during that outbreak. This could theoretically allow for faster and more targeted treatments by only having to know the environmental conditions in order to assume/predict the primary disease factor (instead of waiting for a qPCR or the growth of the isolate on a marine agar plate); although, that potential use may be more of a pie in the sky idea.

5.4.3 *Identification of the virulent ECP in an in vivo mouth rot infection*

Identifying the presence and action of virulence factors is a necessary step for elucidating the pathogenesis of the disease. However, the next step is the identification of secreted product(s) *in vivo* that were responsible for the cytotoxicity demonstrated *in vitro*. If diseased individuals have the bacterial toxins present in systemic circulation, whereas as healthy individuals do not have any signs of bacterial toxins, then this would likely indicate that the soluble virulence factor(s)

contribute to disease progression. Furthermore, this would allow for better pointed research into a vaccine against the toxin, which may be more effective than a vaccine against the bacteria (unsuccessful thus far). Additionally, this proteomics assay can be expanded internationally to other countries for their tenacibaculosis infected salmon as well, not just mouth rot infected Atlantic salmon. This would hopefully help identify problematic secreted products in bacteria such as *T. dicentrarchi* and *T. finnmarkense*, and if fish develop immunity naturally against these secreted products. Further, negative data here would also be useful since it would indicate that the secreted extracellular products are less of a concern regarding virulence factor induced systemic toxicity. Much can be learned from this experiment examining the serum of infected and healthy Atlantic salmon at different points at sea and should be assessed with urgency.

This may also pertain to the M12B Zinc metalloprotease that may be secreted by *T. maritimum.* Identification of the product responsible for the cytotoxicity of salmonid cells would result in easier application of research and treatment methods. An experimental idea is to take the EDTA inactivation a step further and inactivate the M12B metalloprotease with a Zn specific cofactor inactivating products, such as diethylenetriaminepentaacetic acid (DTPA) or N,N,N',N'-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) (Cho et al., 2007). If inactivation of the cytotoxic ECP is seen after Zn chelator addition, then it is likely the product identified in this thesis. Although, more work must follow to confirm.

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Supplementary

Statement of Contributions

Chapter 1 – This chapter provides an overview of the current literature for understanding the topics, goals, and the importance of this thesis to aquaculture industry and the greater scientific community. To this date, this literature review remains unpublished. The writing of this chapter was generated solely by the primary author. Dr. Barreda reviewed, made suggestions, and approved this chapter for publication. No A.I. was used in the generation of this chapter.

Chapter 2 – This chapter contains the methodology and information necessary to understand the experiments and protocols contained in this thesis. Parts of this chapter were published in the paper referenced in the Chapter 3 Preface below. The writing of this chapter was generated by the primary author, but the methods are from the Barreda lab. References to previous work are included where appropriate. Dr. Barreda reviewed, made suggestions, and approved this chapter for publication. No A.I. was used in the generation of this chapter.

Chapter 3 – This chapter dives into the soluble extracellular product characterization of *Tenacibaculum maritimum*. It contains a brief introduction, results, and discussion regarding the cytotoxicity and potential mechanisms of the secreted extracellular products from this bacterium. A large portion of this work is published under "The use of salmonid epithelial cells to characterize the toxicity of *Tenacibaculum maritimum* soluble extracellular products" in the Journal of Applied Microbiology. Fellow manuscript authors were Dr. Shawna L Semple, Reema N Joshi, DVM Patrick Whittaker, and Dr. Daniel R Barreda. Outside of the salinity data collected by Patrick Whittaker, all other data, analysis, and manuscript writing shown in this chapter were generated by the primary author. Dr. Barreda reviewed, made suggestions, and approved this chapter for publication. No A.I. was used in the generation of this chapter.

Chapter 4 – This chapter dives into the comparison between secreted extracellular products from various related and potentially pathogenic *Tenacibaculum* species. It contains a brief introduction, results, and discussion regarding the different bacteria examined (*Tenacibaculum dicentrarchi*, *Tenacibaculum ovolyticum*, and *Cellulophaga baltica*). To this date, this work remains unpublished. Generation of data, figures, and intellectual ideas were in a large part possible with the help of Shawna L Semple. Specifically, the generation of the *T. ovolyticum* and *C. baltica* biofilm data and the *Flavobacteriaceae* ECP cytotoxicity in SHK cells. All other data, analysis, and writing in this chapter was generated by the primary author. Dr. Barreda reviewed, made suggestions, and approved this chapter for publication. No A.I. was used in the generation of this chapter.

Chapter 5 – This chapter consists of a general discussion of the topic. It states the greater scope, conclusions, and future directions assessed using the results obtained in this thesis. This will also include potential implications. To this date, this work remains unpublished. The writing of this chapter was generated solely by the primary author. Dr. Barreda reviewed, made suggestions, and approved this chapter for publication. No A.I. was used in the generation of this chapter.