Investigating the effects of synthetic peptides on aquatic pathogens and rainbow trout immunity

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The following people contributed to the work undertaken in this thesis:

Chapter 1

Sascha R. Brunner helped search for potential peptides within the select criteria.

Chapter 2, 3 and 4

Sascha R. Brunner helped test primers for antimicrobial peptide genes and insulin like growth factor receptor gene via RT-PCR.

Jenny Huynh helped test primers via RT-qPCR and assisted in determining the primer efficiency for each primer set.

Abstract

The demand for fish and seafood has increased exponentially over the past decade and continues to increase. Feeding fish has also become problematic as currently, the industry is dependent on fish meal which is not sustainable. Therefore, alternative protein sources are being investigated. However, some alternative protein sources such as plant-based protein has been shown to cause gut inflammation in salmonids. Aquaculture operations are also in a constant battle with keeping fish free of disease and pathogens have developed resistance to current drugs. Novel drugs are desperately needed to combat aquatic pathogens. The current work explores fish immunology by using novel peptide sequences derived from aquatic organisms and has identified one sequence, rtVWF, to possess antibacterial activity against an important fish pathogen, *S. iniae*. rtVWF was also found to be non-hemolytic which is a good indicator for future *in vivo* use. The monocyte/macrophage-like cell line, RTS11, upregulates tumor necrosis factor alpha in response to *S. ininae*. Besides immune cells, the gut, liver and gill epithelium of fish also play an important role in immune defense. In the current work, the immune response of these cell types: RTgutGC, RTL-W1 and RT-gillW1, respectively, was explored. First, in the presence of another aquatic pathogen, *F. psychrophilum* and subsequently, in the presence of alternative feedstuffs such as soybean protein and mealworm protein. In the presence of *F. psychrophilum*, RTgutGC cells significantly upregulate the proinflammatory cytokine interleukin 1 beta. When the same cells were co-cultured with RTS11, the RTS11 cells significantly upregulated tumor necrosis factor alpha. This suggests the gut epithelium of fish may depend on immune cells to activate tumor necrosis factor alpha signalling during *F. psychrophilum* infection. Exposing RTgutGC cells and RTL-W1 cells to soybean and mealworm protein did not have a significant effect on cell viability, cell adhesion, immune gene expression or wound healing but nevertheless this model provides a new model to study nutrient-liver interactions. Collectively, the results of this study expand our current understanding of host-pathogen interactions in fish immunology but also provide new avenues for investigation in fish physiology.

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Table 1. Aquatic pathogens used in this study and their general characteristics.

List of Abbreviations

ABC - ATP-binding cassette-type ATPases

AMR - Antimicrobial resistance

ATP - Adenosine Triphosphate

BCAAs - Branched chain amino acids

BCWD - Bacterial cold-water disease

Cath2 - Cathelicidin 2

CHSE-214 - Chinook salmon embryo

CPE - Cytopathic effects

EPC - Epithelioma papulosum cyprini

ERD - Enteric redmouth disease

FV3 - Frog virus 3

GALT - Gut-associated lymphoid tissue

GIALT - Gill-associated lymphoid tissue

glD - Gliding motility-associated lipoprotein D

Il1b - Interleukin 1 beta

Il6 - Interleukin 6

IPNV - Infectious pancreatic necrosis virus

IRF1 - Interferon regulatory factor 1

L-15 – Leibowitz’s medium

LPS - Lipopolysaccharide

MALT - Skin-associated lymphoid tissue

MIC - Minimal inhibitory concentration

MTT - Thiazolyl Blue Tetrazolium Bromide

NALT - Nasopharynx-associated lymphoid tissue

PAMPs - Pathogen-associated molecular patterns

PFU – Plaque-forming units

QTL - Quantitative trait loci

RTFS - Rainbow trout fry syndrome

SNP – Single nucleotide polymorphism

SOCS 1-3 - Suppressor of cytokine signaling family genes

$TCID_{50}$/Ml - Tissue culture infectious dose

VHS - Viral hemorrhagic septicemia

VHSV IVb - Viral hemorrhagic septicemia virus

Chapter 1 – Introduction

1.1.1 *Aquaculture*

Fish and seafood are an important source of protein in many developing countries and the demand for these products has increased globally (Reverter et al. 2020). Aquaculture, the practice of rearing or cultivating aquatic animals and plants for food; has rapidly expanded to meet the demand (Naylor et al. 2020). The aquaculture sector contributes $2.1 billion to the gross domestic product of Canada and the annual production volume is 187,026 tonnes, valued at $1.23 billion (Canadian Aquaculture Industry Alliance). Finfish dominate the Canadian aquaculture sector, and 26 species of finish are cultivated, of which salmon is the most significant, followed by trout and Arctic char (Fisheries and Oceans Canada). Some of these species are native to Canada which presents unique challenges to aquaculture, such as management of escapees and the consequences of breeding with wild populations, or transmission of diseases between domestic and wild fish populations. The prevalence of disease depends on a susceptible host, favorable environment, and pathogen.

Environmental DNA from multiple fish pathogens has been observed to be elevated near active salmon farms (Shea et al. 2020). However, acquired resistance to pathogens in domestic populations has been postulated to reduce infection in wild fish, by reducing pathogen load (Alves and Taylor, 2020). Selective breeding of rainbow trout (*Oncorhynchus mykiss*) has led to the development of pathogen resistant animals which may be beneficial to fish farmers but presents a problem if escapees mix with wild fish populations and disrupt gene pools (Wiens et al. 2018). The sustainability of aquaculture has been heavily debated but recent pressures on the industry to adopt sustainability measures has improved the practice (Naylor et al. 2020). Nevertheless, some practices such as the increased use of antibiotics, warrants surveillance (Schar et al. 2020). Enhanced antimicrobial stewardship will aid in decreasing the incidence of antimicrobial resistant pathogens in animal production and will also decrease treatment failure rates (Schar et al. 2020). The application of antimicrobials in aquatic environments and their residues, widens the potential exposure pathway for drug distribution and has important global health consequences (Schar et al. 2020). For this reason, there is a need for antibiotic alternative discovery in aquaculture. In addition

to this, the pathogenesis of some important pathogens is still under investigation (Semple et al. 2020; Mugetti et al. 2020; Barger et al. 2020).

Another important issue faced by the aquaculture industry is maintaining enough fish to meet consumer demands and thus appropriate stocking densities. Stocking density can have a multitude of impacts on fish including growth, feeding and survival (M'balaka et al. 2012). Feeding fish has become a global problem since there are finite resources and producers want to reduce costs; turning to alternative protein sources to feed fish, such as plant-based or insect-based proteins (Naylor et al. 2009). However, the physiological responses by the host are not entirely understood (Naylor et al. 2009). High stocking of fish species also impacts the evolution of pathogens and not only enhances their transmission but can also lead to the development of more virulent strains (Pulkkinen et al. 2010). Future research in aquaculture should focus on strategies to mitigate disease while supporting the growth and survival of various species in a sustainable manner.

1.1.2 *Aquatic Pathogens*

Several aquatic pathogens have been identified to date and surveillance of emerging pathogens continues to be an active area of aquaculture research (Semple and Dixon, 2020; Chen et al. 2017; Lőrincz et al. 2011). The most well characterized pathogens are of bacterial and viral origin but some parasitic and fungal diseases are also well-studied and have significant impacts on fish health (Semple and Dixon, 2020; Di Natale et al. 2019; Igboeli et al. 2013; Crane and Hyatt, 2011; Kales et al. 2007). Controlling pathogens and disease outbreaks remains a major challenge for the aquaculture industry. In this study, a subset of available pathogens was selected to be exposed to the synthetic peptides based on their individual characteristics such as Gram-positive or Gram-negative or enveloped and non-enveloped for bacterial and viral pathogens, respectively (Table 1). Each selected pathogen will be briefly discussed with a focus on proteases and antimicrobial resistance (AMR) mechanisms.

Table 1. Aquatic pathogens used in this study and their general characteristics.

Name	Gram stain/Envelope or non-enveloped	Optimal temperature (°C)	References
Flavobacterium psychrophilum	Gram-negative	12-15	Bernardet and Bowman, 2006
Yersinia ruckeri	Gram-negative	28	Fernández et al. 2007
Streptococcus iniae	Gram-positive	35-40	Zou et al. 2008
Frog virus 3	Enveloped & non-enveloped	26-30	Garvell and Granoff, 1970
Infectious pancreatic necrosis virus	Non-enveloped	10-15	Dobos and Roberts, 1983
Viral hemorrhagic septicemia virus IVb	Enveloped	9-12	Goodwin and Merry, 2011

Flavobacterium psychrophilum (*F. psychrophilum*) is part of the *Flavobacteriacae* family and is considered to be one of the most important pathogens in salmonid aquaculture (Nematollahi et al. 2003). *F. psychrophilum* is the aetiologic agent of rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD) (Takeuchi et al. 2021). Genomic sequencing of *F. psychrophilum* strain JIP02/86 (ATCC 49511) revealed various drug/metabolite efflux transporters (Duchaud et al. 2007). Interestingly, sugar kinase and phosphotransferase system genes used by bacteria for carbohydrate uptake were not identified (Duchaud et al. 2007). It has been suggested that *F. psychrophilum* is unable to use carbohydrates and thus relies on protein and lipids for energy instead (Bernardet and Kerouault, 1989). *F. psychrophilum* also secretes proteases to degrade protein and peptides and may import the resulting mixture of amino acids and oligopeptides through a variety of transporters (Duchaud et al. 2007). Antimicrobial resistance (AMR) and AMR-associated genes have been identified in global isolates of *F. psychrophilum* (Saticioglu et al. 2019; Miranda et al. 2016; Izumi and Aranishi, 2004).

Streptococcus iniae (*S. iniae*) is a Gram-positive bacterial pathogen of fish that belongs to the *Streptococcaceae* family (Numberger et al. 2021). *S. iniae* is also able to cross species barriers and is considered a zoonotic pathogen (Miller and Neely, 2005). Some *S. iniae* proteases degrade host proteins such as chemokines and contribute to dampened immune responses (Zinkernagel et al. 2008). Sequencing of the *S. iniae* SF1 genome revealed various types of

transporters of which ATP-binding cassette (ABC)-type ATPases were most abundant (Zhang et al. 2014). ABC transporters are important in bacterial pathogenicity where they contribute to drug resistance and may play a role in drug efflux (Lewis et al. 2012). However, antibiotic resistance in *S. iniae* isolates is not as well characterized, as in *F. psychrophilum* (Park et al. 2009). Gram-positive bacteria such as *Streptococci*, have a specialized external structure; composed of a thicker peptidoglycan layer, embedded with teichoic acids and capsular polysaccharides which form the outermost layer (Vollmer et al. 2008; Weidenmaier and Peschel, 2008). Together, this structure known as the capsule, provides an extra layer of resistance towards the external environment and contributes to pathogen clearance and virulence (Locke et al. 2007).

Yersinia ruckeri (*Y. ruckeri*) is a Gram-negative bacterial pathogen that belongs to the *Enterobacteriaceae* family (Kumar et al. 2015). Some *Y. ruckeri* strains are motile and two biotypes have been identified; biotype 1 is motile and lipase-positive and biotype 2 is non-motile and lipase-negative (Wrobel et al. 2019). It is the aetiologic agent of enteric redmouth disease (ERD), a type of hemorrhagic septicemia in fish (Kumar et al. 2015). Some strains of *Y. ruckeri* are resistant to various antimicrobial agents, including the antibiotics oxytetracycline and sulphonamide (Rodgers, 2001). In addition, antibiotic resistance genes have been identified in some strains of *Y. ruckeri* (Duman et al. 2017). Certain *Y. ruckeri* isolates demonstrate evidence of genomic mosaicism, along with recombinational exchange; most likely a result of horizontal gene transfer (Ormsby and Davies, 2021). The ABC transport system in *Y. ruckeri* is also used to export proteases, such as *Yersinia ruckeri* protease 1, which plays a major role in pathogenesis by digesting muscle proteins, laminin, fibronectin, actin, and myosin (Fernández et al. 2002; Fernández et al. 2003). Further in-depth characterization of *Y. ruckeri* isolates will improve our understanding of pathogenesis and may identify novel drug targets.

Frog virus 3 (FV3) belongs to the *Iridioviridae* family and is a large double-stranded DNA virus that mainly infects amphibians and reptiles but also some teleosts (Tidona et al. 1998; Chinchar and Waltzek, 2014). Bullfrog farming has been associated with the spread of disease into the natural environment and frogs may harbour the virus, potentially spreading it to other hosts (Ribeiro et al. 2019; Miller et al. 2007). Although the ability of FV3 to replicate and cause disease in fish is limited, there is evidence that demonstrates FV3 can replicate in rainbow trout gill and liver cell lines (Pham et al. 2015). Moreover, it causes limited mortality in other fish species

(Brenes et al. 2014). Therefore, FV3 is considered an emerging pathogen of fish and more research is required to understand its pathogenesis and future impacts on aquaculture. Unlike FV3, infectious pancreatic necrosis virus (IPNV) is of great concern to the aquaculture industry and results in catastrophic mortality events (Dopazo, 2020). It belongs to the *Birnaviridae* family and is a double-stranded RNA virus (Dopazo, 2020; Dobos, 1995). Besides edema and necrosis, IPNV infection results in erratic corkscrew swimming and anorexia (Dopazo, 2020). IPNV is non-enveloped and has a bi-segmented genome which encodes for different features; for example, segment A encodes for VP4, which is a protease (Dobos, 1976; Dopazo, 2020). VP4 has autoproteolytic activity, cleaving polyprotein PP during its translation but it can also stall interferon induction (Dopazo, 2020). IPNV is widespread and infects many fish species, which makes it an important disease to target, in terms of developing treatments but to date; these efforts have had limited success (Frost and Ness, 1997; Tamer et al. 2021).

Viral hemorrhagic septicemia (VHS) is a viral disease of fish with significant mortality; and has serious economic impacts on aquaculture operations (Baillon et al. 2020). It is caused by the viral hemorrhagic septicemia virus (VHSV) (Baillon et al. 2020). VHSV is part of the *Rhabdoviridae* family of viruses and VHSV virions are enveloped (Basurco et al. 1991; Baillon et al. 2020). The most well characterized genotypes include I and IV, of which the later can be further classified as IVa from the Northwest Pacific Ocean and IVb, from the Great lakes (Pham et al. 2013; Einer-Jensen et al. 2004; Elsayed et al. 2006). VHSV IVa and IVb genotypes show differential effects *in vitro*, where rainbow trout gill cells are more susceptible to IVa compared to IVb (Pham et al. 2013). VHSV leads to systemic infection, mainly of the gastrointestinal tract, spleen, kidney, liver and meninges (Lovy et al. 2012). VHSV can also cause corkscrew swimming and darkening of skin (Lovy et al. 2012). Some rainbow trout display some resistance against VHSV; which has been attributed to the detection of quantitate trait loci (QTL) (Verrier et al. 2013). Selective breeding of animals with major QTL may lead to enhanced resistance. The development of vaccines, as well as the use of adjuvants, has shown promising results against certain VHSV genotypes (Encinas et al. 2011; Standish et al. 2016; Chun et al. 2021). More recently, the use of natural products such as algal extracts from *Ecklonia cava*, on VHSV, have been investigated and fish fed *E. cava* extract, are more protected against VHSV (Yang et al. 2018). Furthermore, VHSV exposed to *E. cava* extract *in vitro*, also shows diminished infectivity (Yang

et al. 2018). Therefore, investigating natural products in the future may lead to a potential therapeutic against VHSV.

1.1.3. *Fish Immune System*

The fish immune system is equipped with various cells and molecules to aid in the defense against pathogens. The immune system consists of two subsystems: namely, the innate and adaptive arms. The innate arm is the first to respond to pathogens and includes chemical, cellular and physical barriers (Smith et al. 2019). Such barriers include the skin, gill or intestinal tract which are in constant contact with the external environment and serve as a port of entry for several pathogens. This has led to the development of several cell lines from each of these organs to study host-pathogen interactions *in vitro* (Ossum et al. 2004; Bols et al. 1994; Kawano et al. 2011; Pasquariello et al. 2021). In the current study, the gill epithelial cell line (RTgill-W1), was chosen to study the effects of synthetic peptide and bacterial infection. If a pathogen breaches the physical barrier, the cellular defense system will be activated through various immune cells which will release toxic compounds or even engulf and digest the pathogen in a process of elimination from the host (Smith et al. 2019). Depending on the type of infection, specialized immune cells will become activated to eliminate the threat. One of the most important immune cells in bacterial infections of fish, is the macrophage; which possess phagocytic activity and synthesizes an array of antibacterial compounds (Grayfer et al. 2018). RTS11 is a monocyte/macrophage cell line derived from the spleen of rainbow trout and has been a useful tool to study *in vitro* immune responses, towards various aquatic pathogens (Ganassin and Bols, 1998; Brunner et al. 2020; Semple et al. 2020). Furthermore, macrophages are one of the most well studied immune cells in fish immunology (Grayfer et al. 2018). Therefore, in the current study, the RTS11 cell line was selected to study *in vitro* responses to synthetic peptides and bacterial infection. Fish macrophages also play a role in adaptive immunity and can recruit other immune cells to sites of infection (Grayfer et al. 2018).

Unlike innate immunity, the adaptive arm targets pathogens more specifically and develops memory for future encounters. The adaptive immune system is largely governed by immunoglobulins, T cell receptors and major histocompatibility complex (Smith et al. 2019). The main cells involved in adaptive immunity are B lymphocytes and T lymphocytes (Smith et al.

2019). B cells in the fish intestinal tract produce immunoglobulins that prevent microbes from crossing the epithelium (Parra et al. 2016). Unlike in mammals which have germinal centers and lymph nodes to enhance interaction of B cells with antigen presenting cells; fish lack these structures and therefore respond with a slower antibody response to pathogens (Parra et al. 2016). T cells in fish display similar activity to mammalian T lymphocytes, which are cytotoxic (CD8), helper (CD4) and regulatory (Treg, Th17) (Scapigliati et al, 2018). Furthermore, most of the T cells in fish are found in the gills and intestine (Scapigliati et al. 2018). In the fish gill, T cells are able to proliferate during infection (Nuñez et al. 2014). For this reason, epithelial cells in mucosal organs may play a role in enhancing the interaction between B or T lymphocytes and antigen presenting cells. However, the immune system of fish is very complex and adherent intestinal cells from Atlantic salmon exhibit phagocytic activity while expressing macrophage-specific genes (Park et al. 2020). Therefore, further studies are needed to elucidate the role of intestinal and gill cells during infection. The immunity to bacterial infections in fish are crucial to aquaculture operations and methods to fine-tune or enhance immunity in fish are of great interest (Semple et al. 2020). One of the ways in which fish immunity can be modulated is using peptide molecules which may elicit a biological response in the cells, tissue or organism.

1.1.4. *Antimicrobial Peptides*

AMPs are short, usually cationic, amphiphilic molecules that have direct or indirect effects on the immune system and physiology of animals (Hancock and Scott, 2000). To date, several have been identified in teleosts and are intricately involved in innate and adaptive immunity (Masso-Silva and Diamond, 2014; Brunner et al. 2020). Many fish AMPs have also been shown to be effective against important aquatic bacterial pathogens (Masso-Silva and Diamond, 2014; Brunner et al. 2020). Salmonid AMPs are very diverse and range from 11 – 79 amino acids in length, are generally found in skin secretions but have been detected in many cells and/or tissues, (Brunner et al. 2020). Interestingly, the cationicity of AMPs have been attributed to antimicrobial functions however, Histone 2A, a rainbow trout AMP, has a net charge of 0 and still possesses potent antimicrobial activity (Fernandes et al. 2002; Brunner et al. 2020). This suggests that although charge is important to AMP function, it may be an AMP dependent factor. Modifying the length of salmonid AMPs, such as cathelicidin 2 (Cath2) from grayling, has been shown to

have differential effects on bacterial pathogens; where the longer Cath 2 showed more potent antimicrobial activity than the shorter fragment (D'Este et al. 2016). Studying the structure of salmonid AMPs may lead to the development of new drugs in aquaculture or medicine.

AMPs are expressed constituently in some tissues but may increase in response to a pathogenic threat (Brunner et al. 2020). The expression of AMP genes in salmonids, may also be influenced by a variety of factors in the environment, including diet (Brunner et al. 2020). Nutrigenomics or the interaction of genes with the diet, has been a growing area of research in aquaculture since maintaining and improving the health of animals is important (Dawood, 2020). For example, feeding components of bacterial (peptidoglycan) or fungal (zymosan) origin to rainbow trout increases the expression of AMPs (Brunner et al. 2020). Rainbow trout fed peptidoglycan show increased expression of AMPs in the skin (Casadei et al. 2015), whereas the incorporation of zymosan in fish feed, or direct application to a rainbow trout gut cell line, resulted in increased AMP expression and immunomodulatory effects to enhance intestinal immunity (Schmitt et al. 2015). A better understanding of fish gut immunity is required to use nutrigenomics to its full advantage in aquaculture.

1.1.5 *Plant and Insect-Derived Protein Alternatives*

Feed can be used as a vector to deliver AMPs and some natural sources such as plant and insect meal contain endogenous AMPs but their effects of fish remain unknown. For example, mealworms (*Tenebrio molitor*), produce peptidoglycan repeat protein, an important AMP in response to bacterial infection of the gut (Keshavarz et al. 2020). However, soybean (*Glycine Max* L.) plants also produce potent AMPs (Brand et al. 2012). They may influence fish immunity either by interacting with immune cells or shifting the gut microbiome, but the direct effects of these peptides on fish pathogens and immunity is unknown. Nevertheless, soybean and mealworm meal continue to be investigated for use in the diet of farmed salmonids.

Traditionally, fishmeal is used as a source of protein but this is not sustainable due to increased consumer demands for fish. Instead, aquaculture has been attempting to transition into using alternative protein sources of non-animal origin. Despite these increases in sustainability efforts, teleost gut responses to protein alternatives, such as plant and insect-derived proteins, are

not particularly well characterized. The introduction of soybean meal to fish diets, has led to a significant increase in expression of important AMPs, such as hepcidin (Zhang et al. 2021). The effects of including dietary insect meal in the form of mealworms; for example, is not as clear (Henry et al. 2018). There is growing evidence that supports soybean induced gut inflammation (enteritis) in several fish species (Miao et al. 2018; Yu et al. 2021; Booman et al. 2018; Seibel et al. 2022). In general, the enteritis which is also known as soybean-meal induced enteropathy; is characterized by an upregulation of interleukins, thickening of the muscularis, decreased microvilli height and in some cases a shift in the microbiota (Miao et al. 2018; Booman et al. 2018; Yu et al. 2021). Therefore, investigating the interaction of alternative protein sources is important to improve fish health in aquaculture.

The purpose of the current work was to investigate this interaction between peptides and proteins from various sources and potential interaction with rainbow trout cell lines, to modulate immunity. It is hypothesized that some peptides may possess antimicrobial activity towards aquatic pathogens and that alternative feedstuffs may modulate the immune response in cells. In addition, some of the crosstalk between rainbow trout immune cells and epithelial cells is hypothesized to involve AMPs. To study this, the co-culture of rainbow trout immune cells with epithelial cells was employed in the presence of *F. psychrophilum*. It was hypothesized that epithelial cells may change levels of gene expression, in response to the pathogen, to recruit immune cells. Collectively, these experiments will lead to a better understanding of fish physiology and disease.

Chapter 2– Characterizing the potential antimicrobial and antiviral effects of synthetic peptides on aquatic pathogens

2.1 Introduction

2.1.2 *Drug Discovery in Aquaculture*

The use of drugs in aquaculture is not a new concept and one of the main targets is to prevent disease (Burka et al. 1997). However, the aqueous environment in which fish live and their unique physiology presents a challenge in pharmacology (Burka et al. 1997). To date, only ten drugs are authorized for use in food-producing aquatic animals in Canada (Health Canada – Veterinary Drugs, 2021). The limited options present a challenge for the treating veterinarian or fish farmer. Turning to natural products for drug discovery may be a feasible option to expand the current repertoire. Natural drug discovery has been reawakened due to recent advances in analytical tools, genome mining and engineering,us in addition to advanced microbial culturing techniques (Atanasov et al. 2021; Reverter et al. 2014; Falaise et al. 2016; Herrera et al. 2021). Many plant-derived compounds have been investigated as potential biomedicines for use in the aquaculture industry, mainly due to limited environmental effects (Citarasu, 2010). However, many other compounds such as carbohydrates, lipids and peptides from various sources, have been investigated for potential use as drugs, due to their unique antimicrobial activities against aquatic pathogens (Ahmed et al. 2020; Park et al. 2016; Herrera et al. 2021). Delivery of antibacterial compounds to fish has proved to be a challenge and new methods are needed (Gholipourkanani et al. 2019). One common method of administration is through feed (Herrera et al. 2021). If the compounds are to be used in living animals, assessment of hemolytic and cytotoxic properties are also important considerations (Bridle et al. 2011; Semple et al. 2019). In addition, large collections of such compounds are screened for potential therapeutic functions. For example, in chemogenomics-based drug discovery, which is also used to mine veterinary drugs; large chemical libraries are systemically screened to reveal biological targets or biologically active compounds, in a specific biological system (Bredel and Jacoby, 2004; Huang et al. 2017). Besides screening chemical compounds, the genomes of organisms can also be screened for molecules with potent antimicrobial properties, such as AMPs (Javan et al. 2018; Van Hoek, 2014; Maróti et al. 2011).

2.1.3 *Antimicrobial Peptides as Potential Treatments in Aquaculture*

The use of AMPs as a potential treatment or prophylactic against pathogens in animal production has been investigated in many species, including fish (Kumar et al. 2020; Silveira et al. 2021; Barrosso et al. 2021). In general, AMPs do not have negative impacts on the growth of fish and can have positive effects on homeostasis, in addition to preventing infection or limiting mortality (Herrera et al. 2021; Barrosso et al. 2021). AMPs can be expressed and produced directly by some feed sources such as fish eggs, in addition to being added to feed as a supplement (Tseng et al. 2020; Herrera et al. 2021). Besides being administered orally, AMPs can also be introduced via cloning. For example, transgenic fish have been developed by introducing the moth-derived AMP known as cecropin P1 through a transgene to rainbow trout tissues under the regulation of a CMV promoter, which resulted in enhanced disease resistance (Chiou et al. 2014). The introduction of cecropin P1 can also significantly alter the expression of immune genes in the spleen, kidney and liver of rainbow trout, which may explain its immunomodulatory role (Lo et al. 2014). Probiotics can also be used as a vector to deliver AMPs to animals and the AMPs do not have to be homologous to the host, they can be from a different host but still improve growth and immune responses (Zhang et al. 2016; Tai et al. 2020). However, this has not been investigated in fish. Another challenge with this method is maintaining a consistent dose of AMP production in the host.

Other methods of administration, such as injection, are better at delivering a consistent dose and have had similar immunomodulatory effects in fish but this method is not realistic for large aquaculture operations. For example, injection of Caspian trout hepcidin into healthy specimens resulted in an upregulation of pro-inflammatory cytokine genes in the spleen and kidney which may aid in the immune response against pathogens (Shirdel et al. 2019). Injection of pleurocidin, an AMP found in the skin secretion of winter flounder, into the peritoneal cavity of Coho salmon; significantly reduced mortality when challenged with *Vibrio anguillarum* (*V. anguillarum*) (Jia et al. 2000). Bath immunization methods may also be used to expose fish to AMPs since AMPs can be used as vaccine adjuvants (Kai and Chi, 2008; Fritz et al. 2004; Munshi et al. 2020). This method may work better on juvenile or larval fish where several can be treated at the same time.

AMPs also play an important role in young fish where the immune system is still under development and therefore rely on AMPs as a first line of defense against pathogens (Brunner et al. 2020). Some salmonids continuously produce AMPs at basal levels but can quickly ramp up AMP production, in response to disease (Brunner et al. 2020). Salmonid AMPs also differ in length, charge and hydrophobicity which results in unique functional properties that are able to interact with pathogen structures (Brunner et al. 2020). Salmonid AMPs range from 11 to 79 amino acids in length (Brunner et al. 2020). Even fragmented AMPs can retain antimicrobial activity, suggesting that select AMPs may remain functional even during peptidolysis (Souza et al. 2013). Most salmonid AMPs are cationic, except Histone H2A but nevertheless, it retains its antimicrobial activity and is a potent inhibitor of several aquatic pathogens: *Lactococcus garvieae*, *Aeromonas salmonicida*, *Yersinia ruckeri* and *Ichtyophthirius multifiliis* (Muñoz-Atienza et al. 2019). The hydrophobicity of fish AMPs is important to consider when developing AMP-based therapeutics as there is preliminary evidence to suggest that hydrophobicity may be involved in contributing to hemolysis and influences solubility (Brunner et al. 2020).

The present study aimed to investigate the effects of synthetic peptides selected from a range of aquatic organisms which included rainbow trout, tilapia, and the fish tapeworm (Appendix Table 1). These peptides were selected based on specific criteria that would allow for rapid synthesis, solubility, an overall charge similar to most fish AMPs and specificity (i.e. no sequence overlap with known AMPs). The following were used as filtering criteria: AMP length of less than 50 amino acids, overall charge between 0 and +10, total hydrophobic ratio of less than 50%. The peptides were employed against a range of aquatic pathogens and were found to exhibit differential antibacterial properties. None of the peptides studied inhibited viral infection of the fish cell lines. In addition, one the peptides, rtVWF was also assessed for potential hemolytic activity in rainbow trout erythrocytes and did not cause hemolysis. rtVWF also did not reduce the viability of rainbow trout gill (RTgill-W1) or monocyte/macrophage cells (RTS11); however, it did not have any immunomodulatory effects in these cell lines.

2.2 Materials and Methods

2.2.1. Peptides

Peptides were synthesized and lyophilized to > 95% purity, by Genscript® USA (Appendix Table 1). For each peptide, mass spectrometry, high plate liquid chromatography and peptide solubility tests were also conducted by Genscript® USA. The peptides were stored at -20°C until further use. The peptides were then dissolved in sterile molecular grade water (Thermo Fisher Scientific, USA), to achieve a final concentration of 1000 µM and were stored in 30 µl aliquots at -20°C, until further use.

2.2.2. Growth of bacteria

Flavobacterium psychrophilum FPG101 was grown at 14°C in cytophaga broth with a final pH of 7.2. Each liter of cytophaga broth contained: 0.5 g tryptone, 0.5 g yeast extract, 0.2 g sodium acetate, 0.2 g beef extract (BD Biosciences). *Yersinia ruckeri* and *Streptococcus iniae* were a generous gift from Dr. Mark Fast at the University of Prince Edward Island. *Y. ruckeri* and *S. iniae* were grown at 28°C in BD Tryptic Soy Broth (Soybean-Casein Digest Medium) with a final pH of 7.2, (BD Biosciences). Each liter of BD Tryptic Soy Broth contained: 17 g of tryptone, 3 g soytone, 2.5 g glucose, 5 g sodium chloride, 2.5 g dipotassium phosphate. All bacterial stocks were stored in a 50% (v/v) of glycerol and respective broth solution at -80°C in sterile 1.5 ml cryovials (Thermo Fisher Scientific, USA). Before each experiment, the stocks were plated onto cytophaga plates for *F. psychrophilum* or LB agar plates, for *Y. ruckeri* and *S. iniae* and the plates were incubated for 3 days at the aforementioned temperatures. A single colony from each plate was picked and grown in 3 ml of the respective broth for an additional 3 days, at the aforementioned temperatures and this stock was used for each experiment.

2.2.3. Broth microdilution assay

The broth microdilution assay was used to determine the minimal inhibitory concentration (MIC) of each peptide on the bacteria. Briefly, the bacterial suspensions were prepared as previously described and diluted to a final O.D. of 0.001 in the respective broth (Semple et al.

2019). One hundred microliters of the final suspension was added to each well of a 96-well plate, in triplicate (Thermo Fisher Scientific, USA). Then, the 1000 µM peptide stock was serially diluted to 500, 250, 125, 62.5 and 31.25 µM in sterile molecular grade water and 10 µl of each was added to wells, in triplicate, to reach a final concentration of peptides at 45.4, 22.7, 11.3, 5.7 and 2.8 µM, respectively. Some wells, in triplicate, also received bacteria alone with 10 µl of sterile water, which served as a vehicle control, or 10 µl of a kanamycin sulfate at 45.4 mg/ml (Sigma-Aldrich). Some wells also received 100 µl of broth without bacteria and 10 µl of sterile water which was used as a background control. Plates were sealed with parafilm and experiments with *F. psychrophilum* were incubated at 14°C for 3 days before reading the O.D. 600 nm using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments), whereas plates with *Y. ruckeri* or *S. iniae* were incubated at 28°C and a reading at O.D. 600 nm was obtained every hour for 7 h using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments).

2.2.4. Cell culture and viral production

Epithelioma papulosum cyprini (EPC) and Chinook salmon embryo (CHSE-214) cell lines were maintained at room temperature and ambient air in vented 25 cm^2 cell culture flasks (Thermo Fisher Scientific). The cells were supplemented with 4 ml of Leibowitz's (L-15) medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin-streptomycin (Sigma). EPC and CHSE-214 cells were routinely subcultured at a ratio of 1:2; every four days. Briefly, cell monolayers were rinsed with 1 × Dulbecco's phosphate-buffered saline (Gibco) and a 0.05% trypsin–EDTA (Gibco) solution was applied to detach the cells from the plate. As for viral production, FV3 and VHSV IVb were titered on EPC cells, at 28°C and 14°C, respectively. IPNV was tittered on CHSE-214 cells at 14°C. Briefly, 50, 000 cells were added to each well of a 96-well plate, in L-15 medium with 2% FBS and free of antibiotics overnight. The following day, 10-fold serial dilutions of each viral stock were made and diluted to a final sample dilution of 10^{-8}. Next, 100 µl of each serial dilution was used to inoculate cells in six wells. The edge of the plate was sealed with parafilm, and the plates were incubated at the respective temperature, depending on the virus. Six wells each received 100 µl of fresh media (L-15 with 2% FBS) and no virus, which served as a control. The plates were monitored, and each well was scored for cytopathic effects (CPE) over 10 days. The median tissue culture infectious dose ($TCID_{50}$/mL) was calculated

using the Reed-Muench method (Reed and Muench, 1938). The $TCID_{50}$/mL was converted to plaque forming units (PFU) by using the following equation: PFU = $TCID_{50}$/mL × 0.69.

2.2.5. Virus/peptide preincubation assay

The virus/peptide preincubation assay was used as previously described but with slight modifications (Marcocci et al. 2018). Fifty thousand EPC and CHSE-214 cells were seeded in triplicate, in a 96-well plate. The plate was sealed with parafilm and incubated for 12 h at room temperature. Each peptide (45.4 µM) was preincubated with FV3, IPNV or VHSV IVb (MOI = 1) for 1 h at 28°C for FV3 and 14°C, for IPNV and VHSV in sterile centrifuge tubes. The MOI for each virus was calculated by using the following equation: MOI = PFU/ number of cells. This mixture was used to infect EPC and CHSE-214 monolayers in a 96-well plate and after viral adsorption for 2 h, at the respective temperature, the media was removed, and the cells were gently washed with 1 x PBS. The media was replaced with L-15 and 2% FBS, without antibiotics and incubated at the respective temperatures for 10 days. The cells were monitored, and each well was scored for CPE. The % CPE was calculated and expressed as a ratio of wells showing CPE, compared to the number of wells not showing CPE.

2.2.6. Thiazolyl Blue Tetrazolium Bromide (MTT) assay

RTS11 and RTgill-W1 cell lines were used to evaluate the potential cytotoxic effects of rtVWF. Briefly, 500,000 cells were seeded in triplicate within a 96-well plate. The cells were suspended in 100 µl of L-15 with 15% FBS and without antibiotics. Three wells did not receive cells but did receive medium only as a background control. The plate was sealed with parafilm and incubated at room temperature. The following day, the cells were exposed to 22.7 µM of rtVWF for 24 h. The media was removed and 11 µl of a 0.22 µm filtered MTT (Sigma-Aldrich, Cat no. M5655) solution in 1 x PBS, of 5 mg/ml, was added to each well to reach a concentration of 0.5 mg/ml. The three positive control wells also received a fresh solution of 0.01% (v/v) Triton X-100 (Sigma-Aldrich). The plate was then sealed with parafilm and placed on a shaker at 100 rpm for 1 min. The cells were incubated in this media for 3 h in the absence of light. Next, 150 µl of DMSO was added to each well. Each well was gently mixed by pipetting to release the formazan crystals and then the plate was incubated for an additional 15 minutes at room temperature. Lastly, the

plate was read at an O.D. of 520 nm, using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments). The wells with media only and DMSO, served as a background control and were subtracted from all other values.

2.2.7. Hemolysis assay

Rainbow trout erythrocytes were obtained from fish at the Alma Aquaculture Research Station (Elora, ON). Five millilitres of heparinized blood was centrifuged at 4°C for 5 min at 2000 × g. The supernatant was discarded and the pellet was washed with 1 × PBS (Gibco). The cells were resuspended in a 50 ml sterile solution of 1 × PBS (Gibco) supplemented with glucose (0.2% v/v). Briefly, the erythrocytes were gently resuspended in a 1 x PBS solution with a final concentration of 0.2% glucose. Ninety microliters of the suspension was added to each well of a U-bottom 96-well plate (Nunc) in duplicate. Ten microliters of rtVWF were also added to wells in duplicate, to reach a final concentration of 50, 25 and 12.5 µM. Some wells received a 0.1% SDS solution as a positive control. The plates were incubated at room temperature for 30 minutes and were then centrifuged at 2000 x g for 3 minutes at 4°C, to pellet the cells. In order to measure hemolysis, 70 µl of the supernatant was transferred to a flat bottom 96-well plate along with 70 µl of 1 x PBS, which served as a background control. The plate was read at an O.D. of 405 nm using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments). The percentage of hemolysis was calculated using the formula: % hemolysis = (O.D. 405 nm of erythrocytes exposed × 100/ O.D. 405 nm of erythrocytes exposed to SDS).

2.2.8. Exposure of cells to peptides and *S. iniae*

Five hundred thousand RTS11 and RTgill-W1 cells were seeded in 100 µl of L-15, with 15% FBS, into each well of a 96-well plate (Thermo Fisher Scientific). The plate was sealed with parafilm and incubated overnight. The MTT assay was used to evaluate the cytotoxic effects of peptides and was employed as described in section 1.2.6. However, 10 µl of rtVWF was added to each well in triplicate at final concentrations of 11.3 and 45.4 µM for 24 h before adding MTT. To assess the effects of *S. iniae* on cell viability, the same assay was employed but *S. iniae* was found to not grow well in L-15 media and was therefore heat-killed. The heat-killed bacterial suspension was added to each well, at a low and high dose, and the MTT assay was repeated. Briefly, *S. iniae*

was grown as described in section 1.2.2. but was diluted to a final O.D. 600 nm of 0.017. To kill the bacteria, the bacterial suspension was heated to 95°C and incubated at this temperature for 30 min, after which it was sterilized, by passing the material through a 0.22 µm filter (FroggaBio). The filtered suspension was frozen at -80°C until further use. The suspension was used to stimulate RTgill-W1 and RTS11 cells at a low (final O.D. 600 nm of 0.0017) and high dose (final O.D. 600 nm of 0.017). For gene expression analysis, five hundred thousand RTS11 or RTgill-W1 cells were added to a 24-well plate (Thermo Fisher Scientific) in duplicate, with L-15 and 15% FBS. The plate was sealed with parafilm and incubated overnight. The following day, the media was replaced with L-15 and either 15% or 2% FBS. To examine the effects of peptide and heat-killed *S. iniae*, the cells were then treated with rtVWF for 24 h, which was added to each well at a final concentration of 45.4 µM, in 0.5 ml of media. Some wells did not receive peptides and served as a control. Depending on the experiment, some cells were then exposed to heat-killed *S. iniae* for an additional 24 h. Again, some wells did not receive heat-killed *S. iniae* and served as a control. After 24 h of incubation, total RNA was isolated from pooled duplicates.

2.2.9. Total RNA isolation and cDNA synthesis

Total RNA was extracted from RTgill-W1 and RTS11 cells using the RNeasy Micro Kit by following the manufacturer's protocol (Qiagen). RNA purity and quantity were determined using a NanodropTM 2000 Spectrophotometer. Total RNA samples were treated with DNase I (Thermo Scientific) and stored at -80 °C until further use. cDNA was synthesized on the same day from 400 µg of RNA per reaction, using qScriptTM cDNA SuperMix (Quantabio) and following the manufacturer's protocol. Each reaction was incubated at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The cDNA was stored at -20 °C for future analyses.

2.2.10. qRT-PCR

qRT-PCR was performed with WISENT ADVANCED™ qPCR mastermix (Wisent), by following the manufacturer's instructions on the LightCycler® 480 II (Roche). Briefly, each 10 µl reaction consisted of 1 µg of cDNA (2.5 µl), 0.25 µM of forward and reverse primers (0.5 µl), 2 × WISENT ADVANCED™ qPCR mastermix (5 µl) (Wisent), and DEPC water (2 µl). A no-template control was also included. All trials were conducted in triplicate and each program

consisted of a pre-incubation at 95 °C for 10 min, then 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at the specified primer annealing temperature for 30 s and extension at 72 °C for 20 s. Primer efficiency was between 91.77 – 110.74% (Appendix Table 3). The fold change of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.2.11. Statistical analysis

Antimicrobial activity and gene expression data were analyzed using a one-way ANOVA test with Tukey's post hoc test using R statistical software. The alpha value was set at 0.05 ($P < 0.05$) for all tests. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3 Results

2.3.1. Growth of *Flavobacterium psychrophilum* in the presence of peptides

The peptides did not negatively impact the growth of *F. psychrophilum* after three days of exposure. However, rtVWF appeared to slightly increase the growth of the bacteria at 45.4 and 22.7 µM but this was not statistically significant (Figure 2.1A). On the other hand, Tsvep1 signifcantly increased the growth of the bacteria at 45.4 µM (Figure 2.1B). The observed increase in bacterial growth also appeared to be dose-dependent at 22.7, 11.3 and 5.7 µM but this was not stastically different from the bacteria control cells without peptide.

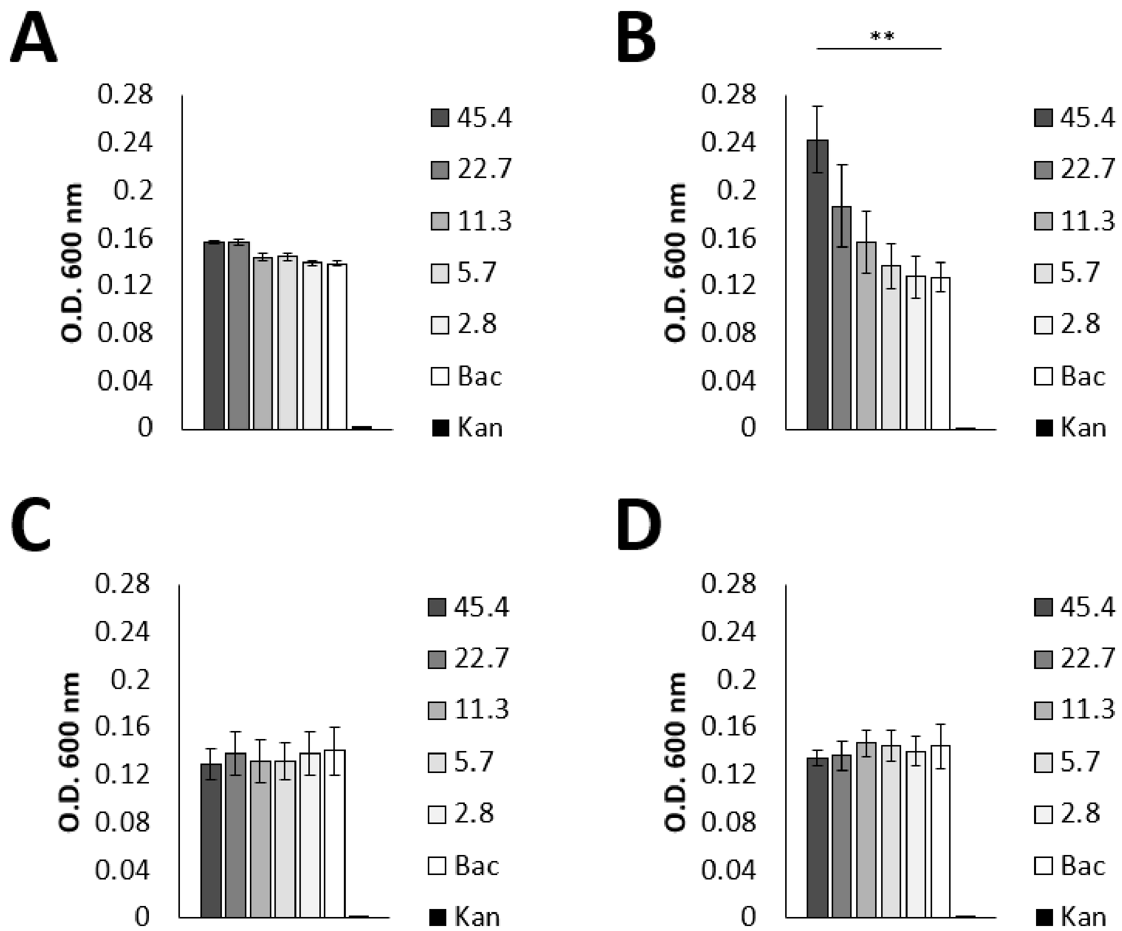


Figure 2.1. Effects of peptides on the growth of *F. psychrophilum*. The data are representative of *F. psychrophilum* growth after 72 h in the presence of (A) rtVWF, (B) Tsvep1, (C) Dpbl-32, (D) Dpbl-48. Where Kan (kanamycin 500 µg/ml) served as a positive control. Means are representative of three independent experiments, ± S.E.M. Asterisk represents a significant difference from the control (where p <0.01 = **).

2.3.2. Growth of *Yersinia ruckeri* in the presence of peptides

The growth of *Y. ruckeri* did not appear to be impacted by peptide treatment over 7 h (Figure 2.1 and Figure 3.1). Tsvep 1 at 45.4 µM appeared to slightly decrease the growth of *Y. ruckeri* (Figure 2.3B) but Dpbl-48 at the same concentration appeared to slightly increase the

growth of bacteria (Figure 2.3 D). Treatment with rtVWF and Dpbl-32 appeared to have no effect on the growth of bacteria (Figure 2.3A and Figure 2.3C).

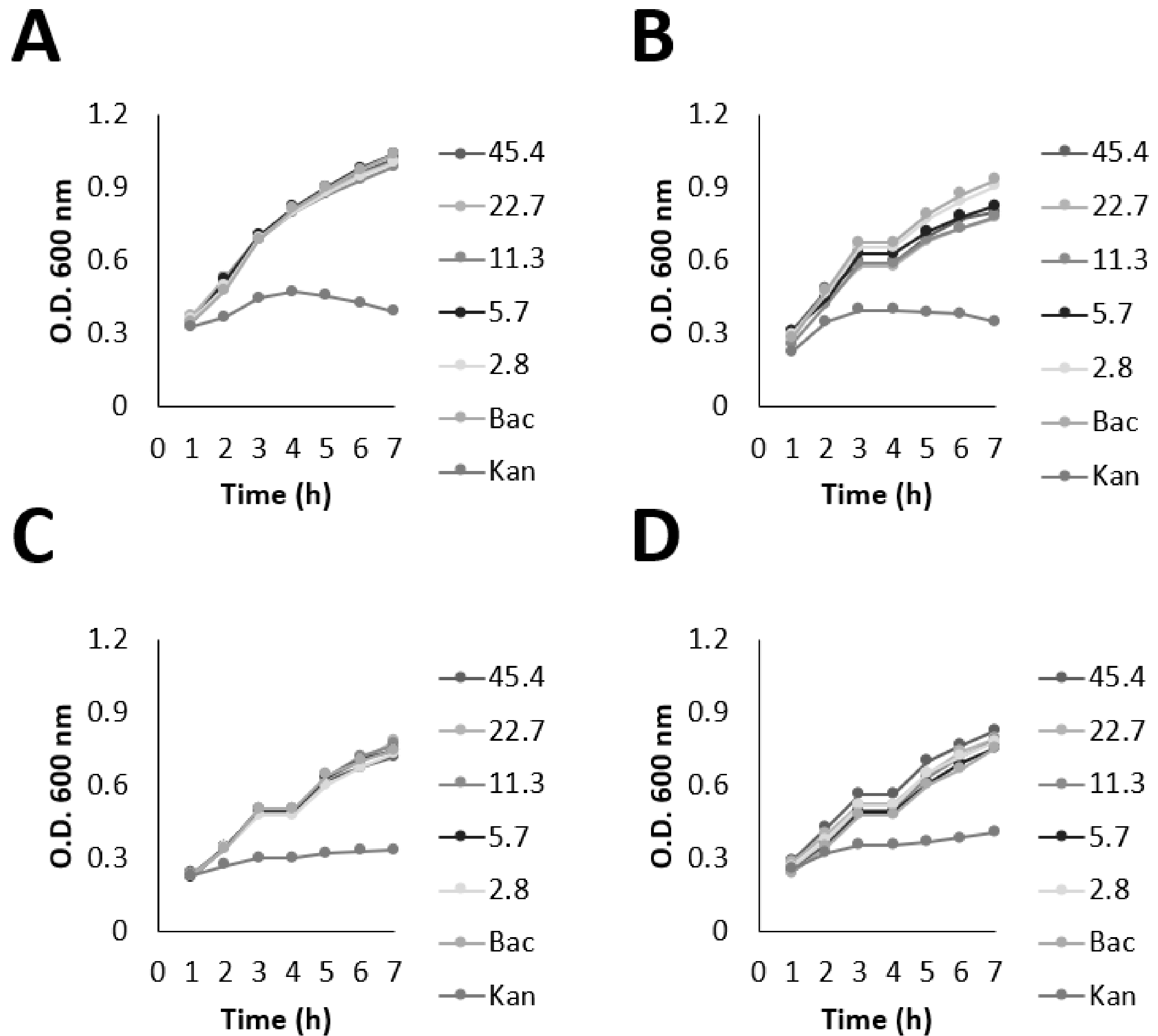


Figure 2.2. Effects of peptides on the growth of *Y.ruckeri*. The data are representative of *Y.ruckeri* growth after 7 h in the presence of (A) rtVWF, (B) Tsvep1, (C) Dpbl-32, (D) Dpbl-48. Where Kan (kanamycin 500 µg/ml) served as a positive control. Means are representative of three independent experiments.

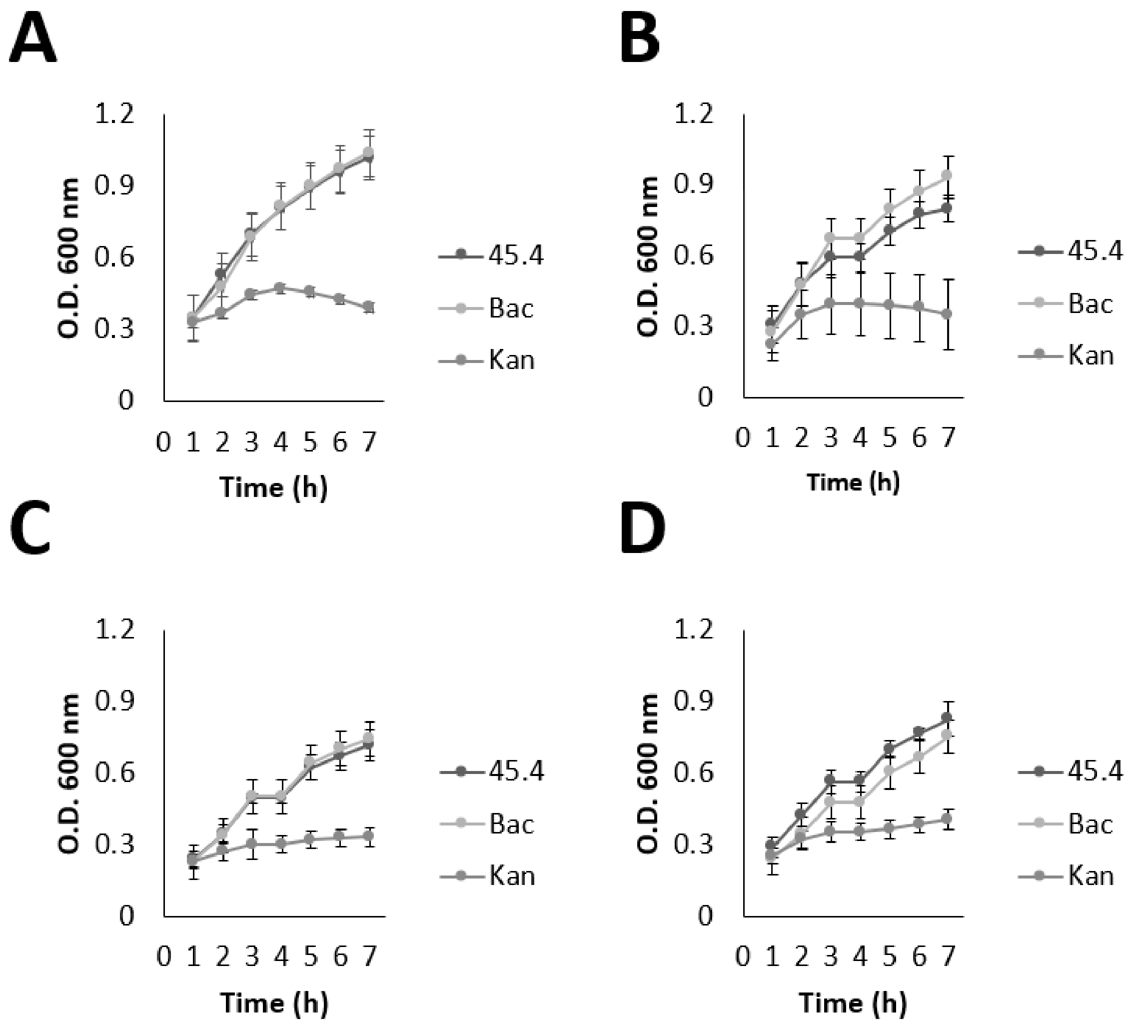


Figure 2.3. Effects of high dose peptides on the growth of *Y.ruckeri*. The data are representative of *Y.ruckeri* growth after 7 h in the presence of (A) rtVWF, (B) Tsvep1, (C) Dpbl-32, (D) Dpbl-48. Where Kan (kanamycin 500 µg/ml) served as a positive control. Means are representative of three independent experiments, ± S.E.M.

2.3.3. Growth of *Streptococcus iniae* in the presence of peptides

The growth of *S. iniae* was significantly reduced by rtVWF at 7 h post exposure (Figure 2.5A) but all other peptides did not impact the growth of bacteria (Figure 2.4).

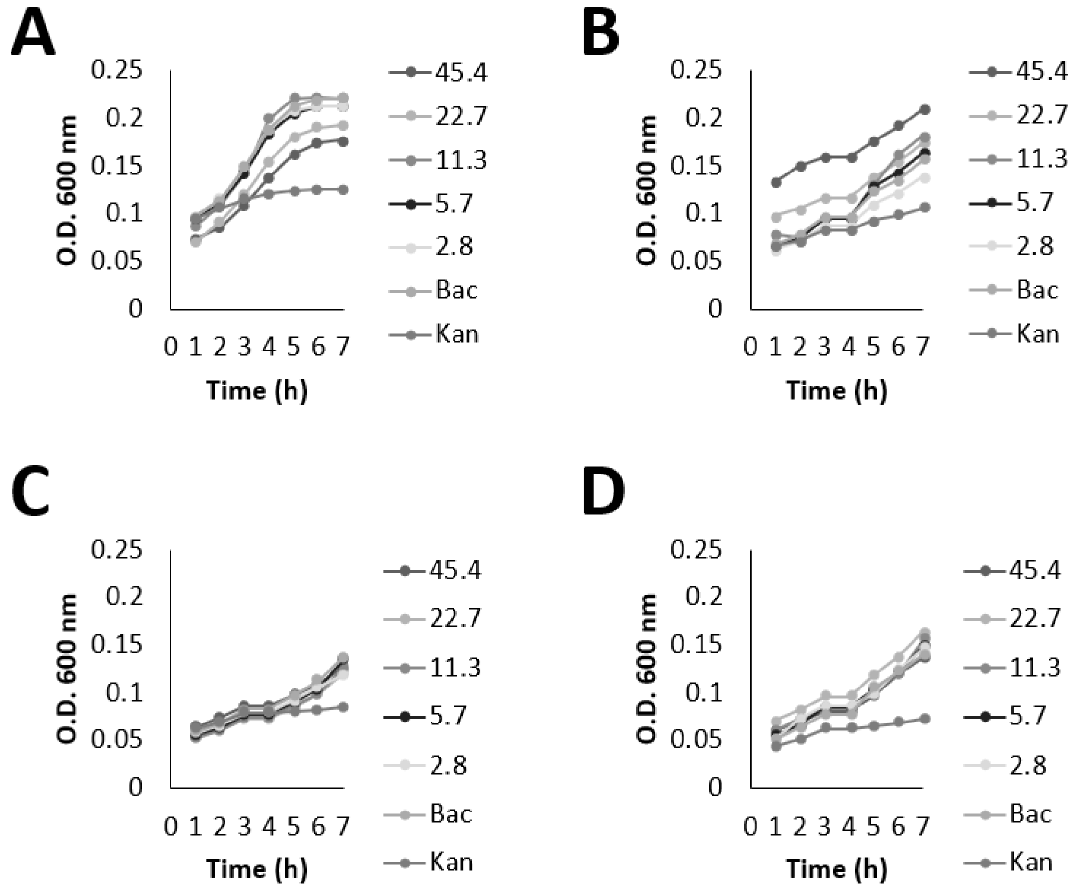


Figure 2.4. Effects of peptides on the growth of *S. iniae*. The data are representative of *S. iniae* growth after 7 h in the presence of (A) rtVWF, (B) Tsvep1, (C) Dpbl-32, (D) Dpbl-48. Where Kan (kanamycin 500 µg/ml) served as a positive control. Means are representative of three independent experiments, ± S.E.M.

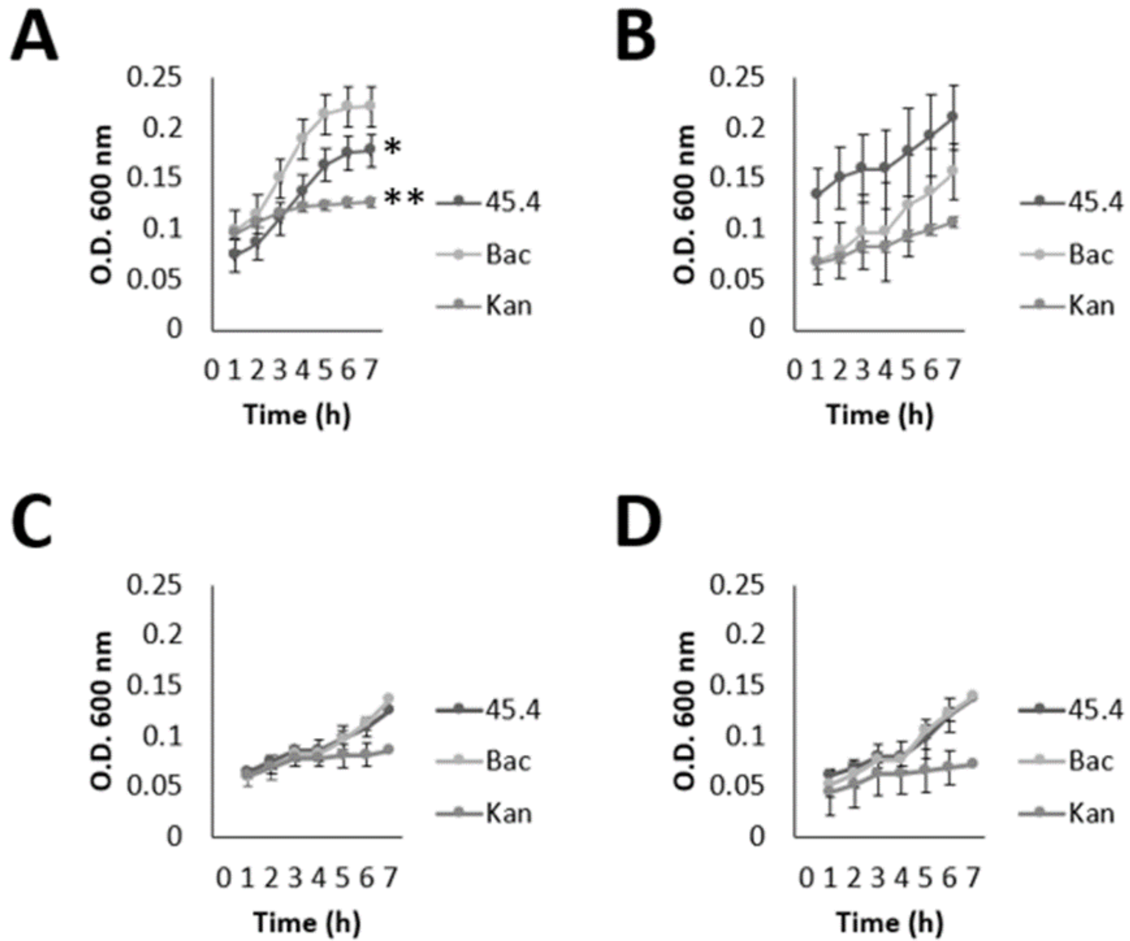


Figure 2.5. Effects of high dose peptides on the growth of *S. iniae*. The data are representative of *S. iniae* growth after 7 h in the presence of (A) rtVWF, (B) Tsvep1, (C) Dpbl-32, (D) Dpbl-48. Where Kan (kanamycin 500 µg/ml) served as a positive control. Means are representative of three independent experiments, ± S.E.M.

2.3.3. The effects of peptides on *in vitro* viral infection

EPC and CHSE-214 showed CPE over the course of 10 days (Figure 2.6). FV3-induced CPEs were observable at 1 day post infection; whereas cells infected with the other viruses only started to show CPE at 3 days post infection (Figure 2.6). Preincubating the peptides with each virus before infecting cells did not inhibit infection as CPEs were observed for each virus (Figure 2.7 – 2.9).

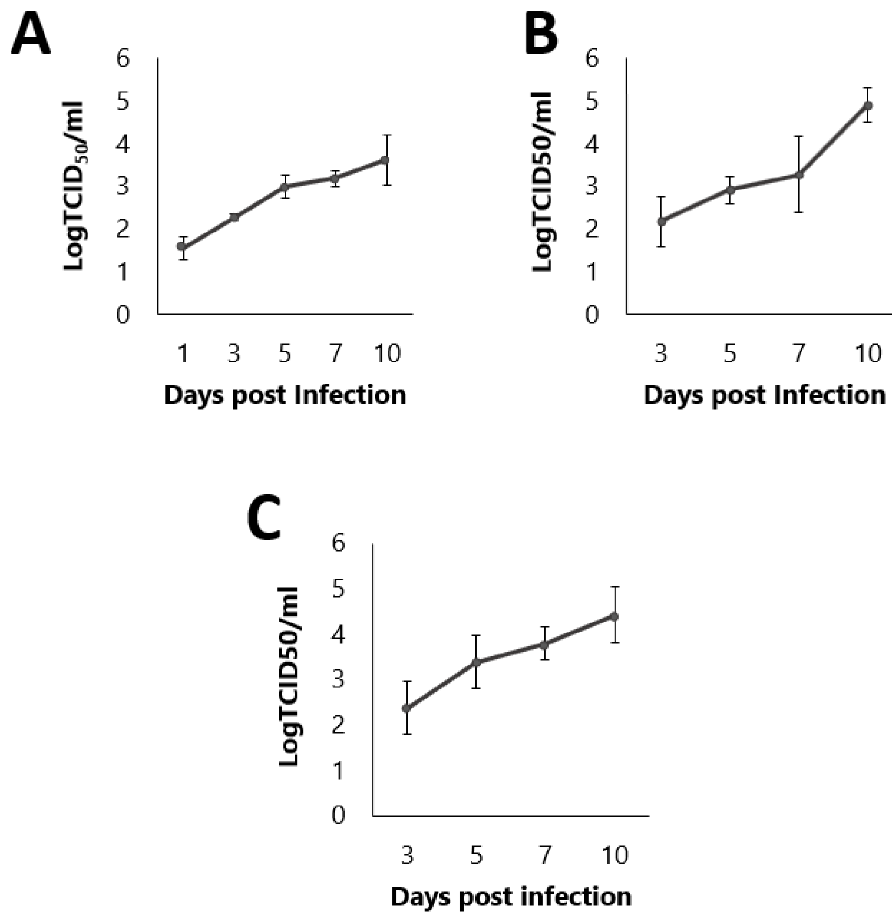


Figure 2.6. Viral titers of FV3, VHSV IVb and IPNV. The data are representative of the days on which cytopathic effects appeared during viral infection and were used to determine the $TCID_{50}$ (A) FV3, (B) VHSV IVb and (C) IPNV. Means are representative of three independent experiments, ± S.E.M.

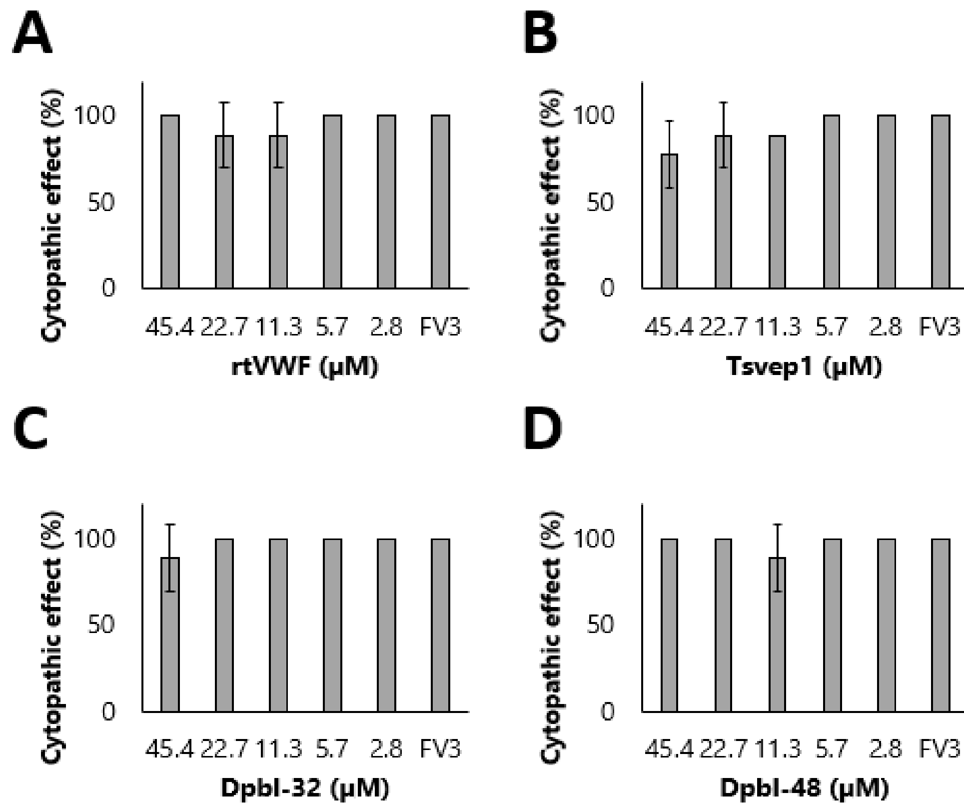


Figure 2.7. FV3 and peptide preincubation assay. Means are representative of three independent experiments, ± S.E.M.

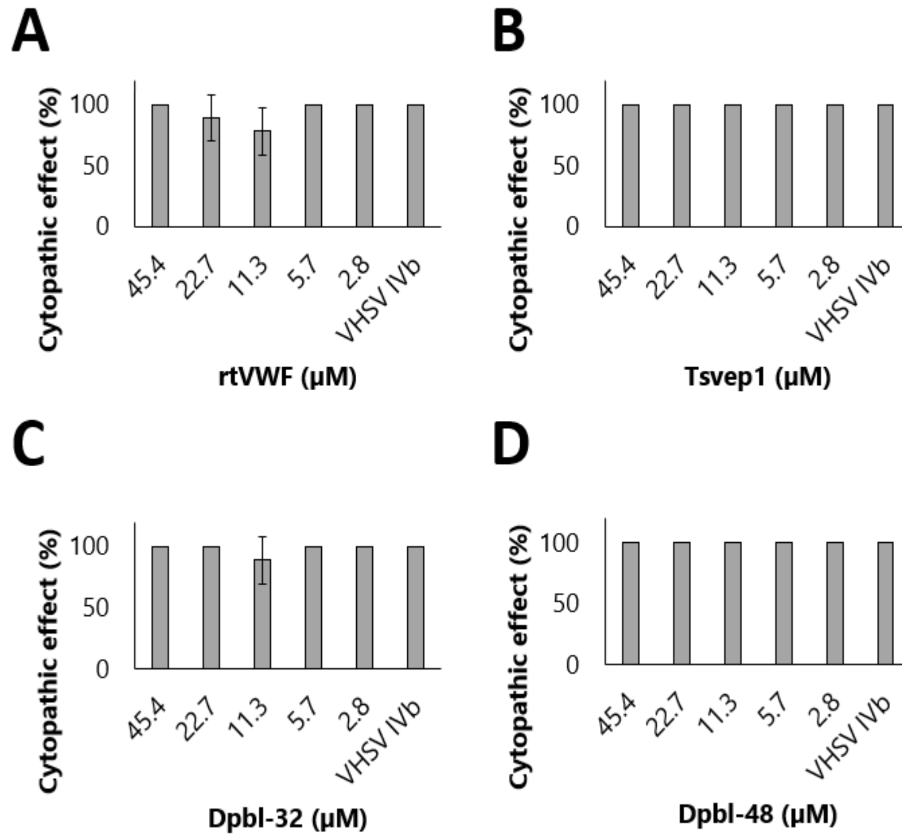


Figure 2.8. VHSV IVb and peptide preincubation assay. Means are representative of three independent experiments, ± S.E.M.

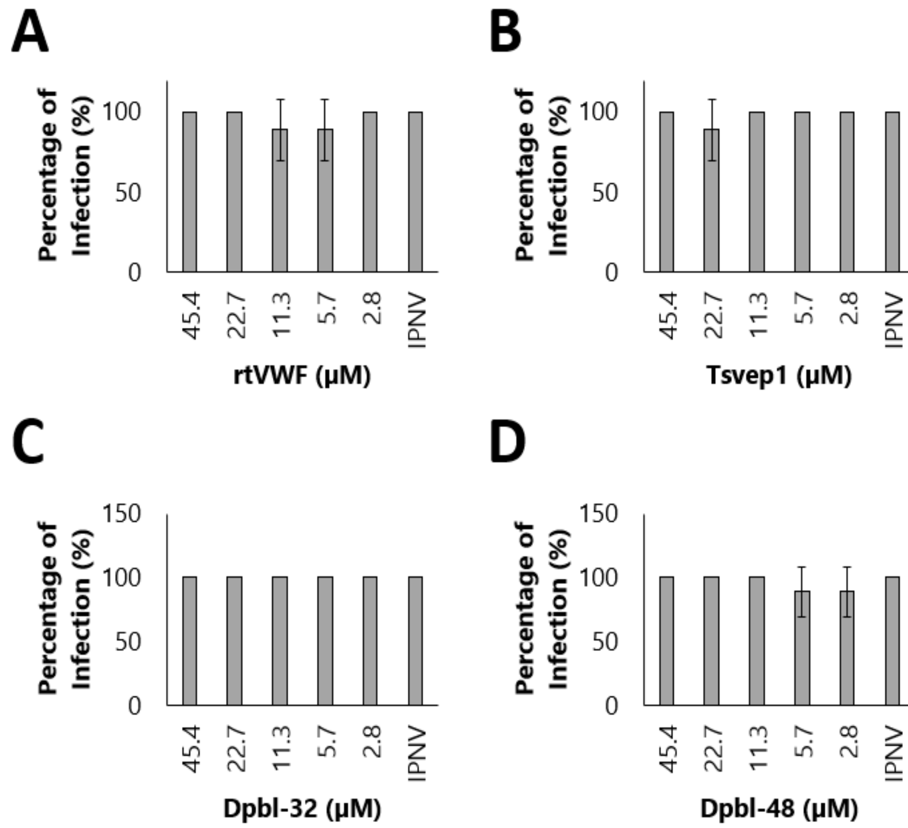


Figure 2.9. IPNV and peptide preincubation assay. Means are representative of three independent experiments, ± S.E.M.

2.3.4. The effects of peptides and *S. iniae* on *in vitro* rainbow trout cellular responses

Overall, treatment of cells with rtVWF did not have any negative effects. RTgill-W1 and RTS11 cells treated with rtVWF remained viable for up to 24 h (Figure 2.10). However, treatment of the same cell lines with heat-killed *S. iniae*, diluted to a final O.D. 600 nm of 0.017 in L-15 and 15% FBS, resulted in a significant reduction of viability in RTS11 and RTgill-W1 cells after 24 h (Figure 2.11). Therefore, the lower dose of heat-killed *S. iniae*, O.D. 600 nm of 0.0017 in L-15 and 15% FBS, was used to stimulate cells for gene expression analysis. Tumor necrosis factor alpha (*tnfa*) mRNA expression was significantly increased in RTS11 cells exposed to heat-killed *S. iniae* but not in RTgill-W1 cells (Figure 2.12A). RTgill-W1 cells did not display any changes in immune gene expression (Figure 2.12B). In addition, rtVWF did not have hemolytic effects on rainbow trout erythrocytes at the concentrations tested: 2.8, 11.3 and 45.4 µM (Figure 2.14B).

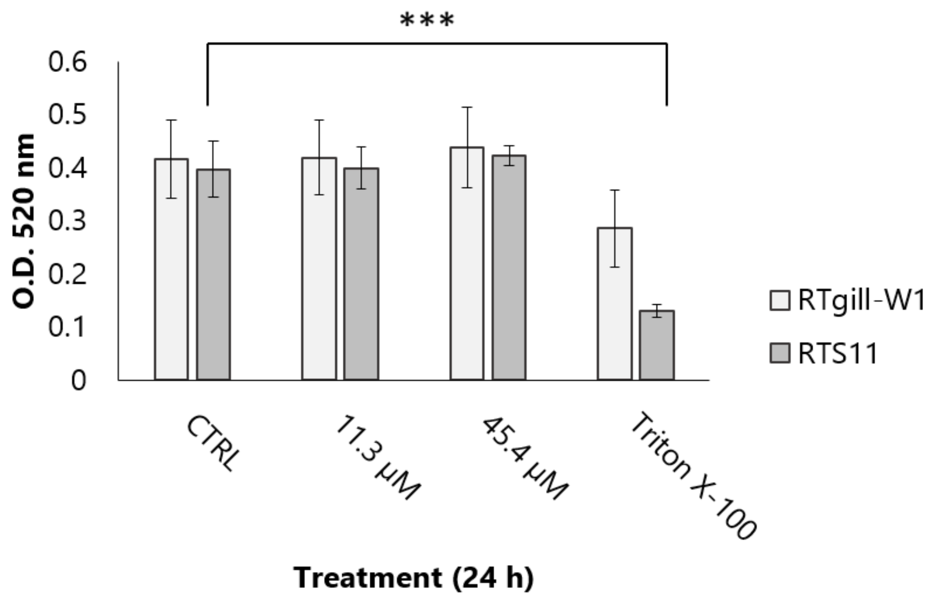


Figure 2.10. Effects of rtVWF on cell viability. The data are representative of MTT conversion in RTgill-W1 and RTS11 cells exposed to rtVWF for 24 h. A 0.01% Triton X-100 served as a positive control. Means are representative of three independent experiments, ± S.D.

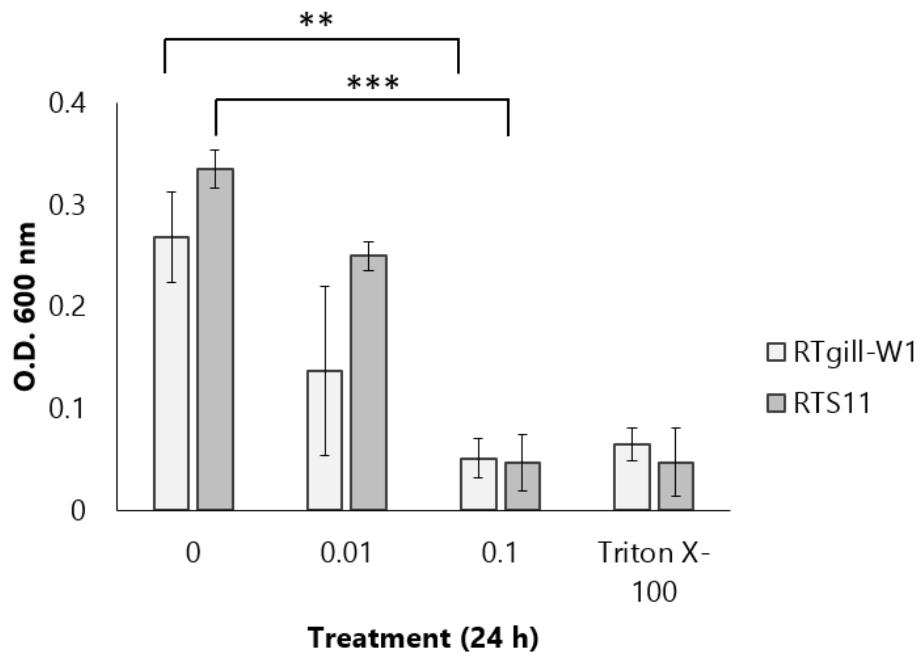


Figure 2.11. Effects of heat-killed *S. iniae* on cell viability. The data are representative of RTgill-W1 and RTS11 cells exposed to heat-killed *S. iniae* (1/100) or (1/10) for 24 h. A 0.01% Triton X-100 served as a positive control. Means are representative of three independent experiments, ± S.D.

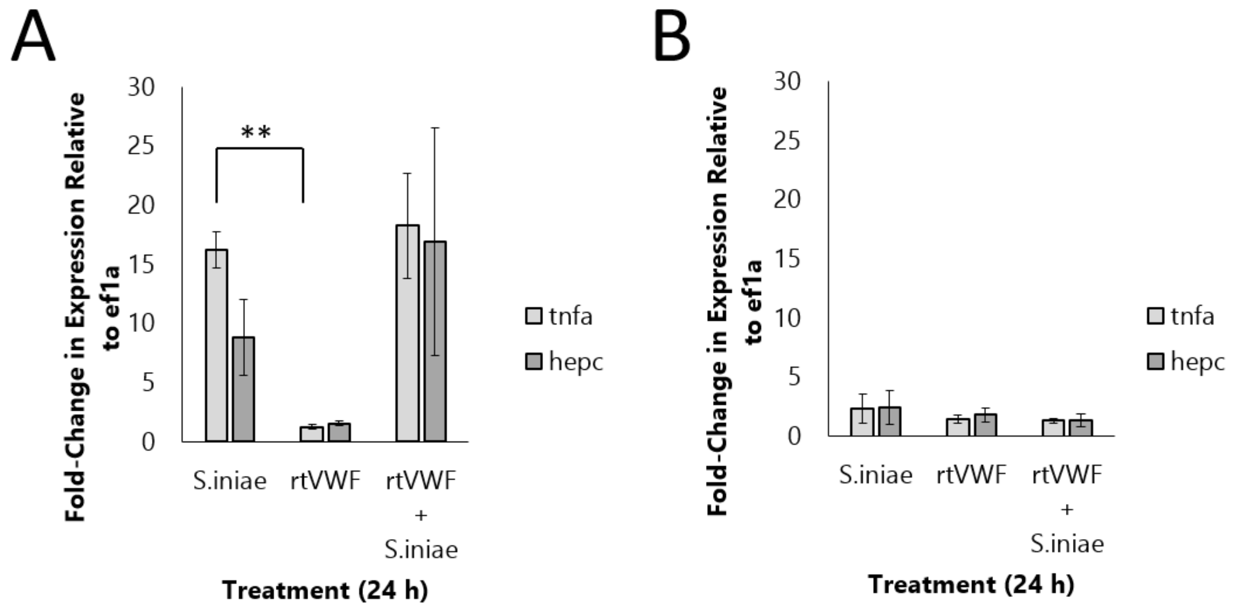


Figure 2.12. Effects of rtVWF pre-treatment and further stimulation with heat-killed *S. iniae* on cellular immune gene expression. The data are representative of immune gene expression in (A) RTS11 and (B) RTgill-W1 cells, exposed to rtVWF for 24 h and then heat-killed *S. iniae* at a dose of (1/100) for an additional 24 h. Means are representative of three independent experiments, ± S.D.

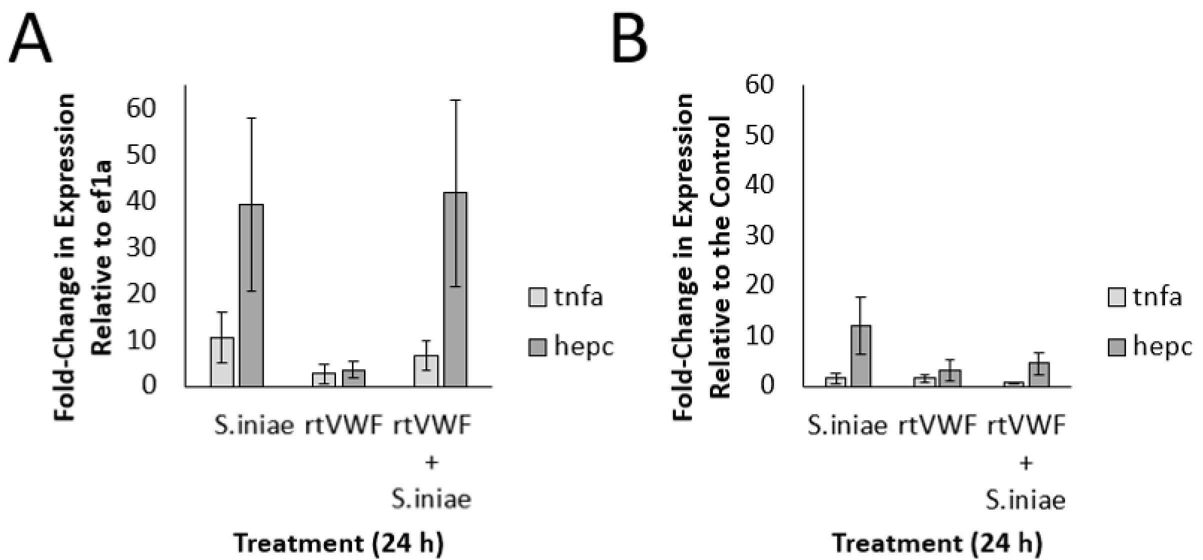


Figure 2.13. Effects of rtVWF pre-treatment in low serum and further stimulation with heat-killed *S. iniae* on cellular immune gene expression. The data are representative of immune gene expression in (A) RTS11 and (B) RTgill-W1 cells, exposed to rtVWF for 24 h and then heat-killed *S. iniae* at a dose of (1/100) for an additional 24 h. Means are representative of three independent experiments, ± S.D.

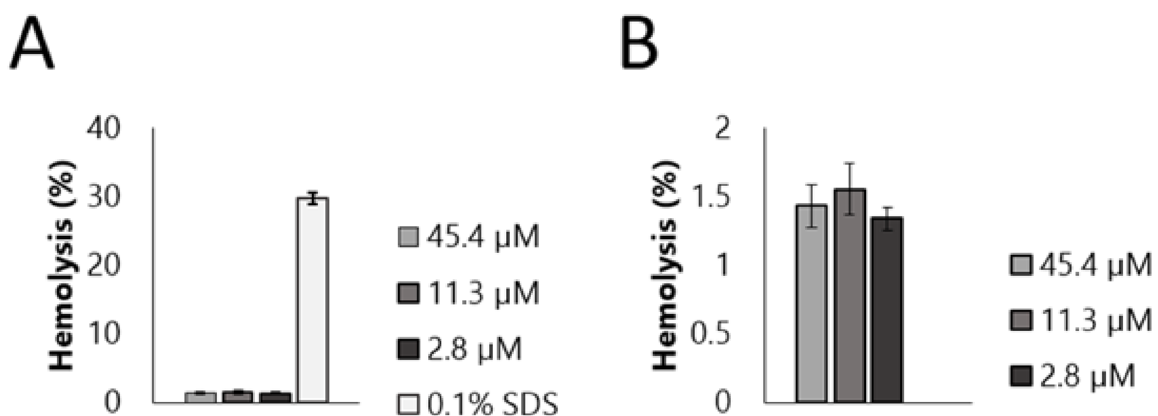


Figure 2.14. Effects of rtVWF on the hemolysis of rainbow trout erythrocytes. The data are representative of rainbow trout erythrocytes exposed to rtVWF for 30 min at room temperature (A) with positive control (0.1% SDS) (B) not showing positive control. Means are representative of three independent experiments, ± S.D.

2.4 Discussion

Investigating the effects of novel peptides on important aquatic pathogens and how they impact host physiology, may lead to the development of future therapeutics. In this study, novel peptides from various aquatic organisms were tested on a subset of aquatic pathogens; in order to better understand their antimicrobial and antiviral properties. In addition, the potential immunomodulatory effects of one peptide, rtVWF, were evaluated in RTS11 and RTgill-W1 cell lines. In general, the peptides did not have much of an effect on *F. psychrophilum* however, Tsvep1 did significantly increase the growth of this pathogen in a dose-dependent manner (Figure 2.1B). This result was unexpected, and it is well established that *F. psychrophilum* uses peptides and lipids for its metabolism (Bernardet and Kerouault, 1989). Tsvep1 was shorter in length, compared to the longer peptides studied, such as Dpbl-48; however, it was still able to stimulate bacterial growth (Appendix Table 1). AMPs that are limited in resistance to bacteria exhibit select physicochemical features including fewer polar and positively charged amino acids and display increased hydropathicity (Spohn et al. 2019). Tsvep1 has a higher proportion of neutral amino acid residues, compared to the other peptides (Appendix Table 2). Furthermore, 10% of the sequence of Tsvep 1 peptides are made up of branched chain amino acids (BCAAs) which are neutral residues such as valine and leucine (Appendix Table 2). BCAAs have been shown to be involved in bacterial physiology by promoting growth and virulence (Kaiser and Heinrichs, 2018). In addition, the peptides may have been susceptible to increased cleavage by metalloproteases which would make BCAAs more available for import. The M43 cytophagalysin metalloprotease genes were upregulated in *F. psychrophilum* cells grown at 8°C compared to 20°C; making this gene a cold-temperature regulated gene (Hesami et al. 2011). In the current study, *F. psychrophilum* was grown and incubated with peptides at 14°C, which may influence the expression of metalloprotease genes. *F. psychrophilum* has also been shown to be resistant to several AMPs of various origin up to 256 µg/ml (Ebbensgaard et al. 2015).

Compared to *F. psychrophilum*, *Y. ruckeri* was more susceptible to the same AMPs (Ebbensgaard et al. 2015). Some AMPs such as the mammalian AMP, Cap18, were highly effective on *Y. ruckeri* with an MIC of 2 µg/ml (Chettri et al. 2017; Ebbensgaard et al. 2015). Interestingly, some *Y. ruckeri* strains such as 392/2003 are not susceptible to a fish-derived peptide, originally from the mucus of hagfish (*Myxine glutinosa* L.), known as myxinidin (Subramanian et

al. 2009; Ebbensgaard et al. 2015). However, other *Y. ruckeri* strains, such 96-4, are highly susceptible to myxinidin at low concentrations (Subramanian et al. 2009). In the current study, none of the peptides were active against *Y. ruckeri*. This may be explained due to strain specificity, or the concentrations tested. Future research should investigate the use of the peptides in this study on various strains of *Y. ruckeri*, in addition to higher doses, to perhaps reveal any potential antimicrobial properties.

On the other hand, *S. iniae* was susceptible to rtVWF, a peptide-derived from rainbow trout. rtVWF significantly reduced the growth of this pathogen after 7 h of exposure when compared to control cells (Figure 2.3A). rtVWF had a higher proportion of acidic amino acid residues in its sequence compared to the other peptides which may have allowed it to interact with the polysaccharides in the capsule of the Gram-positive pathogen (Appendix Table 2, Locke et al. 2007). In addition, rtVWF was the shortest peptide used in the study (Appendix Table 1). Although this was not investigated, the short length of rtVWF may have facilitated its entry into the capsule. To determine if rtVWF was taken up by the capsule, the peptide could be fluorescently labelled and then added to *S. iniae* cells to be examined via microscopy. Scanning electron microscopy may also be useful to determine if the membrane of *S. iniae* is disrupted. The other peptides in this study did not display any antimicrobial effects on *S. iniae* but Tsvep1 did appear to increase the growth of *S. iniae*, similar to the results observed in *F. psychrophilum*; however, this was not statistically significant (Figure 2.5B).

The antiviral activity of salmonid-derived AMPs is not very well investigated (Brunner et al. 2020). However, several antiviral peptides have been isolated from other aquatic organisms and display a wide range of antiviral activity (Sumon et al. 2021). To investigate this direct peptide-virus interaction, each peptide was preincubated with either FV3, IPNV or VHSV IVb; however, none of the peptides prevented the viruses from infecting fish cells at the concentrations studied (Figures 2.7 – 2.9). Therefore, they do not possess antiviral activity at the concentrations tested. This is also common amongst frog skin-derived AMPs which display limited antiviral activity against FV3 (Varga et al. 2019). However, it is possible that the peptides might modulate immunity at the cellular level of the host, to control or prevent viral infection.

Since rtVWF was active against *S. iniae*, it was selected to be further investigated for potential immunomodulatory activities. *S. iniae* did not grow well in L-15 media. Therefore, the

bacteria were heat-killed and applied to RTS11 and RTgill-W1 cells. RTS11 is widely used to investigate *in vitro* teleost immune responses to pathogens but RTgill-W1 was chosen for this study because *S. iniae* uses the gills as a route of entry in fish (Kales et al. 2007; Semple et al. 2020; McNulty et al. 2003). The expression of two immune genes, hepcidin (*hepc*) an endogenous AMP gene and *tnfa*, remained unchanged in response to rtVWF exposure for 24 h (Figure 2.12). Incubation of cells with rtVWF and then heat-killed *S. iniae* also did not impact gene expression significantly but the expression of *hepc* mRNA in RTS11 did appear to increase compared to cells treated with *S. iniae* alone (Figure 2.12A). Tumor necrosis factor alpha gene expression was significantly upregulated in RTS11 cells exposed to heat-killed *S. iniae* for 24 h (Figure 2.12A). However, RTgill-W1 cells were largely unresponsive to the treatment (Figure 2.12B). Under low serum conditions, the cell lines responded in a similar manner but RTS11 cells appear to express lower levels of *tnfa*, in response to *S. iniae* (Figure 2.13). This suggests that low serum may negatively impact the response of cells to this pathogen and does not influence rtVWF. Moreover, *S. iniae* might only be detected by immune cells, such as macrophages, once it enters the gill tissue of fish, since gill epithelial cells appear to be poor sensors of this pathogen. However, only a subset of immune genes were surveyed and there may be others that are modulated during infection.

Peptides from a plethora of organisms have been explored as potential antimicrobial agents in aquaculture and have also been found to improve immunity in several fish species (Herrera et al. 2021; León et al. 2020). Low levels of antibiotics have also been shown to activate teleost immune responses in macrophages but the mechanisms are unknown (Qiu et al. 2020). Administration of AMPs, either prophylactically or therapeutically may be a useful alternative to antibiotics, for which some aquatic pathogens have developed resistance (Reverter et al. 2020). Resistance towards antibiotics amongst microbes usually occurs very rapidly (Yu et al. 2018). However, developing AMP-resistance, is a much slower process and is less likely to occur, due to two main reasons: pharmacodynaic properties and modes of action (Yu et al. 2018). The mutant selection window, an antimicrobial range between minimal concentration required to block growth; spanning to complete inhibition of bacteria, is much narrower for AMPs, than it is for antibiotics (Yu et al. 2018). This is another reason for delayed resistance towards AMPs. In addition, as observed in the current study, some AMPs possess strict antimicrobial properties against selection pathogens; whereas, many antibiotics are broad-spectrum (Reverter et al. 2020). However, the use of AMPs in large-scale aquaculture operations may present some challenges

such as cost and administration. Therefore, research focused on cost effective production and effective administration methods are required. Nevertheless, rtVWF may serve as a promising therapeutic against *S. iniae* in the future and the other peptide sequences in the study can be tested against other organisms, in an attempt to discover novel antimicrobial functions.

Chapter 3 – Investigating the role of rainbow trout gill and gut epithelial cells in *F. psychrophilum* infection

3.1.1. Introduction

Fish aquaculture has expanded to meet consumer demands and is an important source of food and economic activity. However, fish are susceptible to a plethora of pathogens that can result in significant economic losses (Flores-Kossack et al. 2020). A pathogen that is associated with high mortality events is *F. psychrophilum* (Madetoja et al. 2003, Bernadet et al. 2006). To date, no effective treatment has been developed, causing fish farms to rely on antibiotics for *F. psychrophilum* prevention and treatment, which has led to the development of antibiotic resistant strains (Hesami et al. 2010). Unfortunately, the pathogenesis of *F. psychrophilum* is not well understood which may hinder therapeutic developments. Dermal ulcers are thought to be the main route of entry for this pathogen (Miwa and Nakayasu, 2005). However, *F. psychrophilum* has also been found in the gill and gut of rainbow trout but direct host-pathogen interactions at the gill and gut interface remain under investigated (Nematollahi et al. 2005; Nematollahi et al. 2003; Pérez-Pascual et al. 2017).

It is well established that the teleost gill and gut are major sites of mucosal immunity and provide a physical and chemical barrier against pathogens. The epithelium and immune cells work in concert with commensal organisms to fend off invading microbes (Gomez et al. 2013). Moreover, various proinflammatory and anti-inflammatory cytokines in immune tissues are modulated by *F. psychrophilum* infection in rainbow trout (*Oncorhynchus mykiss*) (Kutyrev et al. 2016; Semple et al. 2020). Fish that display resistance against *F. psychrophilum*, exhibit significantly higher basal levels of cytokine receptor expression, especially within the first 2 days of infection (Sakai et al. 2021; Zou et al. 2016; Kutyrev et al. 2016). In addition, treatment of RTS-11 with a recombinant cytokine, tumor necrosis factor alpha 3 (Tnf-α3), upregulates the expression of AMPs (Hong et al. 2013). Collectively, the data suggest that cytokines may be involved in regulating the early immune response during *F. psychrophilum* infection in the gill or gut, by mediating the expression of AMPs in these tissues.

The mucosal tissues of fish are a rich source of AMPs and some peptides have been involved in processes such as commensal colonization and pathogen invasion (Hong et al. 2013; Brunner et al. 2020; Gomez et al. 2013; Schmitt et al. 2015). Many teleost cell types have been shown to express AMPs in response to pathogen challenge and some display potent antimicrobial activity against *F. psychrophilum* (Semple et al. 2019; Brunner et al. 2020). AMPs are also able to engage with receptors and stimulate downstream immune signaling pathways (Brunner et al. 2020; Lee et al. 2019). For example, the human AMP, known as LL-37, is an agonist for insulin-like growth factor receptor and promotes interleukin 1 beta (Il1b) via engagement with purinergic receptors (Girnita et al. 2012; Elssner et al. 2004). Fish AMPs may interact with insulin-like growth factors and purinergic receptors but this remains unknown. Nevertheless, insulin-like growth factor and purinergic receptors play important roles in the immune systems of many species, including teleosts; and are of great interest to aquaculture since they also stimulate growth (Sipos et al. 2017; Tonner et al. 1995; Alzaid et al. 2016; Li et al. 2021).

3.1.2. *Flavobacterium psychrophilum* pathogenesis

F. psychrophilum is a systemic bacterial pathogen that causes fish to develop ulcerative dermal lesions (Miwa and Nakayasu, 2005). However, *F. psychrophilum* has also been observed to associate with the gill and gut of rainbow trout but direct host-pathogen interactions with these tissues remain obscure (Nematollahi et al. 2005, Nematollahi et al. 2003, Pérez-Pascual et al. 2017). Attenuation of genes important in gliding motility such as gliding motility-associated lipoprotein D (*gldD*) and gliding motility-associated lipoprotein G (*gldG*) in the THCO2-90 strain of *F. psychrophilum* impairs bacterial adhesion in the gills of challenged fish (Pérez-Pascual et al. 2017). This suggests that gliding motility, the ability to translocate without external appendages, may be an important factor in the establishment of *F. psychrophilum* infection. Even when glD and gldG are attenuated, the bacterium still reaches the internal cavity and is found in the spleen (Pérez-Pascual et al. 2017). Semple and colleagues have demonstrated that *F. psychrophilum* stimulates the RTS-11 cell line (Semple et al. 2020). RTS-11 responds by increasing the expression of several pro and anti-inflammatory cytokines (Semple et al. 2020). In the living organism, the macrophages would be abundant in tissues such as the spleen which has shown involvement during the pathogenesis of *F. psychrophilum*. Infection with *F. psychrophilum* leads to the development and rapid progression of perisplenitis, splenic necrosis and peritonitis in juvenile rainbow trout

(Marancik et al. 2014). However, more research is warranted to better understand the pathogenesis of *F. psychrophilum*.

3.1.3. Mucosal immunity and *Flavobacterium psychrophilum*

The mucosal immune system of fish consists of gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (MALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas et al. 2015). Mucus is produced by specialized cells, known as goblet cells, in the tissues. The layer of mucus provides a site for bacterial adhesion and supports the colonization of various microbes (Van Tassell and Miller, 2011). Natural species on the skin of fish can outcompete certain pathogens (Peatman et al. 2015). Immunoglobulins secreted by plasma cells near the mucosal barrier also influence what species penetrate the barrier (Peatman et al. 2015). Channel catfish resistant to *Flavobacterium* spp. have increased numbers of goblet cells in the gills (Peatman et al. 2015). The mucosal tissues also contain B and T cells, in addition to other immune cells that work together with commensal organisms and epithelial cells to orchestrate immune responses and confer protection to the host (Salinas et al. 2015). It is likely that all types of mucosal tissues play a role in the pathogenesis of *F. psychrophilum*. There is some evidence to suggest that the microbiome of mucosal sites may play a role. For example, the gut microbiome of fish resistant to *F. psychrophilum*, has been shown to differ in microbial diversity and abundance but the gill microbiomes between resistant and susceptible fish were similar (Brown et al. 2019). Furthermore, the gills of resistant fish had a greater abundance of Proteobacteria and less Firmicutes, compared to susceptible fish (Valdés et al. 2020). This suggests that the microbiome, along with other factors, such as direct gill and gut epithelial-pathogen interactions may be involved in controlling the response to *F. psychrophilum*. Therefore, the purpose of this study was to investigate the roll of RTgill-W1 cells and RTgutGC in the initial immune response to *F. psychrophilum* and to identify potential crosstalk with RTS11 cells. RTgill-W1 and RTgutGC cells remained viable for at least 24 h in the presence of *F. psychrophilum*. Upon exposure to *F. psychrophilum*, RTgutGC cells upregulated interleukin 6 (*il6*) and interleukin 1 beta (*il1b*) gene expression. Furthermore, when RTgutGC cells were co-cultured with RTS11, and exposed to *F. psychrophilum*; RTS11 cells upregulated interleukin 1 beta (*il1b*) and tumor necrosis factor alpha (*tnfa*) gene expression. This suggests that gill and gut epithelial

cells may be important sensors and involved in orchestrating the acute inflammatory response during *F. psychrophilum* infection.

3.2 Materials and Methods

3.2.1. Cell culture

RTgutGC, RTS11 and RTgill-W1 cell lines were maintained at room temperature and ambient air in vented 25 cm^2 cell culture flasks (Thermo Fisher Scientific). RTgutGC and RTgill-W1 cells were supplemented with 4 ml of Leibowitz's (L-15) medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin-streptomycin (Sigma). RTS11 cells were maintained under the same conditions except at 30% (v/v) FBS. RTgutGC and RTgill-W1 cells were routinely subcultured at a ratio of 1:2; every four days and RTS11 cells, every seven days at a ratio of 1:2. Briefly, cell monolayers were rinsed with 1 × Dulbecco's phosphate-buffered saline (Gibco) and a 0.05% trypsin–EDTA (Gibco) solution was applied to detach the cells from the plate.

3.2.2. Growth of bacteria

Flavobacterium psychrophilum FPG101 was grown at 14°C in cytophaga broth with a final pH of 7.2. Each liter of cytophaga broth contained: 0.5 g tryptone, 0.5 g yeast extract, 0.2 g sodium acetate, 0.2 g beef extract (BD Biosciences). All bacterial stocks were stored in a 50% (v/v) of glycerol and respective broth solution at -80°C in sterile 1.5 ml cryovials (Thermo Fisher Scientific, USA). Before each experiment, the stocks were plated onto cytophaga plates and the plates were incubated for 3 days at the aforementioned temperatures. A single colony from each plate was picked and grown in 3 ml of the respective broth for an additional 3 days, at the aforementioned temperatures and this stock was used for each experiment.

3.2.3. Exposure of cells to bacteria

RTgill-W1 and RTgutGC cells were seeded at 300,000 cells/well in 2 ml of L-15 in a 6-well plate (Thermo Scientific) and allowed to incubate at 14°C overnight to develop into a monolayer. Three milliliters of L-15 (Gibco) with or without *F. psychrophilum* and free of antibiotics was applied to each well. The plates were then sealed with parafilm and placed back into a 14°C incubator. Cells were sampled from each well at 1, 6 and 18 h post-infection via trypsinization.

Briefly, adherent cells of one control well and one infected well were re-suspended at each time point using a 0.05% trypsin–EDTA solution and an equal amount of L-15 medium with 10% FBS and 1% penicillin-streptomycin. The trypsinized cells were then used for further analyses.

3.2.4. Co-culture of cells

RTS11 cells were seeded at 300,000 cells/well in 2 ml of L-15 with 2% FBS in a 6-well plate (Nunc). At the same time, polycarbonate inserts with 8 µm pores were added to the centre of each well (Nunc). Three hundred thousand RTgutGC cells were added to each insert in a final volume of 1.75 ml of L-15 with 10% FBS. The plate was sealed with parafilm and allowed to incubate at 14°C overnight to develop into a monolayer. The media from each insert was drained and replenished with 1.75 ml of L-15 with or without *F. psychrophilum* at an MOI of 1. The plates were then sealed with parafilm and placed back into a 14°C incubator. At 1, 6 and 18 h post-infection, the total volume of medium inside the bottom well was collected to capture monocytes and briefly stored at room temperature while any remaining adherent macrophages were removed via scraping and added to the collected suspension. The suspension was centrifuged at 2000 rpm for 2 min at room temperature. The supernatant was removed, and the cell pellet was used for further analysis.

3.2.5. Trypan blue exclusion assay

The trypan blue exclusion assay was used to assess cell viability. Briefly, an equal amount of trypan blue (Sigma) was added to the original trypsinized cell suspension to reach a final dilution factor of 2. Ten microliters of the suspension was loaded onto a hemocytometer and cells were counted via light microscopy. The percent cell viability at each time point was calculated using the following formula: % Cell Viability = total number of live cells/total number of cells × 100. In addition, the media from this experiment was sampled at 24 h to ensure that *F. psychrophilum* remained viable (Appendix Figure 1).

3.2.6. Total RNA extraction and cDNA synthesis

Total RNA was extracted from RTgill-W1 and RTgutGC cells challenged with *F. psychrophilum* using the RNeasy Micro Kit by following the manufacturer's protocol (Qiagen).

RNA purity and quantity were determined using a NanodropTM 2000 Spectrophotometer. Total RNA samples were treated with DNase I (Thermo Scientific) and stored at -80 °C until further use. cDNA was synthesized on the same day from 400 µg of RNA per reaction, using qScriptTM cDNA SuperMix (Quantabio) and following the manufacturer's protocol. Each reaction was incubated at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The cDNA was stored at -20 °C for future analyses.

3.2.7. RT-PCR

The primer designing tool from Primer-BLAST was used to design some of the primers in this study. RT-PCR was performed for each gene of interest and *ef-1α* served as a housekeeping gene. For each 20 µl reaction, the master mix consisted of 2 µl of 10 × buffer, 0.8 µl of dNTP, 2 µl of MgCl2, 0.5 µl of forward and reverse primers; the primers were diluted to achieve a final working concentration of 0.25 µM, 0.04 µl of Taq polymerase, 0.5 µl of cDNA and 13.66 µl of DEPC water. A no-template control was also included. Thermocycling conditions were specific to each primer (Appendix Table 3). Gel electrophoresis of each product was performed on 1% agarose gels with 1 × TAE buffer. The DNA fragment was cut from the gel and the QIAquick® Gel Extraction kit was used to prepare the sample for sequencing by following the manufacturer's protocol. All extracted PCR products were sequenced by The Centre for Applied Genomics and chromatograms were analyzed for each PCR product.

3.2.8. qRT-PCR

qRT-PCR was performed with WISENT ADVANCED™ qPCR mastermix (Wisent), by following the manufacturer's instructions on the LightCycler® 480 II (Roche). Briefly, each 10 µl reaction consisted of 1 µg of cDNA (2.5 µl), 0.25 µM of forward and reverse primers (0.5 µl), 2 × WISENT ADVANCED™ qPCR mastermix (5 µl) (Wisent), and DEPC water (2 µl). A no-template control was also included. All trials were conducted in triplicate and each program consisted of a pre-incubation at 95 °C for 10 min, then 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at the specified primer annealing temperature for 30 s and extension at 72 °C for 20 s. Primer efficiency ranged from 91.77 – 110.74 % (Supplementary Table 1). The fold change of mRNA expression was calculated using the 2-ΔΔCt method (Livak et al. 2001).

3.2.9. Statistics

Viability data was analyzed using an unpaired Student's t-test with unequal variance. Gene expression data were analyzed using a one-way ANOVA test with Tukey's post hoc test using Vassar Stats software (Vassar Stats). The alpha value was set at 0.05 ($P < 0.05$) for all tests. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3 Results

3.3.1. Viability and gene expression in RTgill-W1 and RTgutGC cells exposed to *Flavobacterium psychrophilum*

The viability of RTgill-W1 and RTgutGC cells was not negatively impacted over 24 h of exposure to *F. psychrophilum* (Figure 3.15). However, *F. psychrophilum* exposed RTgutGC cells exhibited a slightly higher viability compared to RTgill-W1 cells (Figure 3.15).

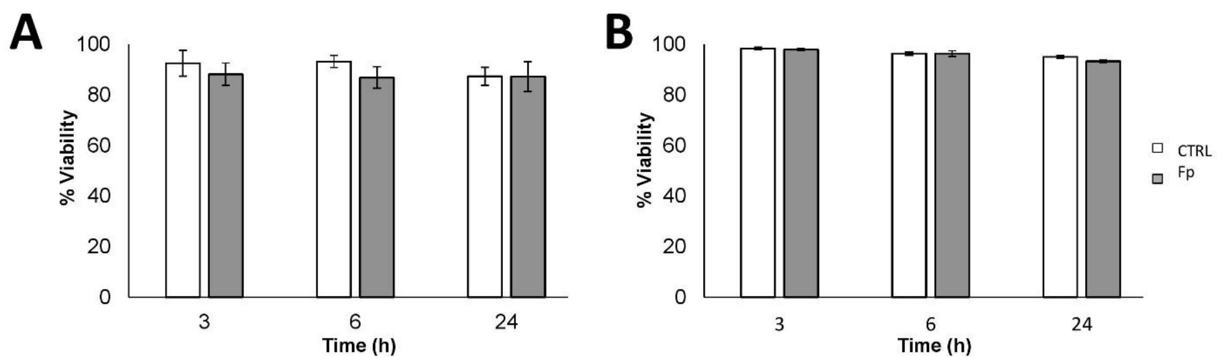


Figure 3.15. Cell viability during exposure to *Flavobacterium psychrophilum*. A: RTgill-W1 cell viability. B: RTgutGC cell viability. Control (CTRL) cells received medium only. Treated (Fp) cells received mediuminteresign with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with an unpaired t-test (unequal variance). Values are representative of the mean ± SEM, $n = 3$.

The expression of receptor genes did not change significantly in RTgill-W1 or RTgutGC cells, exposed to *F. psychrophilum* (Figure 3.16). The expression of *p2x1* appeared to increase at 6 hpi in RTgill-W1 cells but this was not statistically significant (Figure 3.16).

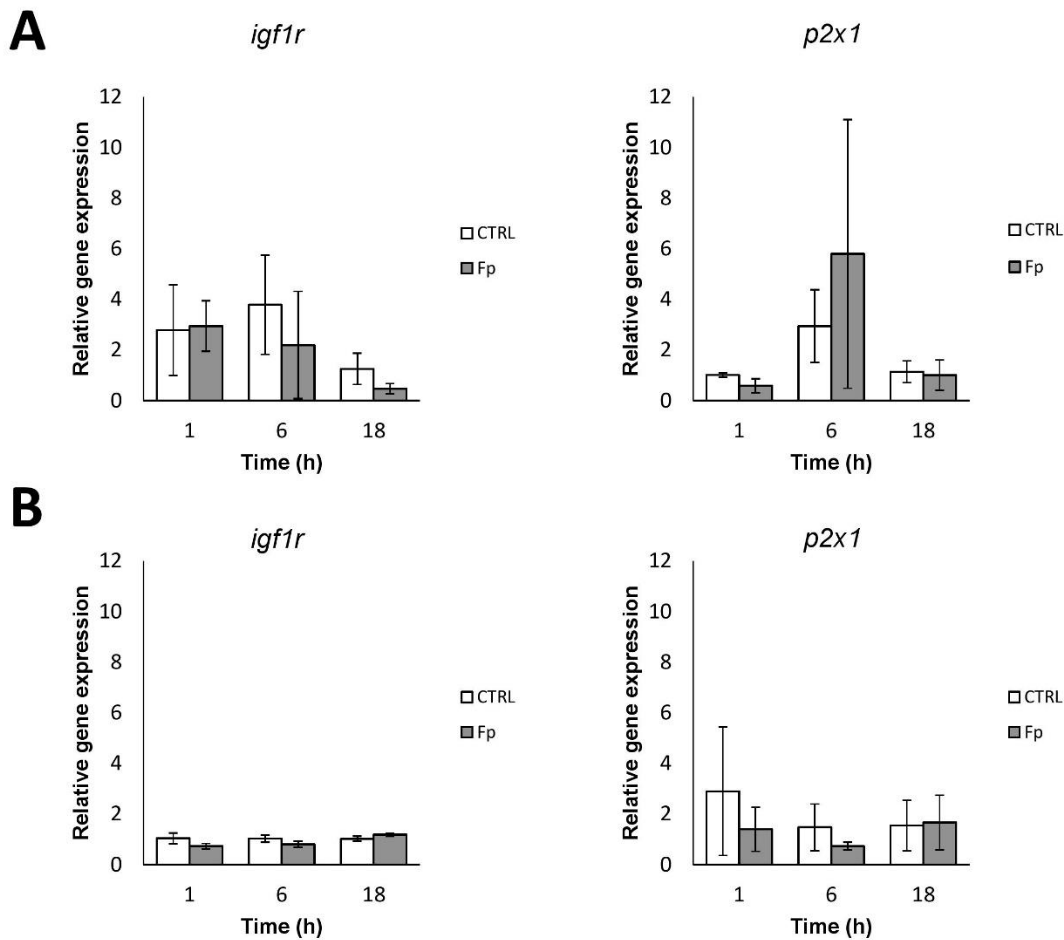
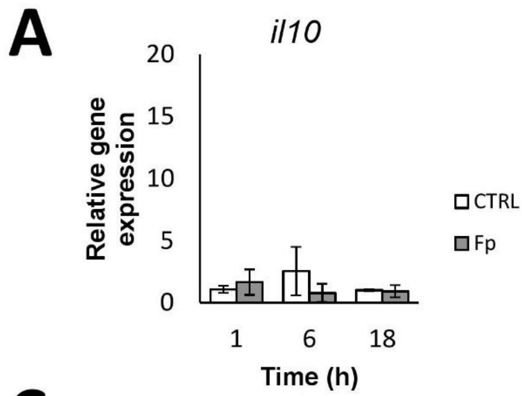
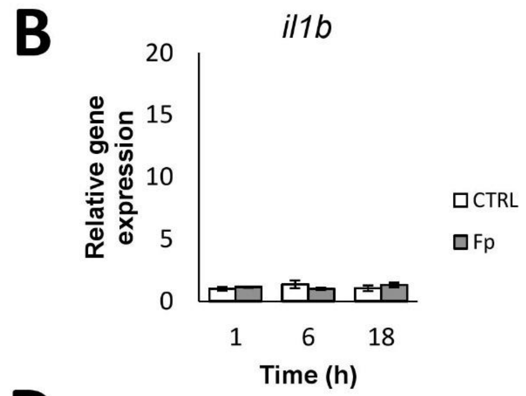
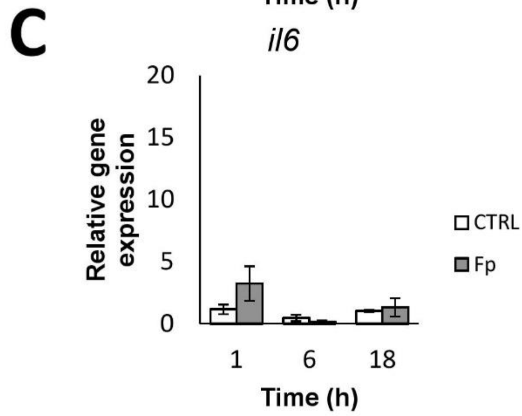


Figure 3.16. Receptor gene expression in response to *Flavobacterium psychrophilum*. (A) RTgill-W1 (B) RTgutGC. Control (CTRL) cells received medium only. Treated (Fp) cells received medium with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

RTgill-W1 cells exposed to *F. psychrophilum* did not exhibit any changes in the expression of cytokine genes over 18 h. However, RTgutGC responded with significant increased expression of *il1b* and *il6* mRNA at 18 hpi (Figure 3.17).


A
il10
Relative gene expression
20
15
10
5
0
1
6
18
Time (h)
CTRL
Fp


B
il1b
Relative gene expression
20
15
10
5
0
1
6
18
Time (h)
CTRL
Fp


C
il6
Relative gene expression
20
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1
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Time (h)
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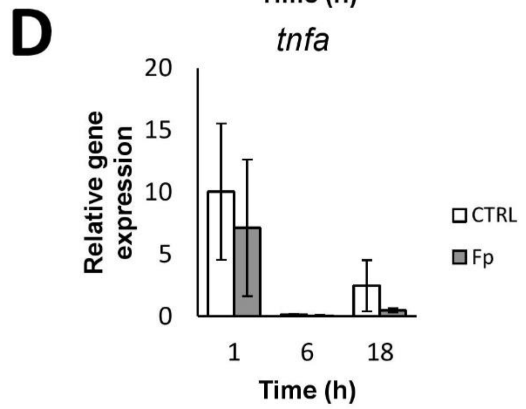
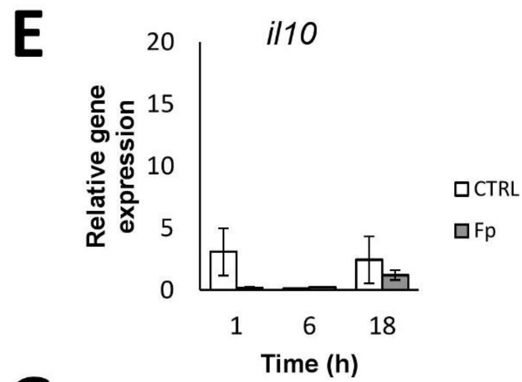
Fp


D
tnfa
Relative gene expression
20
15
10
5
0
1
6
18
Time (h)
CTRL
Fp


E
il10
Relative gene expression
20
15
10
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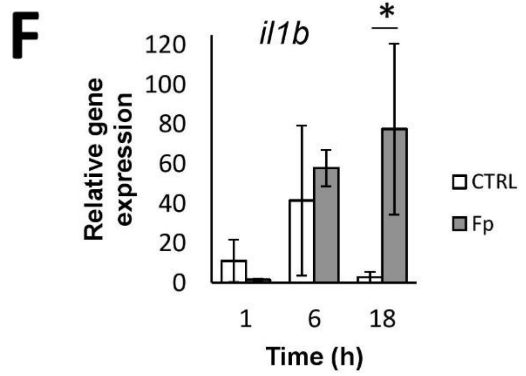
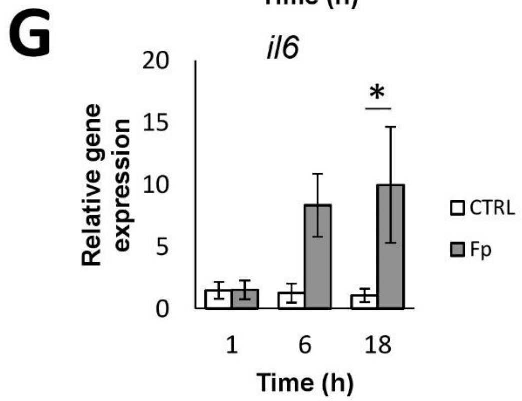
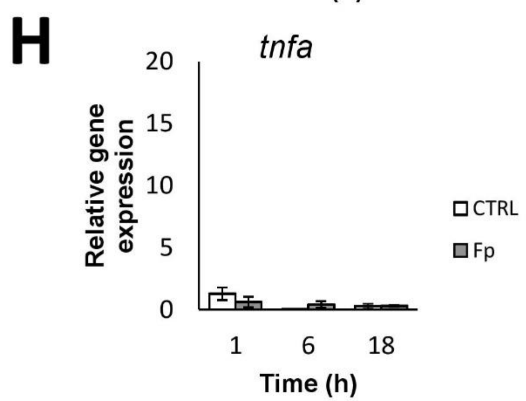
6
18
Time (h)
CTRL
Fp


F
il1b
*
Relative gene expression
120
100
80
60
40
20
0
1
6
18
Time (h)
CTRL
Fp


G
il6
*
Relative gene expression
20
15
10
5
0
1
6
18
Time (h)
CTRL
Fp


H
tnfa
Relative gene expression
20
15
10
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0
1
6
18
Time (h)
CTRL
Fp

Figure 3.17. Cytokine gene expression in response to *Flavobacterium psychrophilum*. (A-D) RTgill-W1 (E-H) RTgutGC. Control (CTRL) cells received medium only. Treated (Fp) cells received medium with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$. Asterisks denote statistical significance ($*P < 0.05$).

The expression of AMP genes did not change significantly after *F. psychrophilum* exposure in RTgill-W1 or RTgutGC. Although *cath1*, *cath2* and *hepc* mRNA appeared to increase in RTgutGC cells; it was not statistically significant (Figure 3.18).

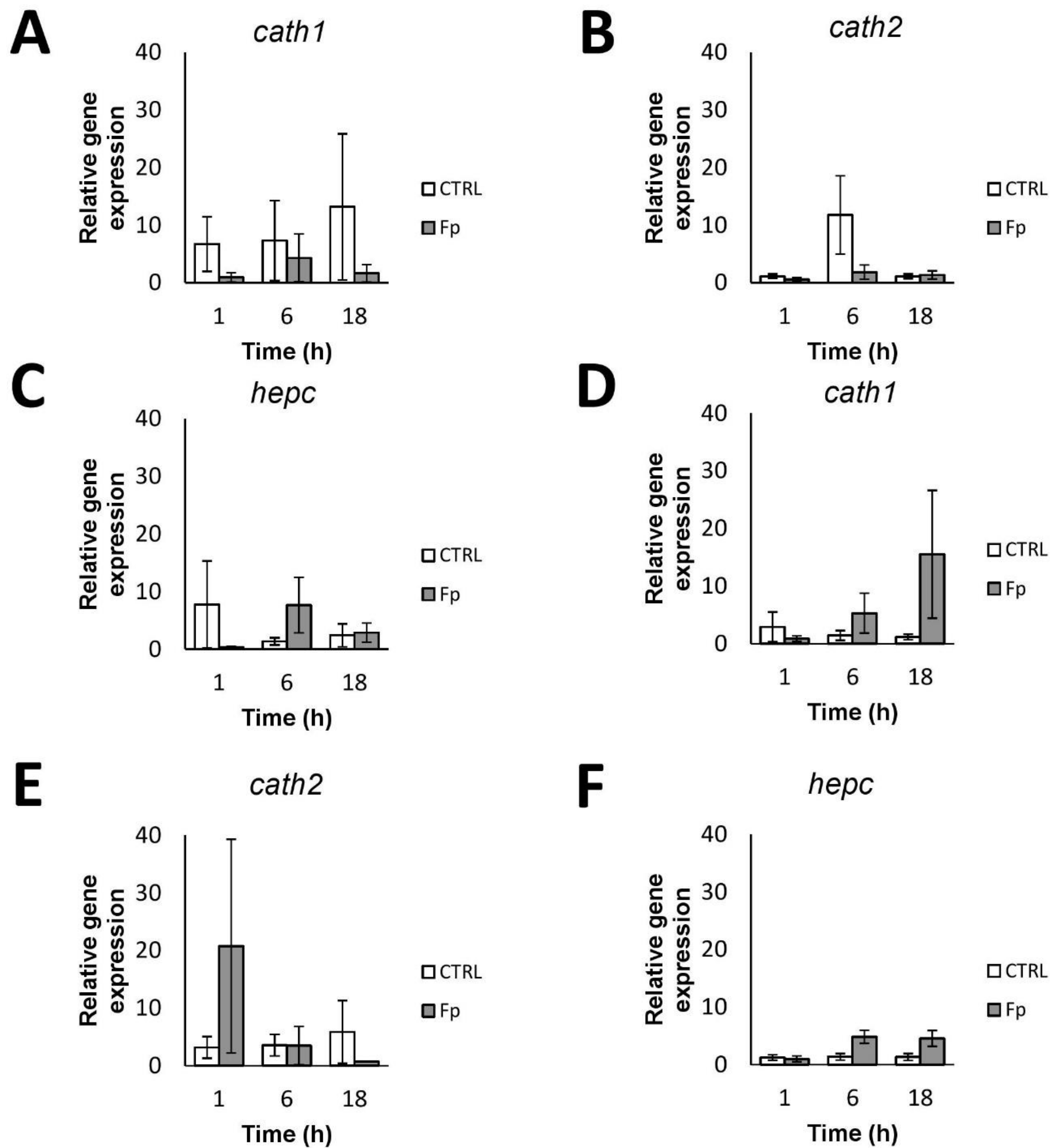

Figure 3.18. Antimicrobial peptide gene expression in response to *Flavobacterium psychrophilum*. (A-C) RTgill-W1 (D-F) RTgutGC. Control (CTRL) cells received medium only. Treated (Fp) cells received medium with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

3.3.2. Gene expression in RTS11 cells exposed to *Flavobacterium psychrophilum*

The expression of *igf1r* or *p2x1* mRNA in RTS11 cells did not change significantly over 18 hpi (Figure 3.19). However, *il1b* and *tnfa* mRNA were significantly upregulated at 6 and 18 hpi (Figure 3.20). Interleukin 6 mRNA also appeared to increase in RTS11 cells exposed to bacteria but this was not statistically significant (Figure 3.20). Similarly, *hepc* mRNA also appeared to increase in RTS11 cells but this was not statistically significant (Figure 3.21).

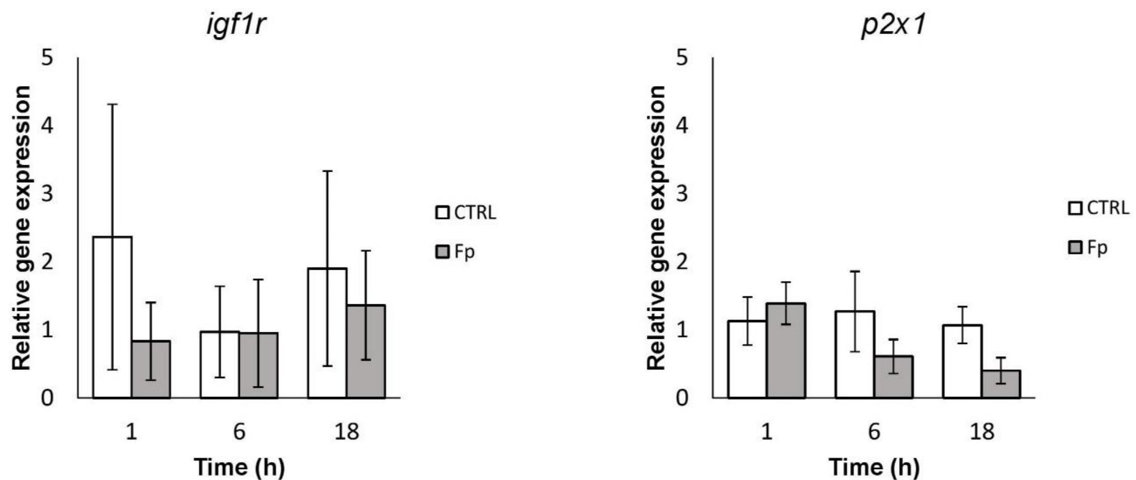

Figure 3.19. Receptor gene expression in RTS11 cells co-cultured with RTgutGC cells and *Flavobacterium psychrophilum*. Control (CTRL) cells received media only. Treated (Fp) cells received media with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

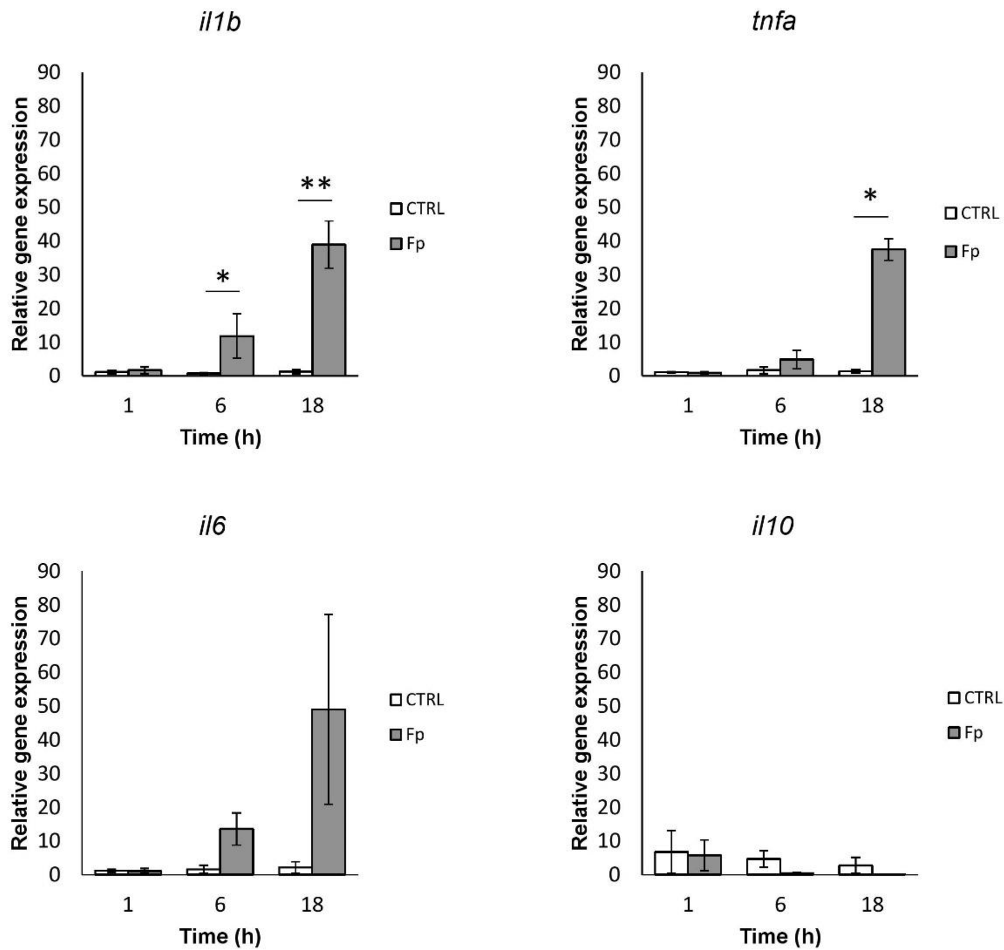

Figure 3.20. Cytokine gene expression in RTS11 cells co-cultured with RTgutGC cells and *Flavobacterium psychrophilum*. Control (CTRL) cells received media only. Treated (Fp) cells received media with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$. Asterisks denote statistical significance (*P <0.05, **P <0.01).

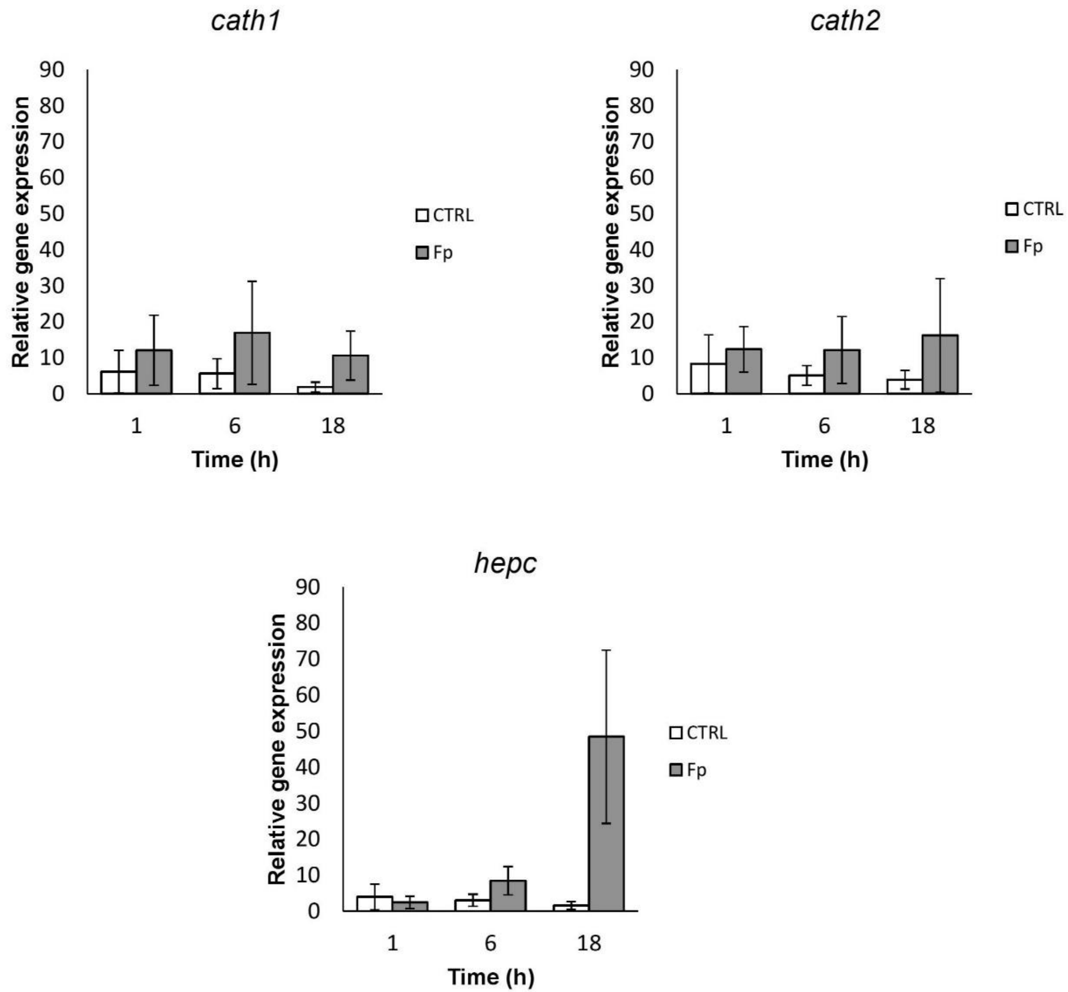

Figure 3.21. Antimicrobial peptide gene expression in RTS11 cells co-cultured with RTgutGC cells and *Flavobacterium psychrophilum*. Control (CTRL) cells received media only. Treated (Fp) cells received media with *Flavobacterium psychrophilum* (MOI 1:1). Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Data are representative of the mean of three independent experiments ± SEM.

3.4 Discussion

The findings of this study demonstrate that RTgill-W1 and RTgutGC are able to survive in the presence of *F. psychrophilum* for at least 24 h. However, RTgutGC appeared to display slightly higher viability compared to RTgill-W1 cells. This is in agreement with other studies, where RTgutGC was challenged with pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). For example, the RTgutGC cell line has been shown to maintain relatively high viability (>80%) even in the presence of LPS (Kawano et al. 2011; Wang et al. 2019). In response to LPS from *Escherichia coli* O111:B4, RTgutGC cells upregulate the expression of several cytokines, especially *il1b*, *il6* and *il8* but also *tnfa* (Wang et al. 2019). *F. psychrophilum* cells contain a low-molecular-mass LPS (~ 16 kDa) which may not be as antigenic as the high-molecular-mass LPS with O antigen, that is found in the supernatant (Crump et al. 2001). Indeed, Semple and colleagues have shown that RTS11 cells are particularly sensitive to the supernatant of *F. psychrophilum*, especially in the first day of exposure (Semple et al. 2020). The underlying mechanism(s) used by RTgutGC cells to sense *F. psychrophilum* remains unknown. The current study did not account for the effect of mucus or commensal microbes, which would be present at the gut-immune interface *in vivo* and may influence gene expression. Using a gut explant model exposed to *F. psychrophilum* may be more representative of this environment.

In the current study, only *il1b* mRNA and *il6* mRNA were upregulated in RTgutGC cells at 18 hpi (Figure 3.17F and Figure 3.17G), respectively. Interleukin 1 beta is produced by many cell types and is an important regulator of immune responses, but interleukin 1 beta gut specific roles are less established (Sakai et al. 2021). Il1b administration in grass carp (*Ctenopharyngodon idella*) results in severe enteritis and stimulates the expression of *tnfa* mRNA (Bo et al. 2015). Zymosan, a fungal mimic, induces the expression of *il1b* mRNA in RTgutGC cells and co-localizes with cathelicidin 2 (Schmitt et al. 2015). In turn, the production of Il1b protein is stimulated by cathelicidin 2 in RTgutGC cells (Schmitt et al. 2015). However, in the current study, *cath2* mRNA did not increase in RTgutGC cells within the first 18 hpi (Figure 3.18E) but perhaps cathelicidin 2 is upregulated during later time points to stimulate Il1b protein production. High levels of Il1b have been shown to be harmful to human intestinal cell barrier function, where high levels increase intestinal cell permeability (Al-Sadi et al. 2007). Il1b has been shown to be upregulated in the intestinal tissue of common carp (*Cyprinus carpio*), infected with *Aeromonas*

hydrophila (Di et al. 2017). Histological sections obtained from infected fish revealed a breakdown of the intestinal mucosa (Di et al. 2017). If Il1b protein production is increased in the gut of fish, it may disrupt the intestinal barrier and allow for more *F. psychrophilum* to enter the gut. Thereby causing further damage to intestinal tissue. Commensal microbes may also regulate intestinal Il1b signaling and production. Cells isolated from the gut of rainbow trout are hyporesponsive, in terms of cytokine mRNA expression within the first 12 h of exposure to probiotics and pathogenic bacteria (Kim and Austin, 2006). However, the results of the current study suggest that intestinal epithelial cells of rainbow trout respond rapidly to the presence of pathogen *in vitro*. Collectively, the results suggest that further research on interleukin 1 beta and its roles in teleost gut immunity are warranted.

Other interleukins involved in gut homeostasis and disease include interleukin 6 (Il6). Il6 has been very well characterized in rainbow trout and mediates cytokine and AMP expression (Costa et al. 2011). For example, recombinant Il6 induces the early and transient expression of suppressor of cytokine signaling family genes (SOCS 1-3) and interferon regulatory factor 1 (IRF1) in RTS11 cells (Costa et al. 2011). Interestingly, a putative IRF1 binding site has been described in the flanking regions of a teleost AMP and this may be conserved in other AMPs (Liu et al. 2010). This suggests that SOCS and IRF1 may be involved in the transcriptional regulation of teleost AMPs. It is interesting to note that Il6 treatment of RTS11 cells by Costa and colleagues led to the induction of *cath2* mRNA and *hepc* mRNA expression but downregulated *il1b* and *tnfa* mRNA (Costa et al. 2011). However, in the current study, only *il1b* and *tnfa* mRNA increased in RTS11 cells (Figure 3.20), despite the *il6* mRNA induction observed at 18 hpi in RTgutGC (Figure 3.17G). Interestingly, *tnfa* mRNA was not significantly upregulated in RTgutGC cells alone. Perhaps the gut epithelium depends on immune cells such as macrophages for this *tnfa* mRNA induction signal. In RTS11 cells treated with LPS, *il6* mRNA expression appears to reach peak levels at 8 h and then decreases (Costa et al. 2011). RTS11 cells exposed directly to live *F. psychrophilum* upregulate *il6* mRNA expression within 24 h of exposure but this begins to decrease two to three days after exposure (Semple et al. 2020). However, in the current study, co-culture of RTS11 with RTgutGC and *F. psychrophilum* appears to increase *il6* mRNA expression in RTS11 cells beyond 6 h, albeit not significantly (Figure 3.20). Il 6 has also been observed to stimulate RTS11 growth and promotes barrier function in the mammalian intestinal epithelium

(Costa et al. 2011; Kuhn et al. 2018). Il 6 is also involved in governing iron status during inflammation and promotes the induction of *hepc* mRNA in mammals; similar to teleost (Nemeth et al. 2004; Costa et al. 2011). However, further research is required to determine the roles of Il6 in rainbow trout gut barrier function and iron hemostasis in fish.

Some strains of *F. psychrophilum* produce siderophores or are small iron-chelating molecules that aid in colonization of the host (Møller et al. 2005). Iron is essential for *hepc* mRNA expression in rainbow trout and *F. psychrophilum* infection may result in the depletion of iron and thus decreased levels of *hepc* mRNA expression (Álvarez et al. 2013). In the current study, *hepc* mRNA was expressed by all cell lines even at 18 hpi. However, *hepc* mRNA expression appeared to decrease at 18 hpi in both control and infected RTgill-W1 cells (Figure 18C). Some host cells in mammals can secrete iron-binding proteins to sequester iron, thus preventing uptake by pathogens (Wilson et al. 2016). The expression of *hepc* mRNA by RTS11 appeared to be higher compared to gill and gut cell lines (Figure 3.21). However, the role of RTS11 cells in iron uptake and sequestration to limit bacterial infections remains unknown. Adenosine Triphosphate (ATP) may have also influenced the results. Given that the membrane integrity of RTgill-W1 and RTgutGC was intact (Figure 2.1), it is unlikely that intracellular stores of ATP were released into the media and therefore the expression of ATP-mediated genes such as *p2x1* remained relatively stable. Nevertheless, this is the first study to identify that RTgill-W1, RTgutGC and RTS11 cells constitutively express *igf1r* and *p2x1* genes.

In conclusion, RTgutGC and RTgill-W1 can tolerate *F. psychrophilum* infection for up to 24 hpi and RTgutGC responds by upregulating the expression of *il6* mRNA and *il1b* mRNA at 18 hpi. Furthermore, co-culture of RTgutGC with RTS11 and subsequent exposure to *F. psychrophilum*, results in the rapid and significant upregulation of *il1b* mRNA and *tnfa* mRNA in RTS11 cells. However, AMP and putative receptor gene expression amongst the cell lines does not change significantly within the first 18 h of exposure to *F. psychrophilum*. Collectively, the results of this study suggest that intestinal epithelial cells of rainbow trout may influence the response of monocytes and macrophages during the early stages of *F. psychrophilum* exposure, by activating the expression of interleukin 6 and interleukin 1 beta genes. Furthermore, the co-culture model employed in this study may be a useful tool to investigate fish gut-immune-pathogen

interactions. This will expand the current understanding of fish intestinal immunity and help to better understand bacterial pathogenesis at the gut barrier.

Chapter 4 – Investigating the effects of plant and insect-derived extracts as potential immunomodulatory agents in rainbow trout gut and liver epithelial cells

4.1 Introduction

4.1.1. *Teleost gut*

The teleost gut plays many roles in homeostasis and disease. The immunology of this organ is well understood, and it has been established as a major influencer of overall health. Mainly, it provides a barrier against pathogens but works together with immune cells and commensal microbes to orchestrate local and systemic immune responses. Enterocytes are the main cell types that form the physical barrier and this is accomplished through tight junction proteins, such as claudins (Minghetti et al. 2017). The gut barrier also uses tight junction proteins to control the passage of some nutrients and solutes. Approximately 63 claudin genes have been discovered in teleosts but the expression of claudins may be tissue dependent (Kolosov et al. 2013). Bacterial infection of rainbow trout has been shown to modulate the expression of cell adhesion proteins in the gut which may influence the immune response (Kumar et al. 2019). Enterocytes are also capable of expressing numerous cytokines and chemokines to recruit immune cells to the gut (Pérez et al. 2010). Feed ingredients may modulate this response since enterocytes also come into direct contact with feed ingredients. The ability to modulate the immune response, through feed, is of great interest in aquaculture research as it can increase revenue.

4.1.2. *Teleost liver*

The immunological role of the liver in teleosts is less understood, when compared to the gut. An intrahepatic immune cell population has been identified in rainbow trout, that accounts for 15-29% of non-hepatocytes and has been shown to respond independently during toxin challenge (Möller et al. 2014). However, there is an emerging idea that the gut communicates with the liver during pathogen surveillance and immune responses; this is known as the gut-liver axis. Many key immune transcripts are shared by mucus and bile in Nile Tilapia (*Oreochromis niloticus*) during bacterial infection (Wu et al. 2016). Some pathogens can also enter the fish gut but colonize the liver and disrupt the microbiota in both organs (Deng et al. 2020). Although the hepatic

immunological responses of teleost are not as well studied; the liver remains a major focus of research in aquaculture, as it is largely impacted by feed ingredients and is important in the metabolism of fish (Véron et al. 2016).

4.1.3. *Nutritional immunity of the teleost gut and liver*

Nutrition is paramount to the health of animals and influences the immune system. Various methods have been used to study the interaction between diet and immunity in fish but more recently, omics technologies have been widely used in nutritional studies (Martin and Król, 2017). For example, a microarray study in rainbow trout fed with low levels of phosphorus, revealed an inhibition of interferon gene expression in the proximal intestine of fish fed the low phosphorus diet (Kirchner et al. 2007). Furthermore, an RNA-seq study of rainbow trout fed a micronutrient deficient diet for 10 weeks, revealed altered gene expression in acute phase proteins in the liver (Olsvik et al. 2013). This suggests that viral immunity may be impacted by low phosphorus in the gut or complement activation in the liver may be dampened to select micronutrients. This approach may help increase our understanding of the specific nutrient-gut and nutrient-liver interaction and may also help to improve the use of sustainable aquaculture feed ingredients (Martin and Król, 2017). Sustainable feeds can also alter the host without impacting immunity against certain pathogens. For example, feeding rainbow trout a sustainable plant-based diet, promotes gut microbiota richness but this does not protect against bacterial infection with *F. psychrophilum* (Pérez-Pascual et al. 2021). In rainbow trout fingerlings, dietary supplementation for 107 days with an aglae, *Chorella peruviana*, improves the growth and innate immune response (Quico et al. 2021). Despite many benefits, further research is required to better understand the overall effects on the host.

Many functional foods, which are food items that are included in the diet of fish; beyond the basal requirements that improve their normal growth and immunity, have been investigated (Martin and Król, 2017). Indeed, functional feed ingredients, such as mannanoligosaccharides and beta-glucan, modulate the expression of gut barrier genes such as claudins in the RTgutGC cell line (Wang et al. 2019). In addition, Oregano (*Origanum onites*) fed rainbow trout, displayed higher lysozyme activity in the plasma and *O. onites* had direct anti-parasitic properties against *Ichthyophthririus multifiliis* (Mathiessen et al. 2021). Dietary inclusion of insect meal, such as mealworm (*Tenebrio molitor*), to rainbow trout diets also increases lysozyme activity (Jeong et al.

2020). Plant and insect-derived feeds are among the most well studied sustainable food sources but still require additional testing to determine benefits, safety and efficacy (Hua et al. 2019).

4.1.4. *Soybean and mealworm protein impacts on fish health*

Salmonids and other fish species often develop enteritis from soybean meal but some fish have been able to recover (Wu et al. 2018). Gut and liver gene signatures from animals that recover from a moderate level of soybean meal display upregulation of genes involved in the acute phase reaction, pattern recognition, inflammatory cytokines, antigen processing, complement system, innate immunity, T/B cell antigen activation, among others (Wu et al. 2018). Soybean meal has also been associated with an initial increase in bile acid synthesis and subsequent decrease with chronic administration (Murashita et al. 2018). Bile acids are important in controlling the absorption of lipids from the small intestine, facilitating digestion and play a role in absorbing cholesterol (Staels and Fonseca, 2009). In addition, soybean meal has also been shown to alter the expression of S100 genes which produce calcium-sensing proteins in the gut, mainly in the distal intestine and in the liver of rainbow trout (Blaufuss et al. 2019). Individual components of soybeans have also been tested on rainbow trout. For example, the soy isoflavones (genistein, daidzein and glycitein) have been fed to fish but have no impact on the reproduction, growth or health (Pastore et al. 2018). Studying specific compounds of soybeans may aid in improving our understanding of fish nutrition.

In general, insect meals, such as yellow mealworm protein, do not produce the same enteritis as plant-based ingredients and are well tolerated by salmonids (Iaconisi et al. 2018; Rema et al. 2019). Significant reductions in omega-3 fatty acid inclusion in rainbow trout fillets have been observed with increases in insect meal (Melenchón et al. 2021). Mealworm meal may be an immunostimulant for trout as it has been found to have an anti-oxidant effect and reduce lipid peroxidation in the intestine (Henry et al. 2018). Rapid antibacterial activity against *E. coli* was also observed in fish fed 25% and 50% mealworm meal (Henry et al. 2018). However, this has not been studied *in vitro*. Therefore, in the current study, soybean protein and mealworm protein were applied to the rainbow trout gut (RTgutGC) and liver (RTL-W1) cell lines. After exposure to the proteins, the viability, adhesion, immune gene expression and wound healing were assessed.

4.2 Materials and Methods

4.2.1. Mealworm and soybean protein extraction

Yellow mealworm larvae were purchased from a local pet store in Guelph, Ontario and protein was extracted using a previously described protocol (Zhao et al. 2016). Briefly, 50 g of mealworms were frozen at -20°C for 12 h. Then the frozen larvae were added to a 305 ml solution of 0.1 M NaOH and stirred for 30 min at room temperature. The supernatant was centrifuged at 4°C for 20 min at 3500 × g. The supernatant was transferred to a new tube and the pH was adjusted to 4.5 using a 2 M HCl solution. The sample was then centrifuged at 4°C for 15 min at 2500 × g. The precipitate was washed twice with deionized water and centrifugation at 4°C and 2500 × g was repeated twice more, for 10 min. The precipitate was frozen at -20°C for 12 h and was then freeze-dried to obtain the final product. The freeze-dried product was dissolved in sterile molecular grade water and the concentration was determined using a BCA assay and manufacturer's protocol (Thermo Fisher). Soybean protein concentrate (90%), was kindly provided by Aminola (Barneveld, Netherlands).

4.2.2. Cell culture

RTgutGC and RTL-W1 cell lines were maintained at room temperature and ambient air in vented 25 cm^2 cell culture flasks (Thermo Fisher Scientific). RTgutGC and RTL-W1 cells were supplemented with 4 ml of Leibowitz's (L-15) medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin-streptomycin (Sigma). RTgutGC and RTL-W1 cells were routinely subcultured at a ratio of 1:2; every four days. Briefly, cell monolayers were rinsed with 1 × Dulbecco's phosphate-buffered saline (Gibco) and a 0.05% trypsin–EDTA (Gibco) solution was applied to detach the cells from the plate.

4.2.3. Thiazolyl Blue Tetrazolium Bromide (MTT) assay

RTgutGC and RTL-W1 cell lines were used to evaluate the potential cytotoxic effects of insect and plant protein. Briefly, 500,000 cells were seeded in triplicate within a 96-well plate. The cells were suspended in 100 µl of L-15 with 15% FBS and without antibiotics. Three wells did not

receive cells but did receive medium only as a background control. Soybean protein and mealworm protein were added to the medium at final concentrations of 0, 5, 10, 15, 25, 50, 75 (% v/v) and 0, 2.8, 4.5, 9.1, 18.2, 36.4 µg/ml, respectively. Chicken albumin was also added as a protein control at 36.4 µg/ml (Sigma). The plate was sealed with parafilm and incubated at room temperature. The following day, the cells were exposed to mealworm protein or soybean protein for 24 h. The media was removed and 11 µl of a 0.22 µm filtered MTT (Sigma-Aldrich, Cat no. M5655) solution in 1 x PBS, of 5 mg/ml, was added to each well to reach a concentration of 0.5 mg/ml. The three positive control wells also received a fresh solution of 0.01% (v/v) Triton X-100 (Sigma-Aldrich). The plate was then sealed with parafilm and placed on a shaker at 100 rpm for 1 min. The cells were incubated in this media for 3 h in the absence of light. Next, 150 µl of DMSO was added to each well. Each well was gently mixed by pipetting to release the formazan crystals and then the plate was incubated for an additional 15 minutes at room temperature. Lastly, the plate was read at an O.D. of 520 nm, using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments). The wells with media only and DMSO, served as a background control and were subtracted from all other values.

4.2.4. Cell adhesion assay

RTgutGC and RTL-W1 cell lines were used to evaluate the potential cell adhesion effects of insect and plant protein. Briefly, 500,000 cells were seeded in triplicate within a 96-well plate. The cells were suspended in 100 µl of L-15 with 15% FBS and without antibiotics. Soybean protein and mealworm protein were added to the media at final concentrations of 0, 5, 10, 15, 25, 50 (% v/v) and 0, 2.8, 4.5, 9.1, 18.2, 36.4 µg/ml, respectively. Three wells did not receive cells but did receive media only as a background control. The plate was sealed with parafilm and incubated at room temperature for 3 h. The wells were then washed with 100 µl of 1 × PBS, and non-adherent cells were removed. The adherent cells were briefly fixed with 100 µl of methanol for 3 min and stained with a 100 µl solution of crystal violet (1% v/v for 2 min). The wells were then gently washed with 100 µl of 1 × PBS and bound crystal violet was extracted by adding 100 µl of acetic acid (10% v/v) to each well. The plate was then read at 490 nm using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments). The wells with media only, served as a background control and were subtracted from all other values.

4.2.5. Total RNA isolation and cDNA synthesis

Total RNA was extracted from RTgutGC and RTgill-W1 cells using the RNeasy Micro Kit by following the manufacturer's protocol (Qiagen). RNA purity and quantity were determined using a NanodropTM 2000 Spectrophotometer. Total RNA samples were treated with DNase I (Thermo Scientific) and stored at -80 °C until further use. cDNA was synthesized on the same day from 400 μg of RNA per reaction, using qScriptTM cDNA SuperMix (Quantabio) and following the manufacturer's protocol. Each reaction was incubated at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The cDNA was stored at -20 °C for future analyses.

4.2.6. qRT-PCR

qRT-PCR was performed with WISENT ADVANCED™ qPCR mastermix (Wisent), by following the manufacturer's instructions on the LightCycler® 480 II (Roche). Briefly, each 10 μl reaction consisted of 1 μg of cDNA (2.5 μl), 0.25 μM of forward and reverse primers (0.5 μl), 2 × WISENT ADVANCED™ qPCR mastermix (5 μl) (Wisent), and DEPC water (2 μl). A no-template control was also included. All trials were conducted in triplicate and each program consisted of a pre-incubation at 95 °C for 10 min, then 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at the specified primer annealing temperature for 30 s and extension at 72 °C for 20 s. Primer efficiency was between 91.77 – 102.56% (Appendix Table 3). The fold change of mRNA expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

4.2.7. Scratch Wound Healing Assay

To determine the effects of soybean and mealworm protein on cell migration, the scratch wound assay was used. Briefly, 600,000 RTgutGC and RTL-W1 cells were seeded in a 12-well plate (BioLite). The cells were suspended in 1 ml of L-15 with 15% FBS and without antibiotics. The plate was sealed with parafilm and was incubated for 12 h. The cell monolayer was scratched with a 200 µl pipette tip and each well was washed twice with 1 × PBS. Wells then received either media alone, soybean or mealworm protein at final concentrations of 50 (% v/v), 36.4 µg/ml and

36.4 μg/ml, respectively (Sigma). Images were captured at 0 h and 24 h after wounding. Wound closure was measured using ImageJ software.

4.2.8. Statistics

Viability data was analyzed using an unpaired Student's t-test with unequal variance. Gene expression data were analyzed using a one-way ANOVA test with Tukey's post hoc test using Vassar Stats software (Vassar Stats). The alpha value was set at 0.05 ($P < 0.05$) for all tests. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3 Results

4.3.1. Viability and cell adhesion of RTgutGC and RTL-W1 cells exposed to soybean and mealworm protein

The incubation of RTgutGC and RTL-W1 cells with soybean or mealworm protein did not result in any significant changes in cell viability (Figure 4.22) or cell adhesion (Figure 4.23). There appeared to be an upward trend in RTL-W1 cells up to (50% v/v) of soybean protein (Figure 4.22B). However, control cells did not differ from treated groups (Figure 4.22 and Figure 4.23).

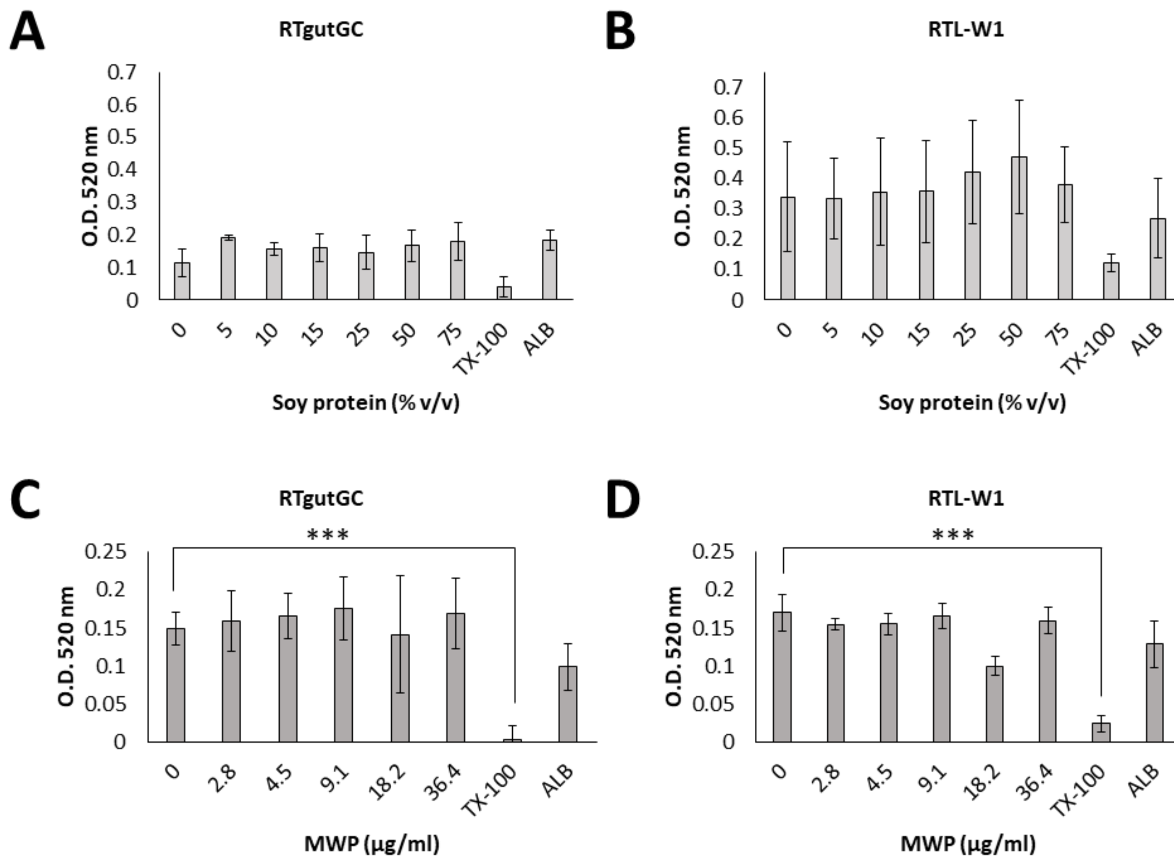

Figure 4.22. The effects of soybean and mealworm protein on cell viability. Cells received either medium alone or media with various concentration of soybean protein (A and B) or mealworm protein (C and D) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$. Asterisks denote statistical significance (*** $p < 0.001$).

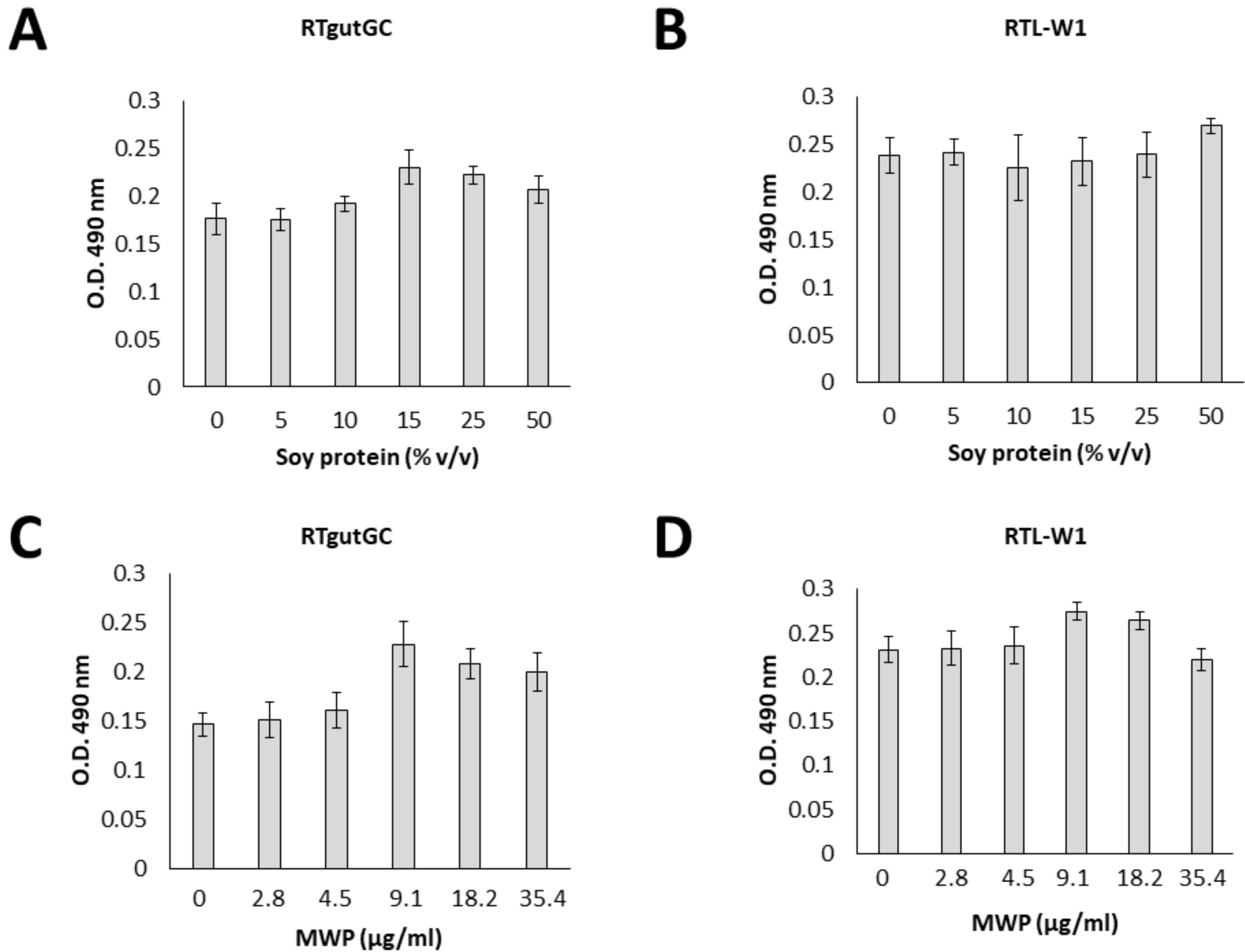

Figure 4.23. The effects of soybean and mealworm protein on cell adhesion. Cells received either medium alone or media with various concentration of soybean protein (A and B) or mealworm protein (C and D) for 3 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

4.3.2. Gene expression in RTgutGC and RTL-W1 cells exposed to soybean and mealworm protein

The incubation of RTgutGC and RTL-W1 cells with soybean or mealworm protein did not result in any significant changes in gene expression. Claudin 3 (*cln3*) was expressed by both cell lines but did not differ significantly from the control. In general, the expression of most genes in

RTgutGC cells exposed to mealworm protein appeared to be more abundant when compared to RTgutGC cells exposed to soybean protein (Figure 4.26). In addition, the expression of *hepc*, *cath1*, *tnfa*, *il6* and *cln3* mRNA appeared to be more abundant in RTgutGC control cells; compared to soybean protein or albumin protein exposed cells (Figure 4.24). RTL-W1 cells appeared to express higher levels of *il6* mRNA under all conditions compared to other genes in the soybean exposure experiment but this was not statistically significant (Figure 4.25). Whereas RTL-W1 cells exposed to albumin appeared to increase the expression of *cath1* and *il1b* mRNA (Figure 4.27).

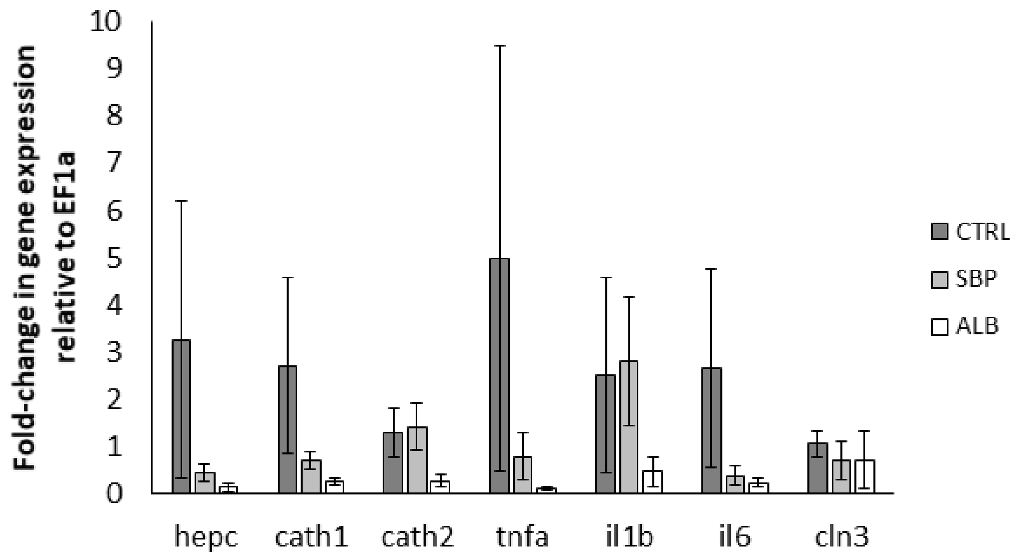

Figure 4.24. The effects of soybean protein on gene expression in gut cells. RTgutGC cells received medium alone, medium with soybean protein (50% V/V), or albumin (50% V/V) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, n = 3.

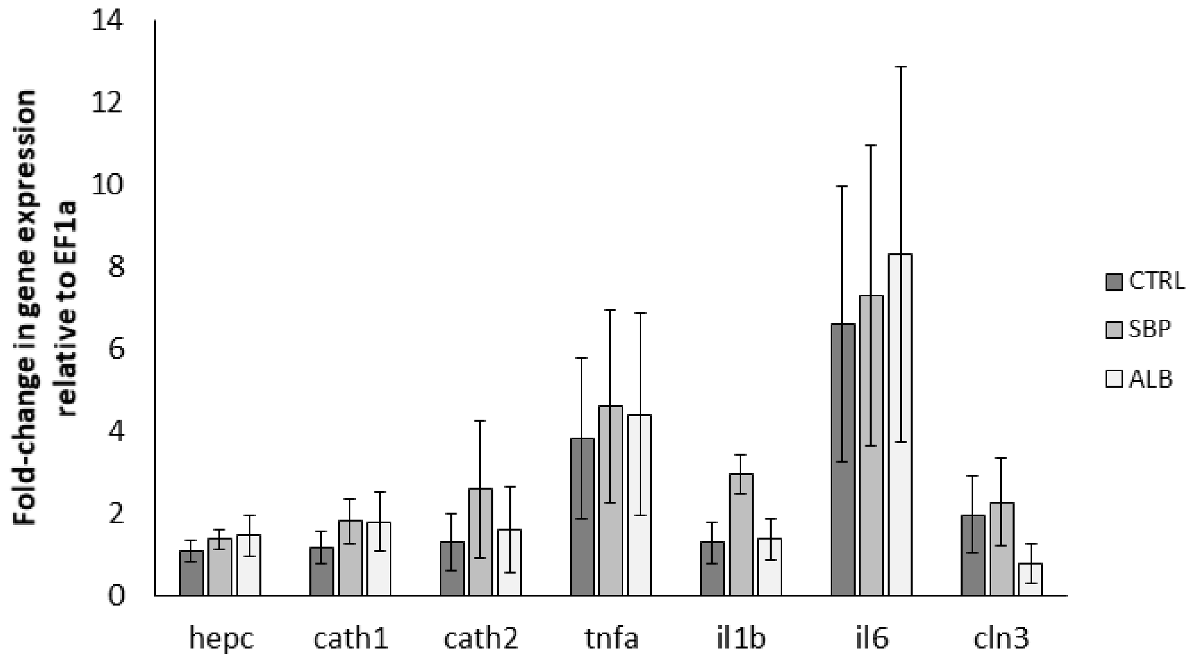

Figure 4.25. The effects of soybean protein on gene expression in liver cells. RTL-W1 cells received medium alone, medium with soybean protein (50% V/V), or albumin (50% V/V) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

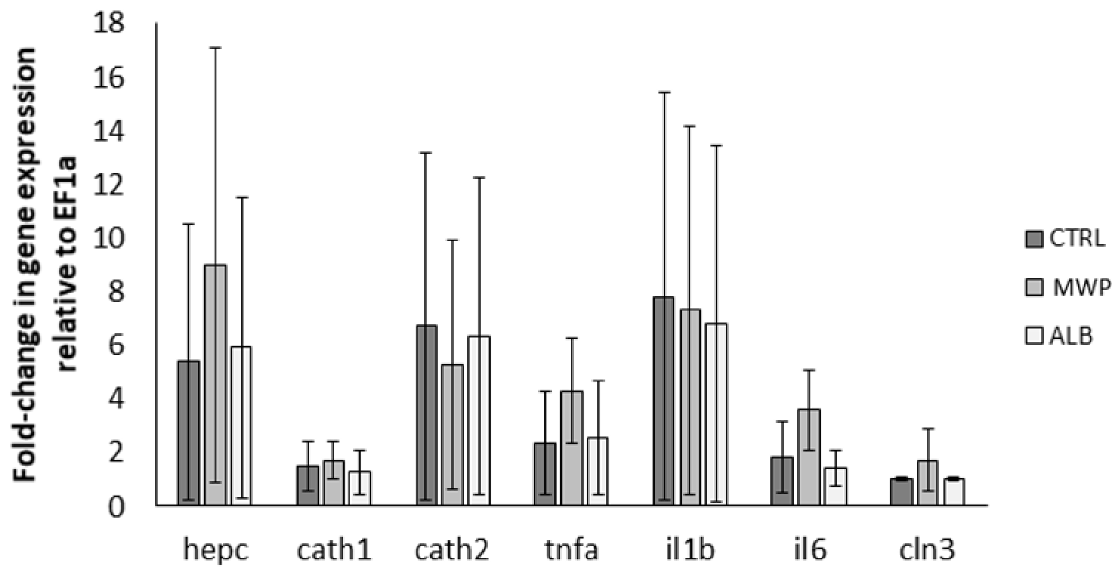

Figure 4.26. The effects of mealworm protein on gene expression in gut cells. RTgutGC cells received medium alone, medium with mealworm protein (36.4 µg/ml), or albumin (36.4 µg/ml) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

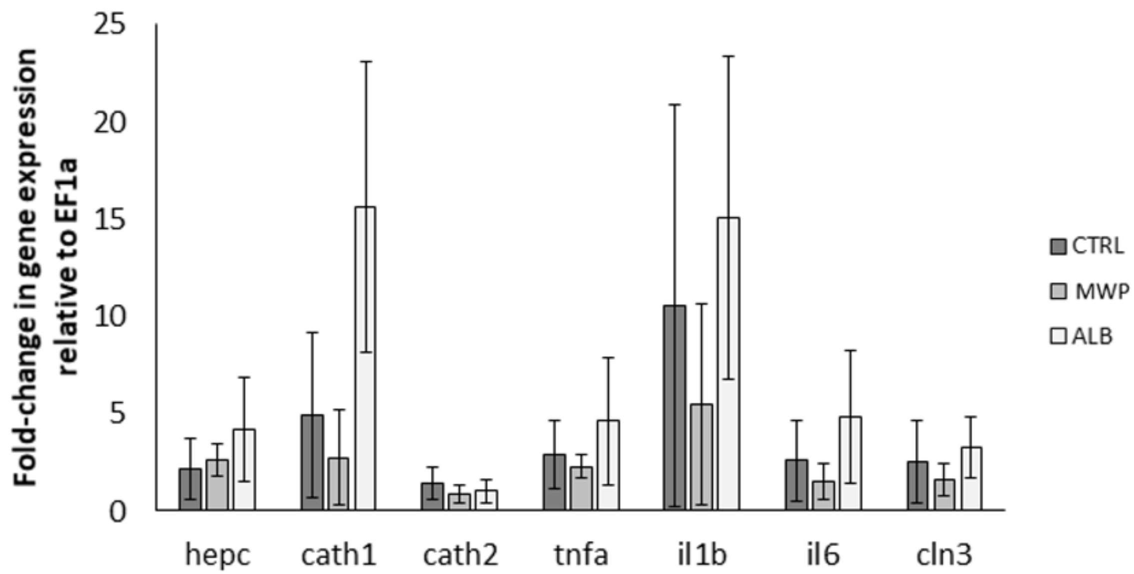

Figure 4.27. The effects of mealworm protein on gene expression in liver cells. RTL-W1 cells received medium alone, medium with mealworm protein (36.4 µg/ml), or albumin (36.4 µg/ml) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

4.3.4. Wound healing in RTgutGC and RTL-W1 cells exposed to soybean and mealworm protein

Wound healing ability did not change significantly in either RTgutGC or RTL-W1 cells (Figure 4.28) in the presence of either protein source. There appeared to be a decrease in wound closure, compared to the control, when RTgutGC cells were treated with soybean and mealworm protein but this was not statistically significant (Figure 4.28A).

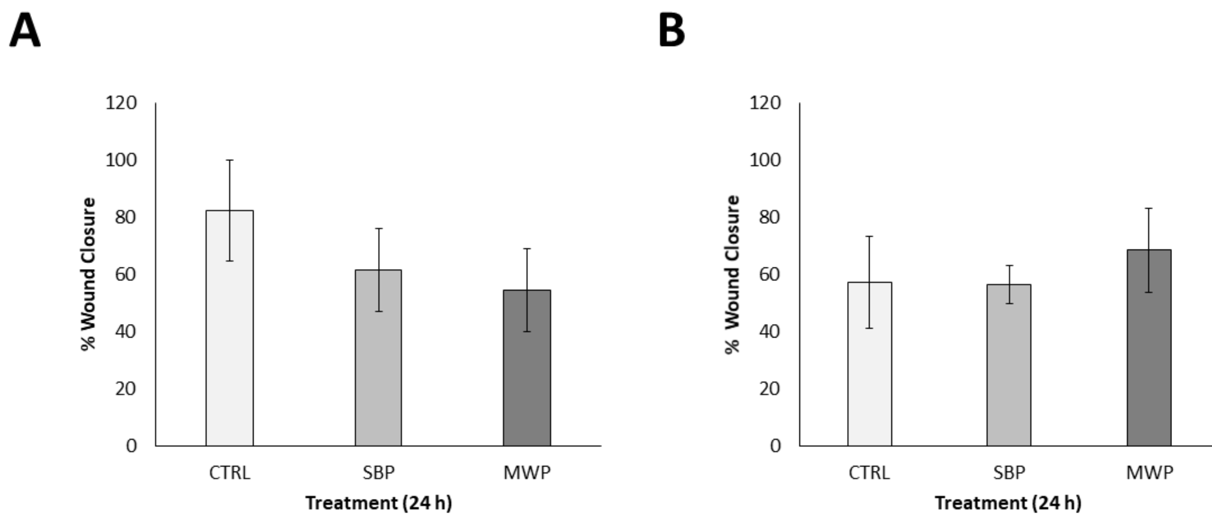

Figure 4.28. The effects of soybean protein and mealworm protein on wound healing in gut and liver cells. After wounding, cells received either medium alone or medium with soybean protein (50% V/V) (A) and mealworm protein (36.4 µg/ml) (B) for 24 h. Data were analyzed with one-way ANOVA and Tukey's *post hoc* test. Values are representative of the mean ± SEM, $n = 3$.

4.4 Discussion

Soybean and mealworm protein were well tolerated by RTgutGC and RTL-W1 cell lines, at all concentrations tested (Figure 4.22). However, many factors may be responsible for this observation. Firstly, proteins would most likely be altered in the gastrointestinal tract through the process of digestion. In addition, the amount of protein remaining in the gastrointestinal tract is unknown. Moreover, the transit time of foodstuffs through the gastrointestinal tract of rainbow trout has been observed to be impacted by temperature and takes approximately 15 h at 18°C (Fauconneau et al. 1983). In the current study, the foodstuffs were applied to cells for 24 h. The current study is also not representative of the gut microbiome which may also influence ingested protein. The gut microbiota of fish has been shown to transform compounds and aid digestion and absorption of nutrients (Nayak, 2010). However, this study demonstrates that gut epithelial and liver epithelial cells from rainbow trout, are not negatively impacted by soybean or mealworm protein *in vitro*.

Given the *in vivo* evidence of soybean induced enteritis in the gut of salmonids, it was hypothesized that this treatment may disrupt cell adhesion or cause an upregulation of proinflammatory cytokines. However, this was not observed in either RTgutGC or RTL-W1 cell lines. The adherence capability of RTgutGC and RTL-W1, were not impacted in the presence of soybean protein or mealworm protein (Figure 4.23). In addition, exposure of cells to both proteins for 24 h did not result in any significant change in the cell adhesion molecule, claudin 3 (*cln3*). Claudin 3 has been detected in RTgutGC cells and the expression of this gene, significantly increases with exposure to functional feed ingredients such as mannan oligiosarccharides or beta-glucan (Wang et al. 2019). However, *cln3* mRNA expression has not been investigated in the RTL-W1 cell line or rainbow trout liver. In the current study, *cln3* mRNA did not change significantly upon exposure to SBP or MWP and was detected in RTgutGC and RTL-W1 cell lines (Figures 4.24 – 4.27). Therefore, soybean protein and mealworm protein do not cause impairments in cell adhesion in the RTgutGC or RTL-W1 cell line under the conditions tested.

The nutritional composition of mealworms has been analyzed and larvae are made up of 13.7% protein; the most abundant amino acid is glutamic acid, at 125.3 mg/g of larvae (Costa et al. 2020). Although the effects of dietary glutamic acid on fish immune responses are unknown;

dietary glutamic acid has been shown to upregulate the expression of some cell adhesion and immune genes in the porcine intestinal tract: *cln3*, *tnfa*, *il1b* and *il6* (Kyoung et al. 2021). However, in the current study the amino acid composition of the mealworm protein sample was not analyzed. Furthermore, mealworms also harbour various types of commensal bacteria, such as yeasts, molds aerobic and anaerobic spore-forming bacteria, *Enterobacteriaceae* and coliforms (Costa et al. 2020). Protein from commensal organisms may have been lost during the extraction process. Bacterial-derived proteins would be expected to produce an inflammatory response in RTgutGC and RTL-W1 but the cells did not display any significant changes in inflammatory gene expression after 24 h of exposure (Figure 4.26 and Figure 4.27). Interestingly, the albumin control cells appeared to express more immune genes and more of the cell adhesion gene, *cln3*, compared to control or mealworm protein treated cells, except for *cath2* (Figure 4.27). Albumin is a protein produced by the liver that carries enzymes, hormones and vitamins. However, the chicken albumin and rainbow trout albumin 1 protein sequence, are only 29.86% identical (BLAST®, National Center for Biotechnology Information). It is possible that the chicken albumin enhanced the metabolism of the RTL-W1 cells but nevertheless this change was not statistically significant.

Although the findings of soybean and mealworm protein on RTgutGC and RTL-W1 wound closure were not statistically significant, this is the first study to use RTL-W1 in this assay. Wound healing studies of the liver in teleosts are scarce but can serve as a model to study liver disease (Pham et al. 2017). The current study also improves our understanding of what influences wound healing in the RTgutGC cell line, as only a subset of compounds has been tested in this model (Wang et al. 2019). One group of wound healing inhibiting compounds, in RTgutGC, is mannan oligosaccharide but this only causes significant decreases in proliferation between 2 – 4 days after wounding (Wang et al. 2019). Mannan oligosaccharides are also found in fish meal and soybean meal (Akter et al. 2019). Therefore, this model may be useful to not only study gut-nutrient interactions but also liver-nutrient interactions, in the future.

Chapter 5 - Conclusion

The current study revealed that mining for peptides using bioinformatic methods may lead to future development of drugs against select bacteria. The current AMP repertoire in rainbow trout has been reviewed and it is well established that AMPs play critical roles in salmonid immunity (Brunner et al. 2020). Although the current work explored peptides from other aquatic organisms, that were not found to have antimicrobial activity against the microorganisms tested here, it expands our understanding of AMPs and these molecules may be tested on other bacterial species in the future. Even salmonid AMPs have been shown to have selective antimicrobial activity (Brunner et al. 2020). Nevertheless, AMPs from other organisms have been introduced into the genome of rainbow trout using transgenic approaches and provide enhanced diseased resistance (Chiou et al. 2014). Since rtVWF was shown to be non-hemolytic against rainbow trout hemocytes, it may be a promising candidate for *in vitro* use. Given that *S. iniae* is also a zoonotic pathogen, the use of rtVWF has implications beyond fish and aquaculture, to perhaps target this pathogen (Miller et al. 2005). Since this pathogen causes high losses in farmed fish, it may be of use in aquaculture operations to control disease (Agnew and Barnes, 2007). It is important to determine the mechanism of action that rtVWF uses to inhibit *S. iniae* growth. Given that biofilm formation is important for the propagation of this pathogen (Heckman and Soto, 2021), it may be useful to investigate if rtVWF has the potential to disrupt biofilm formation. Several AMPs have been shown to disrupt the formation and structure of microbial biofilms (Yasir et al. 2018). If rtVWF cannot be administered directly to fish in aquaculture operations, it may be used as a disinfectant for surfaces used for eggs and young fish (Yasir et al. 2018).

In 2017, three new terms were introduced to the field of *in vitro* biology: invitrome, invitromatics and invitroomics (Bols et al. 2017). Briefly, the invitrome represents the collection of cell lines from a common theme and the current rainbow trout invitrome consists of approximately 55 cell lines (Bols et al. 2017). Invitromatics is the science and history of establishing, characterizing, engineering, storing and distributing cell lines. Invitroomics represents the use of cell lines to study cellular and molecular biology of multicellular organisms or to manufacture useful products (Bols et al. 2017). The current work uses a subset of the rainbow trout invitrome, to interrogate immune responses to peptides, pathogens and traditional functional foods, expanding its invitroomics. For example, RTS11 was used to better understand the

interaction of immune cells with important fish pathogens such as *S. iniae* and *F. psychrophilum*. In the current study, rainbow trout macrophage cells were able to respond to heat-killed *S. iniae* and significantly upregulate *tnfa* mRNA transcripts. The implications of this *in vivo* are unknown but knowledge on the pathogenesis of *S. iniae* in salmonids is scarce. The current study also revealed that RTgill-W1 cells did not respond immunologically to *S. iniae*. Perhaps this response explains why *S. iniae* is able to colonize the gill. In other fish species, such as Nile tilapia (*Oreochromis niloticus*), *S. iniae* is observed to colonize the gills in high numbers (Baums et al. 2013). This suggests that the gill of some fish species, including rainbow trout, may be poor sensors of *S. iniae* infection. In the context of the immune genes investigated in the current work; it was observed that RTgill-W1 cells do not respond immunologically to *F. psychrophilum*. Despite the lack of evidence for an immune response in this case, these findings contribute to our understanding of teleost immunity by providing evidence for a sustained viability of the gill epithelium during early infection. Perhaps gill epithelial cells provide more of a physical barrier against pathogens or scaffold for the mucus but rely on immune cells or other cell types for immune activation. By using fish cell lines to study infection, it reduces the number of animals required for research. Therefore, exploiting the rainbow trout invitrome to interrogate diseases *in vitro*; may yield insightful information for *in vivo* challenge studies.

Furthermore, the invitrome can be used to establish novel co-culture models (Drieschner et al. 2019). In this study, a novel co-culture model was used to study the interactions between *F. psychrophilum* and rainbow trout gut and gill epithelial cells. The immune response of RTS11 cells to this pathogen has been well characterized (Semple et al. 2020). However, its interaction with other cell lines remained unknown. In the current work, it was demonstrated that RTgutGC and RTgill-W1 cells are able to sustain *F. psychrophilum* infection for at least 24 h and RTgutGC cells significantly upregulate *il1b* which provides further evidence for gut-mediated immunity during *F. psychrophilum* pathogenesis (Nematollahi et al. 2005). RTS11 cells also significantly upregulate *tnfa* mRNA transcripts; when co-cultured with RTgutGC cells, in the presence of the pathogen; however, the RTgutGC cells alone, do not upregulate this cytokine. This suggests that the gut epithelium may rely on signals from immune cells to mount a robust immune response against *F. psychrophilum*. A better understanding of the fish gut and how it functions in the presence of *F. psychrophilum*, may lead to preventative measures in aquaculture to control pathogenesis and limit losses due to *F. psychrophilum*. Perhaps the lack of *tnfa* mRNA expression

by gut epithelial cells in crucial to prevent excessive inflammation. For example, administration of microbial feed additives can reduce inflammation in the gut of Atlantic salmon and speed up recovery after inflammatory challenge (Vasanth et al. 2015).

The current study only used one strain of *F. psychrophilum* and it is well established that different strains exhibit various pathogenicity and virulence mechanisms (Sundell et al. 2019). Due to this, it would also be interesting to compare the results with different strains of *F. psychrophilum*. Perhaps certain strains exhibit tropism towards the gut or gill tissues. Resistance towards *F. psychrophilum* may also be studied using this model. A subset of microbial species have also been associated with effective resistance against *F. psychrophilum* infection *in vivo* (Valdés et al. 2020). However, the direct effects of such microbial species on immunity against *F. psychrophilum* in gut or gill epithelial cells, has not been studied. To date, most research on the fish gut microbiome, has been focused on structure and not functional applications that are relevant to aquaculture (Talwar et al. 2018). Harnessing the power of certain microbial species that confer an advantage over pathogens, may be capitalized upon by fish farmers, to limit losses. In general, these studies use many live animals. To reduce the use of animals in research, this co-culture model can be used to study various effects on immunity. For example, it can be used to study the effects of probiotics on immune cells in the gut or gill, without excessive animal use. Therefore, the co-culture model may be useful in future studies to better understand complex gut and gill-pathogen interactions with select species from the microbiome. Nevertheless, it expands our current understanding of *F. psychrophilum* pathogenesis in rainbow trout.

Nutrition also impacts the host response to disease and nutrigenomics, which is focused on how nutrients interact with genes, is a rapidly growing field in aquaculture (Martin and Król, 2017). AMPs have administered through the diet of fish to enhance disease resistance (Herrera et al. 2021). Plants are cost-effective bioreactors for AMPs (Ghidey et al. 2020). Furthermore, AMPs can be modified in plants and crosslinked to other peptides to improve durability or function (Ghidey et al. 2020). For example, plants can be genetically modified to express and synthesize AMPs, which can then possibly be fed to fish (Weinhold et al. 2018). Therefore, certain plants can be used as a vector to deliver AMPs to animals, but their safety needs to be established. The concept of how nutrition influences disease in rainbow trout is not new (Rumsey et al. 1994; Wahli et al. 1998). However, even after the first study of soybean protein on rainbow trout by Rumsey

and colleagues in 1994; many unknowns remain on how this protein impacts rainbow trout immunity, especially at the cellular and molecular level (Rumsey et al. 1994). Despite the unknown impacts, there is a growing interest in turning towards alternative protein sources to feed fish due to the increasing cost and unsustainability of fish meal (Naylor et al. 2020). Therefore, in the current study, a nutrigenomics approach was used to study the interaction between alternative protein sources: soybean and mealworm, in two cell lines, namely RTgutGC and RTL-W1. Unexpectedly, the alternative protein sources exposure study was well tolerated by both RTgutGC and RTL-W1 cells. Soybean or mealworm protein did not have any negative impacts on cell viability, adhesion, immune gene expression or wound healing. This study also expanded rainbow trout invitroomics by revealing that RTL-W1 can be used in the scratch wound assay model to study liver wound healing in the future. In addition, it expands our current understanding of teleost hepatoimmunity; which can be used to study hepatological diseases in other organisms (Forn-Cuní et al. 2015; Wilkins and Pack, 2016). It would be intriguing to look at the impact of other nutrients or perhaps specific compounds, found in soybeans or mealworms, to identify potential immunomodulators that can be used in aquaculture. Given that feed is responsible for 40-75% of aquaculture production cost, it is crucial to identify useful feed ingredients for farmers to capatilize on their investment (Ansari et al. 2021). Despite the lack of benefits uncovered in the current study, there is evidence to suggest that soybean and mealworm protein are well tolerated by gut and liver cell lines *in vitro*.

The current work provides a starting point for this investigation and provides insight into direct nutrient-host interactions. The current study contributes to a better understanding of mucosal immunity in salmonid gill and gut. At these sites, AMPs are abundant and play numerous roles in defence against pathogens (Brunner et al. 2020). Some AMPs such as hepcidin, are important indicators of fish health since they are involved in iron absorption and their expression may be stimulated by cytokines (Brunner et al. 2020). In the current study, hepcidin and tumor necrosis factor alpha gene expression was assessed under various conditions. First, in RTS11 and RTgill-W1 cells exposed to an AMP and *S. iniae*, then during *F. psychrophilum* exposure in RTS11, RTgill-W1 and RTgutGC. Finally, in RTgutGC and RTL-W1 exposed to soybean and mealworm protein. Although the expression of hepcidin did not change significantly under any condition studied, it was expressed by all cell lines, even under basal conditions. Besides playing a role in immunity, hepcidin is also involved in iron metabolism. These cell lines can therefore be used to

test compounds that might disrupt iron homeostasis before they are fed to fish, thus limiting the use of animals used for research. Furthermore, animal feed companies may use these cell lines to test feed ingredients before they are administered to live animals. This will also lead to reduced costs during product development.

Tumor necrosis factor alpha was upregulated in RTS11 cells treated with *S. iniae* and in RTS11 cells exposed to *F. psychrophilum* but not in gill or gut cell lines, respectively. In addition, tumor necrosis factor alpha gene expression did not change under soybean or mealworm protein exposure in gut or liver cells. The level of tumor necrosis factor alpha may be used as an indicator of tissue inflammation (Hong et al. 2013). However, the current study highlights that the cellular composition of tissue may be an important factor to consider when measuring tumor necrosis factor alpha as an indicator of inflammation. Some AMPs may also be induced by tumor necrosis factor alpha (Hong et al. 2013). Although this was not tested in the current study, *F. psychrophilum* was able to induce *il1b* and *il6* mRNA transcripts in RTgutGC cells (Figure 3.17 F, Figure 3.17 G) and this may lead to further downstream signalling to increase AMP transcription. The use of CRISPR/Cas9-mediated gene editing may be useful to study specific gene function in future studies, as it has been successfully used in RTgutGC (Zoppo et al. 2021). However, genetically modifying fish to this end, may not be feasible from an aquaculture industry perspective, as it may limit acceptance by consumers (Bennett et al. 2007).

The current work also contributes to our understanding of fish gill and gut physiology. The mRNA transcripts for *igfr1* and *p2x1* were identified in RTgutGC and RTgill-W1 and were not influenced by *F. psychrophilum* infection. Taking gill clippings from juvenile fish and measuring *igfr1*, may be used as a growth indicator and this approach may be preferable over collecting blood. In addition, *igfr1* mRNA expression may also be of interest to the aquaculture industry since single nucleotide polymorphisms (SNPs) in the *IGF1R* genes of the striped catfish, *Pangasianodon hypophthalmus*, were significantly associated with growth traits (Tran et al. 2021). This is important because fish can reach market size rapidly. Therefore, *IGF1R* may serve as a positive genetic marker for selection in aquaculture. In addition, claudin 3 has been shown to be expressed in the gill of rainbow trout (Chasiotis and Kelly, 2011). The current study adds to this finding and identifies that RTgutGC and RTL-W1 also express *cln3* mRNA transcripts. Therefore, the gill, gut and liver of rainbow trout may use similar mechanisms to control permeability across barriers.

This is important when studying the effects of toxins on aquatic organisms. Fish cell lines have been used as a tool to monitor toxins in environmental samples (Fent, 2001). Although RTgill-W1 and RTL-W1 predominate this literature, the current study suggests that RTgutGC may also be considered as an ecotoxicology tool (Bols et al. 2017). Collectively, by using the rainbow trout invitrome and invirtoomics, the results of this study improve our understanding of fish health and disease and identify new avenues for investigation of host-pathogen interactions, fish nutrition and immunology.

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Appendix

Appendix Table 1. Peptide characteristics

Peptide name	Identified source	Sequence
rtVWF	*Oncorhynchus mykiss*	MQLESAITHTLFK
Tsvep1	*Oreohromis niloticus*	MEGVGRSRCLENGTWTPPPTCRAVCWLQCQNGGVCQRPNTCSCPEGW
Dpbl-32	*Diphyllobothrium latum*	MCPGYAACQARSRVYEQKNRATTIDGAKVSAA
Dpbl-48	*Diphyllobothrium latum*	MSTCKCGLPPSLWHLCGHHSPRTGQLQLCRCRRLRLRCGCLAQEYPCG

Appendix Table 2. Peptide physicochemical properties

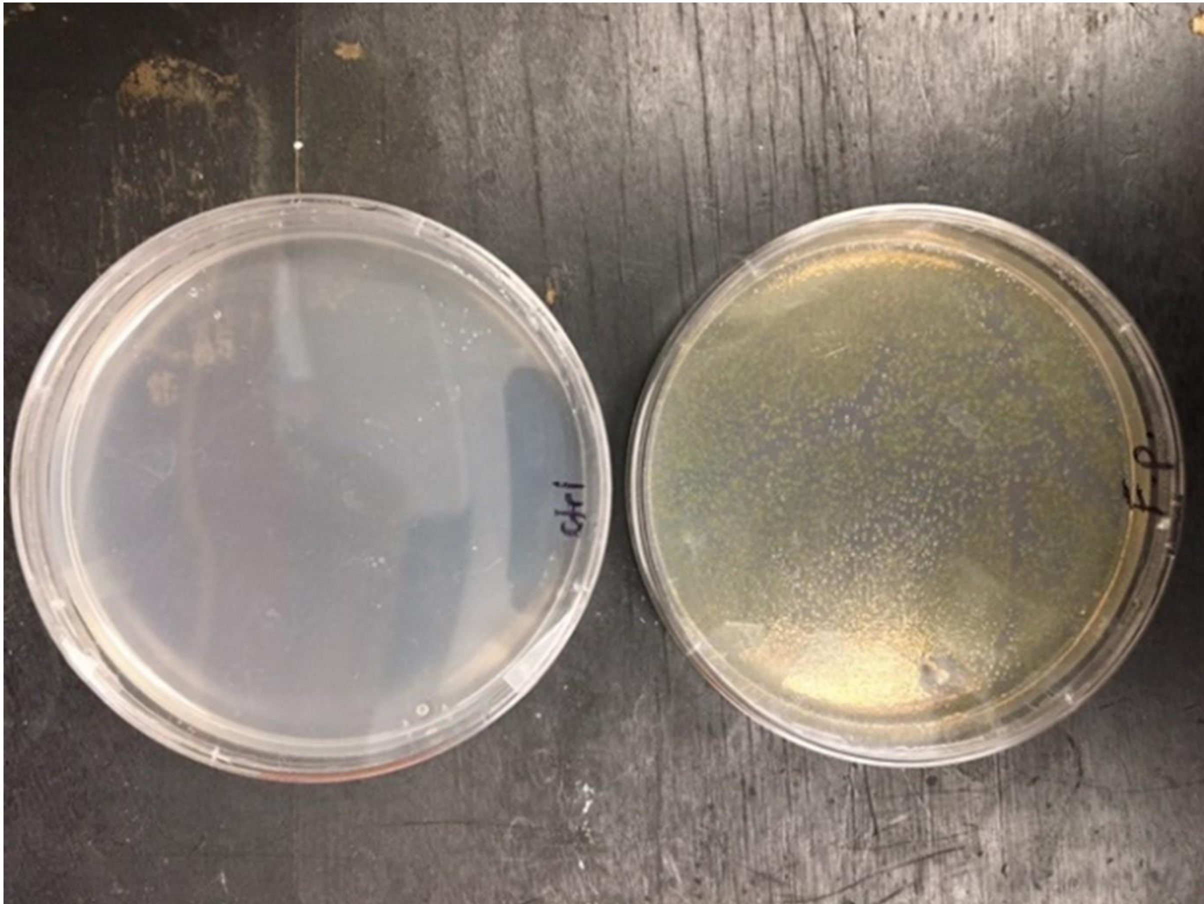
Peptide name	Acidic residue (%)	Basic residue (%)	Neutral residue (%)	Hydrophobic residue (%)	Net charge
rtVWF	7.69	15.38	30.77	46.15	+ 0.25
Tsvep1	6.38	8.51	48.94	36.17	+ 1
Dpbl-32	6.25	15.62	31.25	46.88	+ 3
Dpbl-48	2.08	20.83	35.42	41.67	+ 6.75

This table was generated by using the Antimicrobial Peptide Database: https://aps.unmc.edu/ and BioSynthesis Peptide Property Calculator Ver 3.1: https://www.biosyn.com/peptidepropertycalculator/

Appendix Table 3. Primers

Gene	Sequence (5'-3')	Accession Number	Efficiency (%)	R^2	Reference
cath1	Fw: ACCAGCTCCAAGTCAAGACTTTGAA Rv: TGTCCGAATCTTCTGCTGCAA	NM_001124480	98.99	0.99	Wangkahart et al. 2019
cath2	Fw: ACATGGAGGCAGAAGTTCAGAAGA Rv: GAGCCAAACCCAGGACGAGA	NM_001124463	91.77	0.99	Wangkahart et al. 2019
ef1a	Fw: CGCACAGTAACACCGAAACTAATTAAGC Rv: GCCTCCGCACTTGTAGATCAGATG	NM_001124339	102.56	0.98	Semple et al. 2018
hepc	Fw: GCTGTTCCTTTCTCCGAGGTGC Rv: GTGACAGCAGTTGCAGCACCA	XM_021595153	92.67	0.99	Wangkahart et al. 2019
*igf1r**	Fw: AACCATACACGGCTCACAGG Rv: AGGTTAACGCAGTCAGTCGG	XM_021570658	97.26	0.99	
il1b	Fw: CCACAAAGTGCATTTGAAC Rv: GCAACCTCCTCTAGGTGC	AJ298294	110.74	0.99	Semple et al. 2018
il16	Fw: CTTCTACACGCTATCTCTCACTC Rv: CGTCTGTCCCGAGCT	NM_001124657	98	0.99	Wangkahart et al. 2019
il10	Fw: GCCTTCTCCACCATCAGAGAC Rv: GATGCTGTCCATAGCGTGAC	NM_001245099	106.28	0.98	Semple et al. 2020
*p2x1**	Fw: CAGCTATAGGGTCTGGCGTT Rv: CTCGTCTAAAGGTTCCTGCTCT	XM_021624245	92.29	0.99	
tnfa	Fw: GTGCAAAAGATACCCACC Rv: CACTGCACGGTGTCAG	AJ278085	100.13	0.99	Semple et al. 2019
cln3	Fw: AGGCAACGACGCTACATCAA Rv: GAAACCCAAGCAATGCGTCA	XM_021587920	96.31	0.98	Wangkahart et al. 2019

*The annealing temperature for all primers was 60°C except for igf1r (65°C) and p2x1 (62°C).



Appendix Figure 1. Growth of *Flavobacterium psychrophilum* from viability assay. One hundred microliters of L-15 medium with or without *Flavobacterium psychrophilum*, was sampled and plated onto cytophaga agar plates during viability assays.