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Identification and characterization of a novel peptide from rainbow trout (*Oncorhynchus mykiss*) with antimicrobial activity against *Streptococcus iniae*

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ABSTRACT

The overuse and misuse of antibiotics has led to the emergence of antibiotic-resistant bacterial species which remain a challenge to treat therapeutically. Novel and efficacious drugs are desperately needed to combat pathogens. One method to facilitate these discoveries is the use of *in silico* methods. Computational biology has the power to scan large data sets and screen for potential molecules with antibacterial function. In the current study, an *in silico* approach was used to identify an antimicrobial peptide (AMP) derived from rainbow trout von Willebrand Factor. The AMP was tested against a panel of aquatic bacterial pathogens and was found to possess antibacterial activity against *Streptococcus iniae* (*S. iniae*). Since *S. iniae* is a zoonotic pathogen, this may be useful in other species as well. The peptide was non-hemolytic and non-cytotoxic at the concentrations tested in rainbow trout cells. Pre-treatment of rainbow trout cells with the peptide did not result in an upregulation of immune genes but stimulating the rainbow trout macrophage/monocyte-like cell line, RTS11, with heat-killed *S. iniae*, did result in a significant upregulation of the tumor necrosis factor alpha (*tnfa*) gene. In this study, a new AMP has been identified but its expression, synthesis and role *in vivo* remains unknown. Nevertheless, the findings presented improve our understanding of fish gill and macrophage responses towards this important zoonotic pathogen.

1. Introduction

The increased demand for fish and seafood has led fish farmers to depend heavily on antibiotics to control disease. As a result, increasing antimicrobial resistance in aquatic bacterial pathogens continues to be a problem and has detrimental effects on the environment. As antibiotics continue to lose efficacy, the need for effective therapeutic development is urgent. Bacterial aquatic pathogens, such as *Streptococcus iniae* (*S. iniae*) are not only a danger to aquatic life but can lead to disease in other species (Goh et al., 1998). *S. iniae* can result in high mortality events in farmed and wild fish populations, impacting the economy and biodiversity of natural habitats (Young et al., 2020). Early cases of human infection with the pathogen involved handling of farmed fish caused invasive disease (Weinstein et al., 1997). Although the pathogenesis of streptococcal species in humans has been very well characterized, our understanding of *S. iniae* in rainbow trout, especially at the cellular level, is not well understood (Goh et al., 1998; Weinstein et al., 1997; Glajzner et al., 2021; Lahav et al., 2004). The interaction of *S. iniae* with rainbow trout cell lines has only been investigated in RTS-11 cells but in other fish species, epithelial cells play an important role in pathogenesis by providing a scaffold for adherence and invasion (Eyngor et al., 2010; Locke et al., 2008). Since gill epithelium is a site known to be colonized by the pathogen *in vivo*; we investigated the response of rainbow trout gill epithelial cells to *S. iniae*, by using the RTgill-W1 cell line (McNulty et al., 2003).

S. iniae is a Gram-positive bacterium and can be classified as either serotype I (noninvasive) or serotype type II (invasive) (Zlotkin et al., 2003). Both serotypes cause significant disease but serotype II is more symptomatic and can result in: anorexia, hemorrhage, lethargy, loss of orientation, necrotizing myositis and exophthalmia (Lahav et al., 2004;

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Zlotkin et al., 2003). Some strains of *S. iniae* can survive and even multiply in rainbow trout macrophages, resulting in significant apoptosis of infected cells (Zlotkin et al., 2003). Vaccination efforts against the pathogen have been moderately effective but some *S. iniae* specimens, collected from fish farms, have developed resistance against commonly used antibiotics (Eldar et al., 1997; Heckman et al., 2022; Park et al., 2009). Natural compounds such as thymol, derived from botanical oils, have also been shown to be effective against drug-resistant *S. iniae* and enhance immunity in Channel catfish (*Ictalurus punctatus*) (Yin et al., 2022). Continued exploration of compounds with antibacterial properties against *S. iniae*, is needed to control disease, not only in fish but also in other animals.

Advances in informatics and computational methods have increased the potential to uncover natural products with antibacterial properties (Romano and Tatonetti, 2019). Computational methods may advance the drug discovery process since they take significantly less time when compared to traditional methods and can also identify many compounds in a single screen (Romano and Tatonetti, 2019). However, candidate compounds still require additional testing to assess therapeutic potentials (Romano and Tatonetti, 2019). *In silico* methods can also be used to design molecules, based on signatures with confirmed antimicrobial activity (Maccari et al., 2015). This can also be used to identify such compounds, which can reduce production cost and speed up evaluation of activity and toxicity (Maccari et al., 2015). Antimicrobial peptides (AMPs) are a group of molecules that have been investigated extensively using *in silico* approaches (Maccari et al., 2015; Cardoso et al., 2020; Madrazo and Campos, 2022). AMPs are small and typically cationic and amphipathic molecules (Büyükkiraz and Kesmen, 2022). However, some AMPs can also be negatively charged which influences the range of antimicrobial activity (Wang et al., 2017). AMPs are also ribosomally synthesized, with crucial roles in innate and adaptive immunity (Büyükkiraz and Kesmen, 2022). Salmonid AMPs have a broad range of antibacterial activity against various aquatic pathogens and can also enhance host immunity (Brunner et al., 2020). Despite broad antimicrobial activity, no salmonid AMPs are known to have antimicrobial effects against *S. iniae*. In the current study, an *in silico* approach was used to screen potential AMPs of the rainbow trout proteome. A candidate peptide with the sequence: MQLESAITHTLFK, was identified and shared sequence similarity to salmonid von Willebrand Factor D protein. The peptide was therefore named rainbow trout von Willebrand Factor (rtVWF). It was synthesized and subsequently evaluated for potential antimicrobial and immunomodulatory activity in rainbow trout cell lines. rtVWF was found to have antimicrobial activity against *S. iniae* and did not impact the viability of the rainbow trout cell lines. Furthermore, rtVWF did not have an immunomodulatory effect on the cell lines and was non-hemolytic to rainbow trout red blood cells (RBCs).

2. Methods

2.1. Bioinformatic identification and synthesis of peptide

We previously developed a computational pipeline called AMPminer to enable proteome-wide AMP candidate discovery. The pipeline combines previous sequence motif-based machine-learning methods for AMP prediction with similarity-based approaches that scan sequences with HMM libraries of known AMPs. Particularly, we used the Database of Anti-Microbial peptides (ADAM) (Lee et al., 2015), which is an amalgamation of other databases including APD (Wang et al., 2016), Bactibase (Hammami et al., 2010), CAMP (Thomas et al., 2010), and PhytAMP (Hammami et al., 2009). The full AMPminer application is available at: https://github.com/danielm710/AMP-webserver.

In order to identify potential candidate AMPs in rainbow trout, we used AMPminer to scan the *O. mykiss* proteome (uniprot proteome accession # UP000694395) for potential high-scoring AMP candidates with a probability score of >80%. Sliding windows were used to score peptide regions for AMP-like characteristics, and the full-length protein sequences were also scanned against reference AMP proteins with p-score > 60%. From an initial set of 5000 candidates, we further selected short sequences of length <100 amino acids, narrowing our list to 33 such AMP candidates, one of which was manually selected for further analysis. This protein was labeled as "unknown protein product" (NCBI accession CDR18389). We then selected the C-terminal AMP-like segment (residues 81–93) of this peptide, resulting in the following sequence: MQLESAITHTLFK (accession number: CDR18389.1). MQLESAITHTLFK, was synthesized and lyophilized to >95% purity, by Genscript® USA. Mass spectrometry, high plate liquid chromatography and peptide solubility tests were also conducted by Genscript® USA. The peptide was then dissolved in sterile molecular grade water (Thermo Fisher Scientific, USA), to achieve a final concentration of 1000 μM and were stored −20 °C, until further use.

2.2. Bacterial culture

Flavobacterium psychrophilum FPG101 was grown in 3 ml of cytophaga broth (0.5 g tryptone, 0.5 g yeast extract, 0.2 g sodium acetate, 0.2 g beef extract, with a total volume of 1 L and pH = 7.2) at 14 °C. *Yersinia ruckeri* (*Y. ruckeri*) and *Streptococcus iniae* (*S.iniae*) were cultured at 28 °C in 3 ml BD Tryptic Soy Broth (17 g tryptone, 3 g soytone, 2.5 g glucose, 5 g sodium chloride, 2.5 g dipotassium phosphate, with a total volume of 1 L and pH = 7.2) (Soybean-Casein Digest Medium). Bacterial stock cultures were stored in a 50% (v/v) glycerol and respective broth solution at −80 °C in sterile 1.5 ml cryovials until further use. Prior to experimentation, *F. psychrophilum* was plated onto cytophaga plates and cultured for 3 days at 14 °C. Whereas *Y. ruckeri* and *S. iniae* were plated onto and LB agar plates and incubated at 21 °C for 3 days. Depending on the type of bacteria, single colonies were selected and grown in either 3 ml of cytophaga broth or 3 ml of Tryptic Soy Broth for an additional 3 days. This final bacterial culture was used for subsequent experiments.

2.3. Broth microdilution assay

The minimal inhibitory concentration (MIC) for rtVWF was evaluated using the broth microdilution assay. Briefly, the bacterial cultures were diluted to a final O.D. 600 nm of 0.001 in respective broth. One hundred microliters of the diluted bacterial cultures were added to each well, of a 96-well plate, in triplicate. rtVWF was diluted in sterile molecular grade water to 500, 250, 125, 62.5 and 31.25 μM. Then 11 μl of each solution were added to wells in triplicate, resulting in a final concentration of rtVWF at 45.4, 22.7, 11.3, 5.7 and 2.8 μM. Some wells received bacteria alone with 11 μl of sterile water, which served as a vehicle control, or 11 μl of kanamycin sulfate (45.4 mg/ml). Some wells also received 100 μl of broth and 11 μl of sterile water which served as a background control. All 96-well plates were sealed with parafilm and incubated at either 14 °C for 3 days (*F. psychrophilum*) or 28 °C for 7 h (*Y. ruckeri* and *S. iniae*). This panel of important fish bacterial pathogens was selected due to differences in Gram-negative and Gram-positive properties. The hourly growth of bacteria was monitored using a spectrophotometer at an O.D. of 600 nm (BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Instruments). To establish growth curves, the background values were averaged and subtracted from each reading.

2.4. Thiazolyl blue tetrazolium bromide (MTT) assay

Five hundred thousand RTS-11 and RTgill-W1 cells were seeded into each well of a 96-well plate in triplicate. The cells were suspended in 100 μl of Leibovitz's L-15 Medium with galactose (L-15) (Thermofisher Scientific, Cat no. 11415064) with 15% fetal bovine serum (FBS) (Gibco®) and no antibiotics. Some wells also received medium only which served as a background control. The plate was sealed with parafilm and incubated at 21 °C for 24 h. The cells were then exposed for an additional 24 h, to a low and high concentration range of peptide: 11.3

μM and 45.4 μM, respectively. The medium was removed and 100 μl of L-15 with 15% FBS and no antibiotics was added to each well. Then, 11 μl of a filtered 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, Cat no. M5655) solution in 1 x phosphate buffered saline (PBS) was added to each well for 3 h. A 0.01% (v/v) solution of Triton X-100 (Sigma-Aldrich) was also used as a positive control and was added to wells in triplicate. The plate was sealed with parafilm and placed on a shaker at 100 rpm for 1 min. Then 150 μl of dimethyl sulfoxide (DMSO) was mixed into each well and the plate was allowed to rest for 15 min at 21 °C. A spectrophotometer at O.D. 520 nm was used to obtain a reading of each well (BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Instruments). The wells with medium only and DMSO, served as a background control. These values were averaged and subtracted from all other values to analyze the results.

2.5. Hemolysis assay

Rainbow trout erythrocytes were obtained from fish blood provided by the Alma Aquaculture Research Station (Alma, ON; University of Waterloo Animal Care Permit 40589). 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 × 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 45.4, 11.3 and 2.8 μM. Some wells received a 0.1% SDS solution as a positive control. The plates were incubated at room temperature for 30 min and were then centrifuged at 2000×*g* for 3 min 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 × 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 to rtVWF × 100/O.D. 405 nm of erythrocytes exposed to SDS). For the control cells, the percentage of hemolysis was calculated using the formula: % hemolysis = (O.D. 405 nm of erythrocytes × 100/O.D. 405 nm of erythrocytes exposed to SDS).

2.6. Exposure of cells to peptide and Streptococcus iniae

RTS11 and RTgill-W1 cells were maintained at 21 °C, under ambient air, and in T-25 vented flasks. The cells were passaged every 7 and 4 days, respectively. 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 2.4. 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 2.3. 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. After 24 h of incubation, total RNA was isolated from pooled duplicates.

2.7. Total RNA isolation and DNA synthesis

Total RNA extraction was performed on RTgill-W1 and RTS11 cells using the RNeasy Micro Kit by following the manufacturer's protocol (Qiagen). RNA purity and quantification were assessed using the NanodropTM 2000 Spectrophotometer. Total RNA samples were exposed to DNase I (Thermo Scientific) and stored at - 80 °C for future use. The qScriptTM cDNA SuperMix (Quantabio) was used to synthesize cDNA, using 400 μg of RNA/reaction. Briefly, 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 use.

2.8. qRT-PCR

qRT-PCR was performed using the WISENT ADVANCED™ qPCR mastermix (Wisent), by following the manufacturer's instructions on the LightCycler® 480 II (Roche). Briefly, each 10 μL reaction contained 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 included for each experiment and all trials were conducted in triplicate. Thermocycling conditions were as follows: pre-incubation at 95 °C for 10 min, then 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 20 s. Primer sequences for *tnfa* and *hepc* were previously described (Wangkahart et al., 2019; Semple et al., 2018). The primer sequences were as follows: *tnfa* forward: 5′-GTGCAAAAGATACCCACC-3'; *tnfa* reverse: 5′-CACTGCACGGTGTCAG-3'; *hepc* forward: 5′-GCTGTTCCTTTCTCCGAGGTGC-3'; *hepc* reverse: 5′-GTGACAGCAGTTGCAGCACCA-3'. Efficiency for *tnfa* primers was found to be 100.13% and for *hepc*, efficiency was 92.67%. The fold-change in gene expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

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: $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***).

3. Results

3.1. Antimicrobial and hemolytic effects of rtVWF

rtVWF displayed significant antimicrobial activity against *S. iniae* after 7 h of exposure at a concentration of 45.4 μM (Fig. 1E). The growth of *F. psychrophilum* and *Y. ruckeri* was not impacted by these conditions (Fig. 1). The hemolytic effects of rtVWF were also examined and the peptide did not display any hemolytic effects up to 45.4 μM, in rainbow trout red blood cells (Fig. 2).

3.2. The effects of rtVWF and S. iniae on in vitro rainbow trout cellular responses

The viability of RTS11 and RTgill-W1 cells was not negatively impacted via incubation with rtVWF for 24 h (Fig. 3). Triton X-100

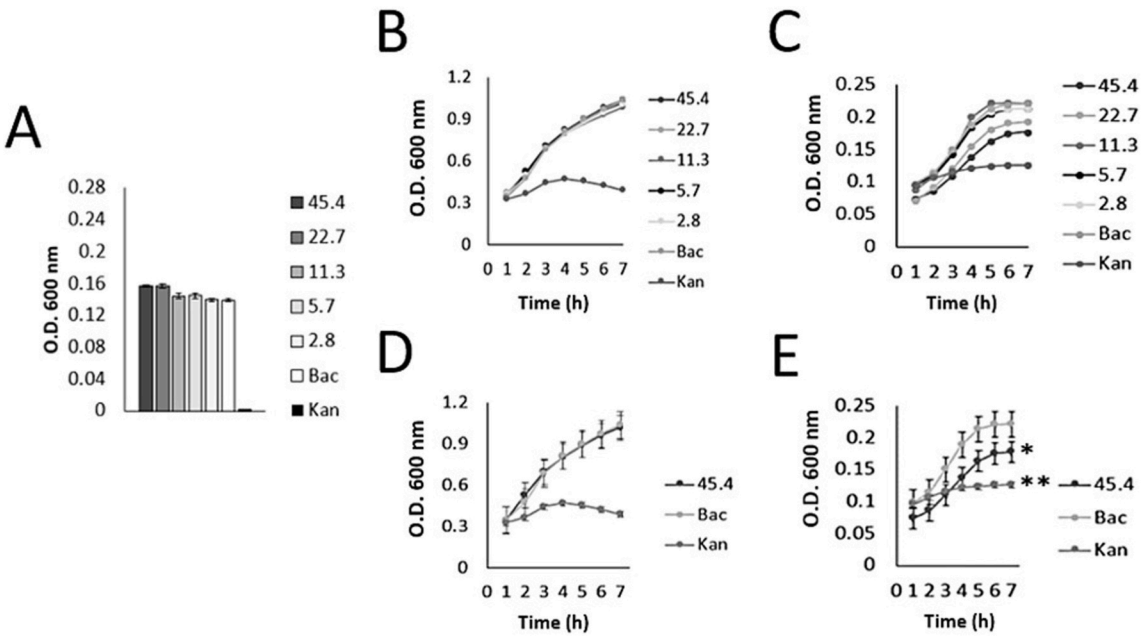

Fig. 1. Effects of rtVWF on aquatic bacterial pathogen growth. The data are representative of bacterial growth for (A) *F. psychrophilum*, (all concentrations: B and just 45.4 μM: D) *Y. ruckeri* and (all concentrations: C and just 45.4 μM: E) *S. iniae*. rtVWF concentrations ranged from 2.8 μM–45.4 μM. The growth at each time point was measured and peptide treated groups were compared to bacteria alone (Bac) as well as kanamycin sulfate (Kan) groups, which served as a positive control. Means are representative of three independent experiments, ± S.D.

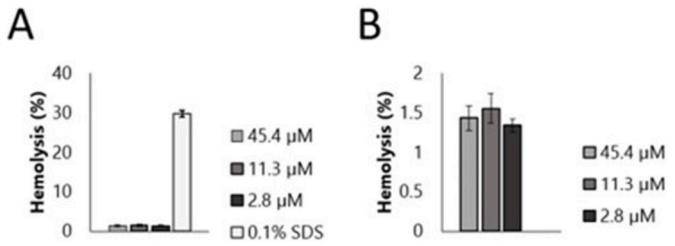

Fig. 2. Effects of rtVWF on the hemolysis of rainbow trout erythrocytes. The data are representative of rainbow trout erythrocytes exposed to rtVWF for 30 min at room temperature (A) with positive control (0.1% SDS) (B) not showing positive control. Means are representative of three independent experiments, ± S.D.

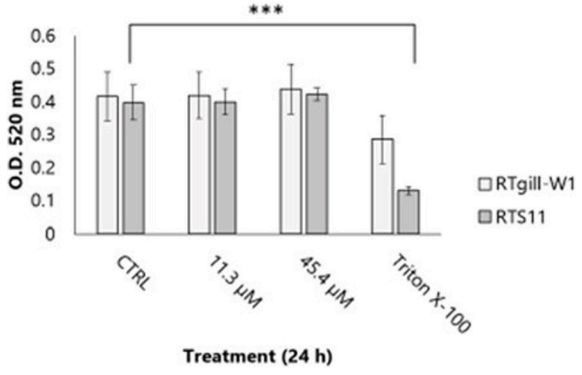

Fig. 3. Effects of rtVWF on cell viability. The data are representative of MTT conversion in RTgill-W1 and RTS11 cells exposed to rtVWF (at 11.3 μM and 45.4 μM) for 24 h. A 0.01% Triton X-100 served as a positive control. Means are representative of three independent experiments, ± S.D.

incubated RTS11 cells showed a significant reduction in cell viability at 24 h (Fig. 3). Since *S. iniae* did not grow well in the cell culture medium, it was heat-killed to stimulate cells. The higher dose of heat-killed *S. iniae*, significantly reduced the viability of RTgill-W1 and RTS11 cells compared to controls (Fig. 4). The reduction in cell viability was similar between the cell lines (Fig. 4). However, the lower dose (1/100), of heat-killed *S. iniae* did not result in a significant reduction in cell viability and was therefore used to stimulate cell lines in future experiments.

3.3. The effects of rtVWF pre-treatment and S. iniae stimulation on in vitro rainbow trout immune gene expression

To evaluate the potential immunomodulatory effects of rtVWF on rainbow trout cell lines, RTS11 and RTgill-W1 were pre-treated for 24 h with the peptide before bacterial stimulation. The expression of immune genes; tumor necrosis factor alpha (*tnfa*) and hepcidin (*hepc*), were

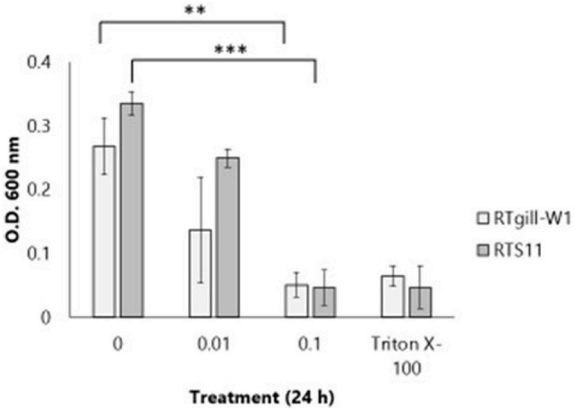

Fig. 4. Effects of heat-killed *S. iniae* on cell viability. The data are representative of RTgill-W1 and RTS11 cells exposed to heat-killed *S. iniae* (1/100) or (1/10) for 24 h. A 0.01% Triton X-100 served as a positive control. Means are representative of three independent experiments, ± S.D.

examined in the cells. Heat-killed *S. iniae* a significantly upregulated the gene expression of *tnfa* in RTS11 cells when compared to rtVWF pre-treated cells (Fig. 5A). rtVWF pre-treatment combined with subsequent exposure to heat-killed *S. iniae* appeared to slightly increase the gene expression of *tnfa* and *hepc* in RTS11 cells but this was not statistically significant (Fig. 5A). Compared to RTS11 cells, RTgill-W1 cells were hyporesponsive to heat-killed *S. iniae* and the gene expression of *tnfa* and *hepc* remained relatively stable between stimulated and pre-treated groups (Fig. 5B). To investigate the influence of serum on pre-treatment with rtVWF and *S. iniae* stimulation, the experiments were repeated under low serum conditions. Low serum did not have any significant effects on either RTS11 or RTgill-W1 cells (Fig. 6A and Fig. 6B).

4. Discussion

4.1. rtVWF is antimicrobial to S. iniae and non-hemolytic to rainbow trout erythrocytes

Incubating rtVWF with *S. iniae* significantly reduced bacterial growth at 7 h (Fig. 1E). The MIC was found to be 45.4 μM (Fig. 1E). Other fish AMPs, such as piscidin 4, moronecidin and chrysophsin also display antimicrobial activity towards *S. iniae* at various concentrations but are found to be ineffective against some bacterial species (Noga et al., 2009; Lauth et al., 2002; Iijima et al., 2003). Antimicrobial activity was not observed when rtVWF was tested against the Gram-negative bacterial pathogens, such as *Y. ruckeri* and *F. psychrophilum*; suggesting that rtVWF may be more effective towards Gram-positive bacteria. This is in agreement with other findings, where AMPs that share sequence similarity to rtVWF also share this antimicrobial signature. Such peptides, include the naturally occurring Temporin-1SPa and Temporin-1SPb from the mink frog (*Rana septentrionalis*) (Bevier et al., 2004; Mishra et al., 2018). When tested against a panel of Gram-negative and Gram-positive bacteria, Temporin-1SPa displayed antibacterial activity only towards Gram-positive pathogens: *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*) (Mishra et al., 2018). Whereas Temporin-1SPb only displayed antibacterial activity towards *S. aureus* (Bevier et al., 2004). The main region of similarity conserved between rtVWF, Temporin-1SPa and Temporin-1SPb, shares the amino acid sequence: SAIT, within the central region of the peptides (Supplementary Fig. 1). Another AMP, known as buCATHL4G from the water buffalo (*Bubalus bubalis*) also shares some sequence similarity to rtVWF but is lacking some amino acids in this region (Supplementary Fig. 1). Unlike rtVWF, Temporin-1SPa and Temporin-1SPb; BuCATHL4G displays antibacterial activity against Gram-negative and Gram-positive bacteria

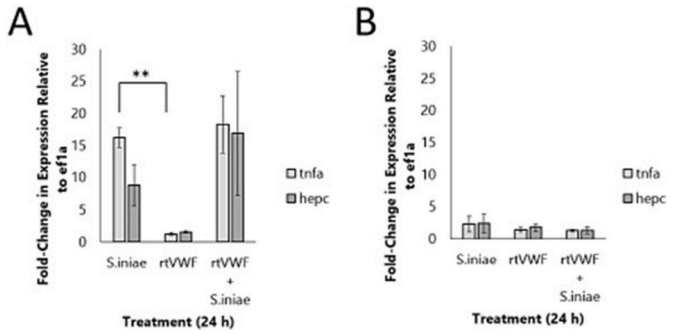

Fig. 5. Effects of rtVWF pre-treatment and further stimulation with heat-killed *S. iniae* on cellular immune gene expression. The data are representative of immune gene expression in (A) RTS11 and (B) RTgill-W1 cells, exposed to rtVWF for 24 h and then heat-killed *S. iniae* at a dose of (1/100) for an additional 24 h. Means are representative of three independent experiments, ± S.D.

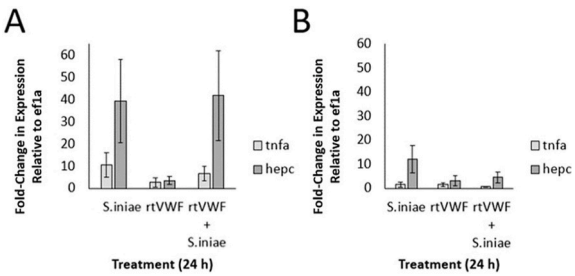

Fig. 6. Effects of rtVWF pre-treatment in low serum and further stimulation with heat-killed *S. iniae* on cellular immune gene expression. The data are representative of immune gene expression in (A) RTS11 and (B) RTgill-W1 cells, exposed to rtVWF for 24 h and then heat-killed *S. iniae* at a dose of (1/100) for an additional 24 h. Means are representative of three independent experiments, ± S.D.

(Brahma et al., 2015). Perhaps synthesizing peptides with alterations in the SAIT sequence motif, or including repetitions of this sequence, would help identify the potential antimicrobial role of this putative peptide domain. The temperature at which the bacteria were cultured may have also influenced antibacterial activity of rtVWF, since some AMPs have been shown to be impacted by changes in temperature (Heymich et al., 2021). In addition, rtVWF did not display hemolytic effects in rainbow trout erythrocytes (Fig. 2). However, Temporin-1SPb was found to exhibit hemolytic activity towards human erythrocytes (Bevier et al., 2004). Further research is necessary to characterize the effects of rtVWF in the future and may involve expanding the bacterial pathogen panel to include non-fish pathogens.

4.2. rtVWF does not induce cytotoxic effects and does not have an immunomodulatory effect on RTgill-W1 or RTS11

The incubation of RTgill-W1 and RTS11 cells with rtVWF peptide was well tolerated even at 24 h post exposure (Fig. 3). However, heat-killed *S. iniae* was cytotoxic to RTgill-W1 and RTS11 cells at the higher dose (Fig. 4). Since the lower dose was well tolerated by both cell lines, this was selected as a potential immunostimulant in further experiments (Fig. 4). Stimulating the cell lines with the lower dose, significantly increased *tnfa* gene expression but only in RTS11 cells (Fig. 5A). Live and heat-killed *S. iniae* have been shown to increase *tnfa* gene expression in RTS11 cells and transcript levels peak within the first 12 h of exposure (Eyngor et al., 2010). Interleukin-1 beta and interleukin 6 transcript levels also peak within this time (Eyngor et al., 2010). Some strains of *S. iniae* can produce large amounts of extracellular polysaccharide which is released by the bacteria into the medium and may lead to the immune response observed in RTS11 cells (Eyngor et al., 2010). Besides RTS11, the response of other fish cell lines to *S. iniae* is unknown; therefore, it was decided to investigate the response of RTgill-W1 cells to this pathogen. Unlike RTS11, RTgill-W1 cells did not display any significant changes in immune gene expression (Fig. 5B). This lack of immune gene expression observed over 24 h, may be attributed to the colonization of fish gill tissue by the pathogen and ultimately its systemic dissemination (McNulty et al., 2003). Furthermore, even though *hepc* gene expression levels did not change significantly in the current study; hepcidin has been shown to significantly increase in the liver of Nile tilapia (*Oreochromis niloticus*) infected with *S. iniae* (Abdelkhalek et al., 2020).

Pre-treament of the cell lines with rtVWF before heat-killed *S. iniae* stimulation does not influence *tnfa* or *hepc* gene expression (Fig. 5). In addition, serum level does not have an effect on this observation (Fig. 6). Tumor necrosis factor alpha gene expression appears to be lower in the low serum groups when compared to original serum levels (Figs. 5 and 6). Serum depletion may cause the cells to invest more energy into transcriptional responses associated with metabolism instead of immune

responses (Novoa-Herran et al., 2016). Starvation of fish has also been shown to increase hepcidin gene expression in the liver, which may have a protective effect against infection, but this rapidly decreases (Mohapatra et al., 2015). Nevertheless, rtVWF does not have an immunomodulatory effect in terms of stimulating the gene expression of *tnfa* and *hepc*. The reasons for this are unknown but it is interesting to note that von Willebrand factor; the same protein that rtVWF is derived from, is rapidly taken up by human macrophages and subsequently degraded to prevent excess clotting (van Schooten et al., 2008). It is unknown whether this mechanism is conserved in fish. However, it would be intriguing to examine it using a fluorescently labelled rtVWF peptide. Von Willebrand factor is well known for its important roles in hemostasis, but has recently been identified as a mediator of vascular inflammation (Gragnano et al., 2017). Perhaps studying the effects of rtVWF in a fish vascular inflammation model, would be useful to examine this potential role.

5. Conclusion

rtVWF was found to be antibacterial towards *S. iniae*, a zoonotic pathogen and was also discovered not to have hemolytic effects against rainbow trout erythrocytes. *S. iniae* may be considered for future use *in vivo* and may also benefit humans with *S. iniae* infection. The current study also revealed that rainbow trout gill cells do not respond immunologically, in terms of tumor necrosis factor alpha and hepcidin gene expression, to heat-killed *S. iniae*. The lack of immune response in the gill cells may explain why the gill is a common route of entry for this pathogen. Collectively, these results expand our understanding of the fish immune response to *S. iniae* and identify a new molecule with the ability to reduce its *in vitro* growth.

Declaration of interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104518.

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