PACAP Analogues with Potential Applications in Finfish Aquaculture

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

Chapter 1 – Tania Rodríguez-Ramos assisted with the performance of permeability assays.

Lowia Al-Hussinee assisted with bacterial growth.

Chapter 2 - Tania Rodríguez-Ramos assisted with the performance of the IL-1β and IFN-γ ELISAs.

Manuel Soto Dávila assisted with the performance of qPCR data analysis.

Valentina Wong Benito assisted with *in-silico* studies.

Abstract

Global aquaculture industry has significant losses each year due to disease outbreaks, parasitic, viral, and bacterial infections. Common methods to treat fish infections include antibiotics, but prolonged use can lead to the emergence of resistant strains. *Aeromonas* spp. infections are a common and problematic disease in fish, and members of this genera are susceptible to the production of antibiotic resistant strains. Antimicrobial peptides (AMPs) have emerged as an alternative method to treat and prevent infections. Pituitary adenylate cyclase activating polypeptide (PACAP) is a prominent member of this family. PACAP has versatile effects including neural development, anti-tumor activity, metabolism, growth, antimicrobial activity, and modulation of immune responses. Thus, the general objective of this research was to study PACAP direct antimicrobial activity and immunomodulatory effect in rainbow trout cell lines.

Analysis of the experimental results and reviewed literature agree on the direct antimicrobial activity of PACAP on *A. salmonicida*, *A. hydrophila*, and *Y. ruckeri* growth. It was also observed that the direct antimicrobial effects of PACAP is dependent on the culture broth used, but not related with the presence of NaAc in the broth or serine protease secretion. Furthermore, results suggest that PACAP direct antimicrobial activity underlying mechanism include a bacterial membrane permeabilizing effect. This study also demonstrated that the five PACAP variants evaluated showed no toxicity at concentrations lower than 25 µM *in vitro* and they can be considered safe for using in aquaculture, especially PACAP 1 and 5.

The effect of TNF-α, IL-1β, IL-8, IFNγ, MyD88, and TGFβ cytokine expression on RTgutGC and RTS11 cell lines infected with *A.salmonicida* and incubated with either PACAP 1 or 5 (200 nM) was evaluated. RT-qPCR results showed that *il1b* and *il8* transcript expression in RTgutGC was significantly downregulated while *tgfb* expression was upregulated. Importantly, IL-1β and IFNγ protein concentration, was also tested with a significant increase of IFNγ protein levels in the

conditioned media of RTS11 cells incubated with PACAP 1 and exposed to *A. salmonicida*. IL-1β protein concentration was also increased. In general, results showed a poor correlation between gene expression and protein amount, suggesting a stimulation of the translation of IL1-β protein from previously accumulated transcripts.

In-silico studies of PACAP-receptor interactions has also showed a turn of the peptide characteristic of PACAP-PAC 1 interaction, correlated with the higher number of interactions observed with this specific receptor, which is also in agreement with the higher PACAP specificity described for PAC1 compared to VPAC1 and VPACA2. Finally, nine amino acids were selected as most related with the receptor associated PACAP functionality (HIS1, SER2, THR7, ASP8, SER11, ARG12, TYR13, ARG14, TYR22).

The highly conserved sequence of PACAP between species prevents its fast clearance. Furthermore, PACAP stimulates energy metabolism, avoiding antibiotic resistance related to ATP depletion and metabolic collapse. All the results obtained in this thesis also demonstrate that PACAP has a direct antimicrobial effect related to a bacterial membrane permeabilization mechanism, which also reduces the probability of generating resistant bacterial strains. It was also possible to corroborate the immunostimulant properties of PACAP and to identify the amino acids most related to the functionality mediated by its interaction with receptors. The low toxicity also demonstrated in these studies supports the possible oral administration of the peptide. Therefore, PACAP is an environmentally friendly alternative and very promising for use in the aquaculture sector.

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I want to especially thank those who became almost family for every minute they gave me inside and outside the laboratory. I am grateful for the friendships that we have developed along the way. Whether it's grabbing coffee together or brainstorming ideas, these individuals have made my experience truly memorable and enjoyable. Because life wanted it, you got to meet my ashes first. Thank you for the unwavering support and belief in my abilities, even during moments of self-doubt. Your trust in me has been instrumental in my personal and professional growth. I am forever grateful for the strong bonds we formed, which extended beyond the laboratory walls and made this journey feel like a second family.

Thanks also to my mother for bearing the physical distance as a warrior and supporting my dreams as always. Her strength and resilience have been a constant source of inspiration for me, reminding me to never give up on my aspirations. I am incredibly fortunate to have such a loving and supportive mother who continues to be my biggest cheerleader in life. Thanks to my father for his unconditional help, for giving me a hand every time I staggered and encouraging me to keep going at the same time. His solid belief in my abilities has instilled in me a sense of determination and perseverance.

Thank you all because you helped me grow and be stronger, because you were part of my path and who I am.

Dedication

To Nature because it is everything in my life, because it gives me shelter when I need it and the energy I require. To Nature that has taught me resilience, patience, and the importance of living in harmony with the world around me. For the feeling of peace and connection with something bigger that it gives me every single day. For the desire to work daily to continue discovering its magical mysteries.

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List of abbreviations

AMPs - Antimicrobial peptides

cDNA - Complementary deoxyribonucleic acid

CFU - Colony forming units

DNA - Deoxyribonucleic acid

DNase I - Deoxyribonucleic nuclease

ELISA - Enzyme linked immunosorbent assay

FBS - Fetal bovine serum

HSP70 - Inducible heat shock protein of 70 kDa

IL - Interleukin

IFNγ - Interferon gamma

L15 - Leibovitz's media

MHB - Mueller Hinton Broth

MIC - Minimum inhibitory concentration

MOI - multiplicity of infection

mRNA - Messenger ribonucleic acid

MyD88 - Myeloid differentiation primary response protein

LPS – Lipopolysaccharides

OD - Optical density

PACAP - Pituitary adenylate cyclase activating polypeptide

PAC1 - Pituitary adenylate cyclase-activating polypeptide type I receptor

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PI - Propidium iodide

qRT-PCR - Quantitative reverse transcriptase PCR

RNA – Ribonucleic acid

RT - Room temperature

RTS11 - Rainbow trout monocyte/macrophage-like cell Line

RTgutGC - Rainbow trout intestinal epithelial cell line

SEM - Standard error of the mean

TBS - Tris-buffered saline

TBS-Tween - TBS with Tween 20

TSA -Tryptic soy agar

TSB - Tryptic soy broth

TNFα - Tumor necrosis factor alpha

VIP - Vasoactive intestinal peptide

VPAC1 - Vasoactive intestinal polypeptide receptor 1

VPAC2 - Vasoactive intestinal polypeptide receptor 2

I. Chapter 1: PACAP analogs with potential antimicrobial application in aquaculture

A. Introduction

1. Antibiotic resistance problem focused on *Aeromonas* spp. and Rainbow trout.

Aquaculture ensures a reliable source of high-quality food products for humanity (Akazawa *et al.*, 2014). Nevertheless, the global aquaculture industry loses approximately 6 billion USD each year due to disease outbreaks with the main risk at the farm level (Akazawa *et al.*, 2014). These losses are related to parasitic, viral, as well as bacterial infections. For instance, Canadian losses are estimated to be $180 000 CAD per crop in a farm with 200 000 fish due to just sea lice infections (Mustafa *et al.*, 2001). In addition, $50 000 000 CAD was lost by producers of Atlantic salmon (*Salmo salar*) in 2002 due to the parasite *Kudoa thyrsites* in British Columbia (Rodger, 2016). Moreover, in a comparison between 2019 and 2020 it was reported that fish sales decreased 56.2% in Newfoundland and Labrador, and 33.6% in New Brunswick, due to the lower finfish production. The sales reduction in these provinces was consequence of challenging biological conditions like infectious salmon anemia (S. C. Government of Canada, 2021). Losses due to bacterial diseases are not less important, as the "Fish health events sites report" by Fisheries and Oceans Canada (DFO) stated that 91% of the marine finfish aquaculture facility infections in British Columbia were caused by bacterial infections (D. F. O. Government of Canada, 2020).

The stocking of catchable-sized rainbow trout is an important part of many cold-water fisheries programs (Branigan *et al.*, 2021; Johnson *et al.*, 1995). Moreover, the Canadian rainbow trout industry is supported by hatcheries, from which all cultivated rainbow trout are sourced (Ingmer & Brøndsted, 2009).These trout also are farmed for food in almost all Canadian provinces (*Products & Regions Index*, n.d.). Therefore, it is very important to protect this species from infectious bacterial outbreaks.

Common methods to treat fish infections generally include the use of antibiotics in both a prophylactic and therapeutic manner. However, the prolonged use of antibiotics can lead to the emergence of resistant bacterial strains (Hossain *et al.*, 2022). For instance, the overuse of antibiotics to treat bacterial infections of the genus *Aeromonas*, which has a high pathogenicity in fish (Wamala *et al*., 2018), can lead to the emergence of resistant strains (Harikrishnan & Balasundaram, 2005; Saavedra *et al.*, 2004).

Aeromonas spp. can grow well in a range of temperatures from 4 °C to 42 °C, and a range of pH from 5 to 10 which makes them a ubiquitous pathogen, with a widespread distribution that includes freshwater, seawater, estuaries, and chlorinated water. *Aeromonas* spp. infections are one of the most common and problematic diseases of fish raised in recirculating ponds (Harikrishnan & Balasundaram, 2005). They are responsible for Motile Aeromonas Septicemia, and they are able to infect numerous fish species, including catfish, salmon, carp and trout (Cao *et al.*, 2020). Members of this genus are Gram-negative bacterium with several antibiotic resistant strains (Cao *et al.,* 2020; Harikrishnan & Balasundaram, 2005).

Bacteria of the genus *Aeromonas* are regular residents of the gut microbiota of fish (Azzam-Sayuti *et al.*, 2021), and they can act as opportunistic pathogens (Azzam-Sayuti *et al.*, 2021; Cao *et al.*, 2020; Ganesan *et al.*, 2023). The meaning of this term (opportunistic pathogen) is that when the opportunity is given this pathogen has the potential of causing disease (Semwal *et al.*, 2023). *A. hydrophila*, for example, is considered an opportunistic invader that infects fish previously infected with another infection or under stressed conditions. It has been even used as an efficient biomarker of stressed aquatic environment (Semwal *et al.*, 2023).

Alternatively, *A. salmonicida* has been catalogued by some authors as opportunistic pathogen (Charette, 2021) while others reported as a primary fish pathogen (Park *et al.*, 2020). It is known to be the only non-motile specie in the genus *Aeromonas* (Park *et al.*, 2020). It is facultative anaerobic,

non-motile, and bacillus shaped bacterium (Soto-Dávila *et al.*, 2019). Typical strains were characterized for first time in 1890, cause furunculosis in salmonids and produce a distinguishing brown pigment. The are five official subspecies, four psychrophiles (*salmonicida, achromogenes, masoucida* and *smithia*) and one mesophilic, *pectinolytica*. Atypical strains did not produce the pigment but are also able to infect salmonids and other species of fish (Charette, 2021). Specifically, it has been reported by Charette (2021) that *A. salmonicida* represent an important challenge for the fish farming industry due to the common occurrence of multi resistance that in some cases include all accepted antibiotics and therefore, it is responsible for important economic losses in the global aquaculture industry, particularly in salmonid culture systems (Charette, 2021).

Likewise, Rainbow trout (*Oncorhynchus mykiss*), is an important part of several cold-water fisheries programs (Branigan *et al.*, 2021). It is farmed in almost all Canadian provinces (*Products & Regions Index*, n.d.) and it is vulnerable to *Aeromonas* infections. Saavedra *et al.* evaluated the sensitivity to ß-lactam antibiotics like penicillin, cephalosporins, monobactams, and carbapenems in 51 strains of *Aeromonas* from 20 rainbow trout, and found a high rate of resistance to ampicillin, carbenicillin and ticarcillin (Saavedra *et al.*, 2004).

Tetracyclines are very often used in aquaculture, particularly to treat furunculosis in salmonids (Rhodes *et al.*, 2000). The low cost and broad-spectrum activity of oxytetracycline make it one of the most used antibiotics in global aquaculture industry (Payne *et al.*, 2022). Specifically, oxytetracycline is a bacteriostatic and it is the most usual treatment of furunculosis and enteric red mouth disease in rainbow trout infected with *A. salmonicida* and *Y. ruckeri* (Payne *et al.*, 2022). However, *A. salmonicida* resistance to oxytetracycline and tetracyclines in general has been reported with an increased frequency from 4% of isolates in 1981 to greater than 50% of isolates in the early 1990s (Rhodes *et al.*, 2000). Other more recent investigations also refer to the resistance of *Aeromonas* spp to oxytetracycline in Atlantic salmon and Senegalese sole (Payne *et al.*, 2022). *A.*

hydrophila isolates have been also reported as resistant to lincomycin and oxytetracycline in a 91.67 % of the isolates from sea trout (Revina *et al.*, 2017). Resistance to florfenicol in *Aeromonas* spp. was also observed in the 14% of isolates from rainbow trout, in a study of Hayatgheib *et al.* (2021). Thus, preventing and controlling infectious outbreaks, with the increasing antibiotic resistance, makes difficult for fish farmers to treat bacterial infections (Mohanty & Sahoo, 2007; Semple *et al*., 2019).

2. Antimicrobial Peptides as a solution to this problem

This continuing rise of multidrug resistance has led to the development of new, non-antibiotic based, methods to prevent and control outbreaks. Methods in pursuit of this aim include, the use of probiotics, enzymes, oligosaccharides, minerals, herbs, vaccinations, selective breeding, immunopotentiators and novel peptides (Health Canada, 2002). Some of these alternatives, especially immunopotentiators and novel peptides, belong to the family of antimicrobial peptides (AMPs) (Semple *et al.*, 2019).

AMPs, also known as Host Defence Peptides (HDPs), are mostly cationic and amphipathic. They are expressed constitutively, or can be induced by pathogen-associated molecular patterns (PAMPs) and cytokines, during infections or inflammation (Drayton *et al*., 2021). These features make them ideal for the interaction with the negatively charged membranes of microorganisms (Mahlapuu *et al.*, 2016). AMPs were named for the first function discovered for them, antimicrobial activity against viruses, fungi, and bacteria. However, they also have immunomodulatory properties (Mahlapuu *et al.*, 2016; Rodríguez *et al.*, 2021). They are able to protect host tissues against proteases while also regulating innate and adaptive immunity through immune cell activation, chemoattraction and regulation of immunomodulatory molecules like cytokines and complement (Tyrrell, 2023). They have been classified, according to their secondary structure, as: β-sheet, α-

helix, extended and loop with β-sheet and α-helix (Bahar & Ren, 2013), or according to their function, as: membrane disruptive AMPs and nonmembrane disruptive AMPs *(Kang et al.*, 2017). In general, AMPs operate as a first line of defense against an extensive variety of bacteria, including multidrug resistant pathogens (Semple *et al.*, 2019).

3. Pituitary adenylate cyclase activating polypeptide

Pituitary adenylate cyclase activating polypeptide (PACAP), originally discovered as a stimulator of adenylate cyclase activity in ovine pituitary cell cultures (Lugo *et al.*, 2013), is a highlighted member of the wide family of AMPs due to its versatile effects (Semple *et al.*, 2019). The prepro-PACAP is the result of the expression of human PACAP gene, a protein of 176 amino acids, which is metabolized by signal proteases releasing the 25 amino acid signal peptide, and pro-PACAP. This second part is metabolized by pro-hormone convertases and carboxypeptidases to generate a small amino- terminal fragment and PACAP-related peptide (PRP), amino acid residues 82 to 129. The resulting C -terminal peptides are processed by the peptidylglycine alpha-amidating monooxygenase enzymes to produce two C-terminal amidated peptides: one from residues 132 to 170 and other from residues 132 to 159, PACAP38 and PACAP27 respectively *(Hirabayashi et al.*, 2018). Figure 1.1 summarize this pathway. However, according to Li *et al.* (2000), Prohormone Convertase 4 (PC4) is the only processing enzyme of the PACAP precursor in testis and ovary (Li *et al*., 2000).

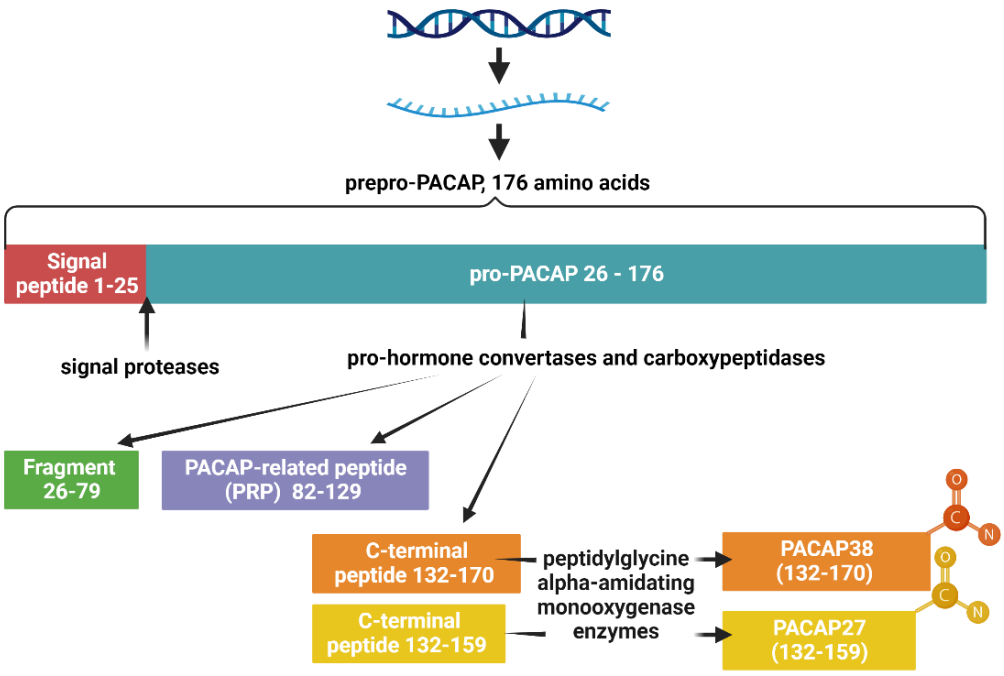

Figure 1.1. Human PACAP expression pathway according to Hirabayashi *et al.*, 2018. Created with BioRender.com

PACAP38 has 100 to 1000 times higher potency than PACAP 27 in processes such as cell proliferation stimulation, DNA synthesis and inositol phospholipid turnover in cells (Semple *et al.*, 2019). PACAP has shown direct antimicrobial activity against Gram-negative and Gram-positive bacteria in mammals and fish (Rodríguez *et al*., 2021). PACAP treatments have also been described to increase the growth rates of fish (Lugo, Carpio, *et al.*, 2010; Lugo, Oliva, *et al.*, 2010), an added value for aquaculture. Furthermore, PACAP has a conserved amino acid sequence between different species (figure 1.2; (Cardoso *et al.*, 2020)), which reduces the risk of being eliminated by the host immune system as a non-self peptide. In addition, the cell penetrating proprieties of PACAP (Debbabi *et al.*, 2018), as well as its impact on bacterial cell wall permeability and on the induction of immune responses (Semple *et al.*, 2019) mean that it would be extremely difficult for bacteria to create resistance to PACAP, making treatments with this peptide an even more environmentally friendly alternative for aquaculture. Also, PACAP stimulates energy metabolism

(Rudecki & Gray, 2016), which is favorable for avoiding antibiotic resistance related to ATP depletion and metabolic collapse, as well as for a sensitization of antibiotics with a metabolic disruptive mechanism (Beam *et al.*, 2021).

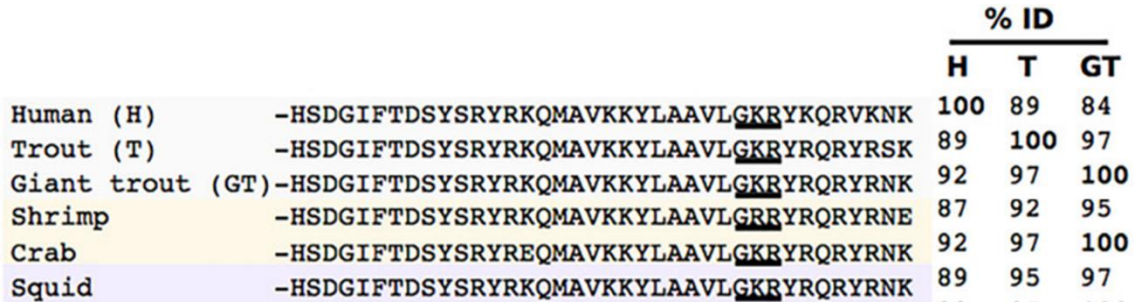

Figure 1.2 Sequence conservation of the invertebrate PACAP mature peptides. Adapted from Cardoso *et al.* (2020)

Summarizing, PACAP is an AMP with potential use in aquaculture, reducing costs through the prevention and control of infection and it is a good candidate for reducing antibiotic resistance problems in fish farming (Rodríguez *et al*., 2021; Semple *et al.,* 2019). Also, rainbow trout culture is an important part of Canadian fisheries (Branigan *et al.*, 2021; *Products & Regions Index*, n.d.) and this species is vulnerable to this pathogen. In addition, Rodríguez *et al.* (2021) previously published that oral administration of PACAP lowers stress compared to delivery by injection or immersion. Thus, the application of PACAP in trout feeding seems to be a promising alternative. Therefore, there are two *hypotheses* to validate in this chapter. The first one is to test *if PACAP peptide analogs have antimicrobial activity with low or no toxicity to host cells*, using cell viability test and minimum inhibitory concentration (MIC) assays. The second hypothesis is to evaluate *if membrane permeability is one of the mechanisms underlying PACAP antimicrobial activity,* by means of bacterial membrane permeability tests.

There are three main *objectives* to assess the research hypotheses. Objective number one is to determine toxicity of PACAP analogs to RTgutGC (rainbow trout intestinal epithelial cell line,

CVCL_DE13), using MTT and Tryan blue assays. Objective number two is to test the antimicrobial effect of PACAP analogs on *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Yersinia ruckeri* (control for non-genus specific antimicrobial activity), using MIC assays. Finally, objective number three is to test the membrane integrity of *A. hydrophila* and *A. salmonicida* cultures incubated with PACAP variants at a high and a low concentration, using the membrane permeability Blacklight kit.

B. Materials and Methods

1. Peptides

Five different peptides were obtained from chemical synthesis. These were the native *Clarias gariepinus* PACAP sequence and four variants modified for stability and a randomized sequence control. These peptides were obtained from Bio Basic with a reported minimum of 95% of purity. Table 1.1 summarizes the name, sequence, and structure of these peptides.

Table 1.1. PACAP analogues synthetic peptides. *Clarias gariepinus* PACAP sequence (PACAP1), three other peptides modified for increased protease stability (PACAP2-4) and a fifth variant that was a scrambled sequence (PACAP5).

PACAP variant name	Sequence
PACAP1 (PACAP38)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
PACAP 2 (PIP3_PCACP)	HS[pipecolic acid-G]IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
PACAP 3 ([N-acetyl-His1,Pip3]PACAP38)	[N-Acetyl-H]S-[pipecolic acid-G]IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
PACAP 4 ([Pip3,Aib16,28,Ala17,Lys34,D-Lys38]PACAP38)	HS[pipecolic acid-G]IFTDSYSRYRK[a-aminoisobutyric acid-A]AVKKYLAAVL[a-aminoisobutyric acid-R]RYRQKFRN(d-K)
PACAP 5 (PACAP38_Random)	AVLGIFTDSRVKYSRYRKQMAFRKYLAGRRYRQHSDNR

2. PACAP analogs toxicity to the RTgutGC cell line:

The toxicity of PACAP analogs was determined by calculating RTgutGC cell viability after exposure to the various peptides, by means of a modification of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay(Mosmann, 1983). Cell viability was also determined by Trypan blue method. Concentrations of PACAP analogs from 3.12 to 200 nM were tested. Triton 1X was used as positive control and cells without treatment (non-treated cells) were considered as 100% viable.

a) MTT assay

Flat-bottomed sterile 96-well culture plates (Thermo-Fisher Scientific, BioLite) were seeded with the RTgutGC cell line (10^4 cells/well) in 100µL of L15 culture media with 2% FBS. Plates were incubated at room temperature for 24h to allow the cells to attach and take the usual morphology. Then, PACAP peptides were added and incubated for 24, 48 and 72 h. Subsequently, at each time point, media was removed, and cell viability was measured by the addition of MTT at a final concentration of 1mg/mL, followed by 4h incubation at room temperature in the dark. The supernatant of the reaction was carefully removed, and formazan crystals were dissolved by the addition of 100µL of DMSO per well for the further quantification in a microplate spectrophotometer (Synergy H1 Hybrid Reader BioTek) by recording absorbance at 540 and 630nm. Data were analyzed using GraphPad software. Each of the two independent experiments was performed in triplicate. The percentage of viable cells was calculated using the following formula:

viable cells (%) = ((O.D. 540nm - O.D. 630 nm of treated cells) / (O.D. 540 nm - O.D. 630 nm of non-treated cells)) × 100.

b) Trypan blue assay

PACAP toxicity was also determined by calculating the cell viability of RTgutGC, by means of a modification of the Trypan Blue Exclusion Test of Cell Viability, (Strober, 1997). Flat-bottomed 96-well microculture plates (Thermo Fisher Scientific) were seeded with the Rtgut cell line (10^4 cells/well) in 100µL of L15 culture media (Cytiva) with 2% FBS. Plates were incubated at room temperature for 24h letting the cells attach and take the usual morphology. Then, PACAP treatments were added and incubated for 24, 48 and 72 h. Afterwards, the cells were washed with PBS and detached with 50 µL of trypsin/well (Multicell). Cells were collected in50 µL of L15 with 10% FBS for trypsin inactivation and a final volume of 100µL. After a dilution 1:2 with trypan blue,

viable cells were quantified with a Neubauer counting chamber in a light microscope, at100X magnification. The percentage of viable cells after treatments was calculated using the following formula:

viable cells (%) = ((number of viable treated cells)/ (number of viable non-treated cells)) × 100. Data were analyzed using GraphPad software. Two independent experiments with triplicates were performed.

3. *A. hydrophila* and *A. salmonicida* growth

Each bacterial species was cultured in trypticase soy agar (TSA) from their respective glycerol stocks, obtained from Dr. Mark Fast's Laboratory in the University of Prince Edward Island. Plates were incubated at room temperature for 48h until colonies were visible. Later, one single colony was transferred to Trypticase soy broth (TSB) and allowed to grow for 24h at room temperature before use in the experiments.

4. Antimicrobial effect of PACAPs in *A. hydrophila*, *A. salmonicida*, and *Y. ruckeri* cultures

The antimicrobial effect of PACAP analogs was tested by determining the lowest concentration at which no bacterial growth was detected (OD_{600nm} = 0; (Semple *et al.*, 2019), by means of the minimum inhibitory concentration (MIC) assay, according to the protocol described by Otvos and Cudic (Otvos & Cudic, 2007a; Semple *et al.*, 2019). Growth inhibition of *A. hydrophila* and *A. salmonicida* were tested after 24 hours of PACAP variants treatments. A single colony of each bacteria previously grown in Tryptic Soy Agar (TSA) was inoculated into 4mL of Tryptic Soy Broth (TSB) and allowed to grow for 24h at room temperature.

After that time, 1mL of the culture was centrifuged at 5 000 g for 5 min and the pellet was resuspended in 4 mL of fresh TSB. Bacteria culture OD_{600nm} was determined and diluted to reach a

final OD_{600nm} of 0.001. 90 µL of bacterial suspension were added to each well of a sterile flat-bottom 96-well plate (Thermo Fisher Scientific BioLite). Later, 10 µL of PACAP variants were added, at final concentrations from 0.05 to 50 µM. This 10ul was replaced by 10mg/mL Ampicillin as positive control for growth inhibition and 1X PBS as negative control. The bacterial growth (OD) was measured after 24h of incubation. PACAP concentrations and controls were tested in triplicates. Growth inhibition percentages were determined using the formula showed below.

Growth inhibition = 100 – ((bacteria+ treatment) OD_{600nm} / (bacteria +PBS) OD_{600nm})

5. Permeabilizing effect of PACAP on the membrane of *Aeromonas* cultures

Both bacteria (*A. hydrophila* and *A. salmonicida*) were growth as described previously and 90uL of bacteria (OD = 0.001) were added per well in a sterile 96-well plate (Thermo-Fisher Scientific BioLite). As positive controls 100 µL of the heat-killed bacteria were added, as a replacement for the live bacteria. Heat killed bacteria was obtained by exposing 1mL of the live culture to 80˚C for 1h. 10 µL of the five different variants of PACAP were added at two different final concentrations (0.1 and 20 µM). Broth without bacteria was used as negative control. Plates were incubated for 24 h at room temperature. Following this, 100 µL of 2X BacLight solution (Thermo-Fisher Scientific, Invitrogen, L13152) was added to each well and plates were incubated in the dark for 15 min. The BacLight kit uses two different dyes, SYTO 9 (6 µM) is membrane-permeable while propidium iodide non- membrane permeable (30 µM). SYTO 9 fluorescence is green and propidium iodide fluorescence is in the red spectrum. Therefore, measurements were taken by reading fluorescence with an excitation of 485 nm and an emission of 530 nm for SYTO 9, and with an excitation of 485 nm and an emission of 630 nm for propidium iodide. Bacterial membrane permeability was calculated as a ratio of 530/630 fluorescence intensities and presented as the green/red fluorescence ratio. Experiments were conducted in triplicate.

C. Results

Due to the potential of applying PACAP as an oral treatment in aquaculture, it is important to test its toxicity to host cells, its direct effect on bacterial cultures as well as the possible underlaying mechanisms of these effects. To accomplish that, the results of experiments intended to test PACAP analogs (PACAPs) toxicity to the RTgutGC cell line, antimicrobial effects of PACAPs in *A. hydrophila* and *A. salmonicida* cultures, and the permeabilizing effect of PACAPs on bacterial membrane of *A. hydrophila* and *A. salmonicida* cultures will be presented in this chapter.

1. PACAP analogs toxicity on RTgutGC cell line

Two experiments were designed to evaluate the cytotoxic effect of the five variants of PACAP (PACAP 1, 2, 3, 4 and 5) on the RTgutGC cell line. MTT assays were conducted at 24h and 72h after treatment with concentrations from 0.78 µM to 50.00 µM of the five variants of PACAP, (Figure 1.2). For the five PACAPs, none of the studied concentrations decreased the RTgutGC cell viability more than a 50% at 24hours of incubation. In addition, for all PACAP variants, only concentrations of 50 µM were able to reduce cell viability to 50% or less, at 72 hours of treatment.

Similar experiments were conducted with lower PACAP concentrations (0.003 µM - 0.2 µM) and no toxic effect was detected. However, in the results of these assays which are included in Figure 1.2, it was interesting to note that cell viabilities rose higher than 100%. To follow up on this, a second experiment with a non-metabolic indicator was performed in parallel with the MTT assay (Figure 1.3), because of the well-defined role of PACAP in increasing cellular metabolic rates (Bozadjieva-Kramer *et al.*, 2021; Inglott *et al.*, 2011; Sherwood *et al.*, 2000).

MTT assays evaluate cytotoxicity based on 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT) reduction to MTT-formazan crystals, a purple precipitate. An indirect indicator of mitochondrial dehydrogenases activity, enzymes that catalyze the reaction. Therefore, this method

has been related with mitochondrial respiration and in general with cellular energy capacity (Chacon *et al.*, 1997; Kuete *et al.*, 2017; Patravale *et al.*, 2012). Alternatively, Trypan blue is a simple method based on the ability of viable cells to exclude the dye. While non-viable cells are permeable to the dye turning the cytoplasm blue (Hussein & Mohsin, 2019; Strober, 1997). Thus, this method is independent of the metabolic activity of the cell.

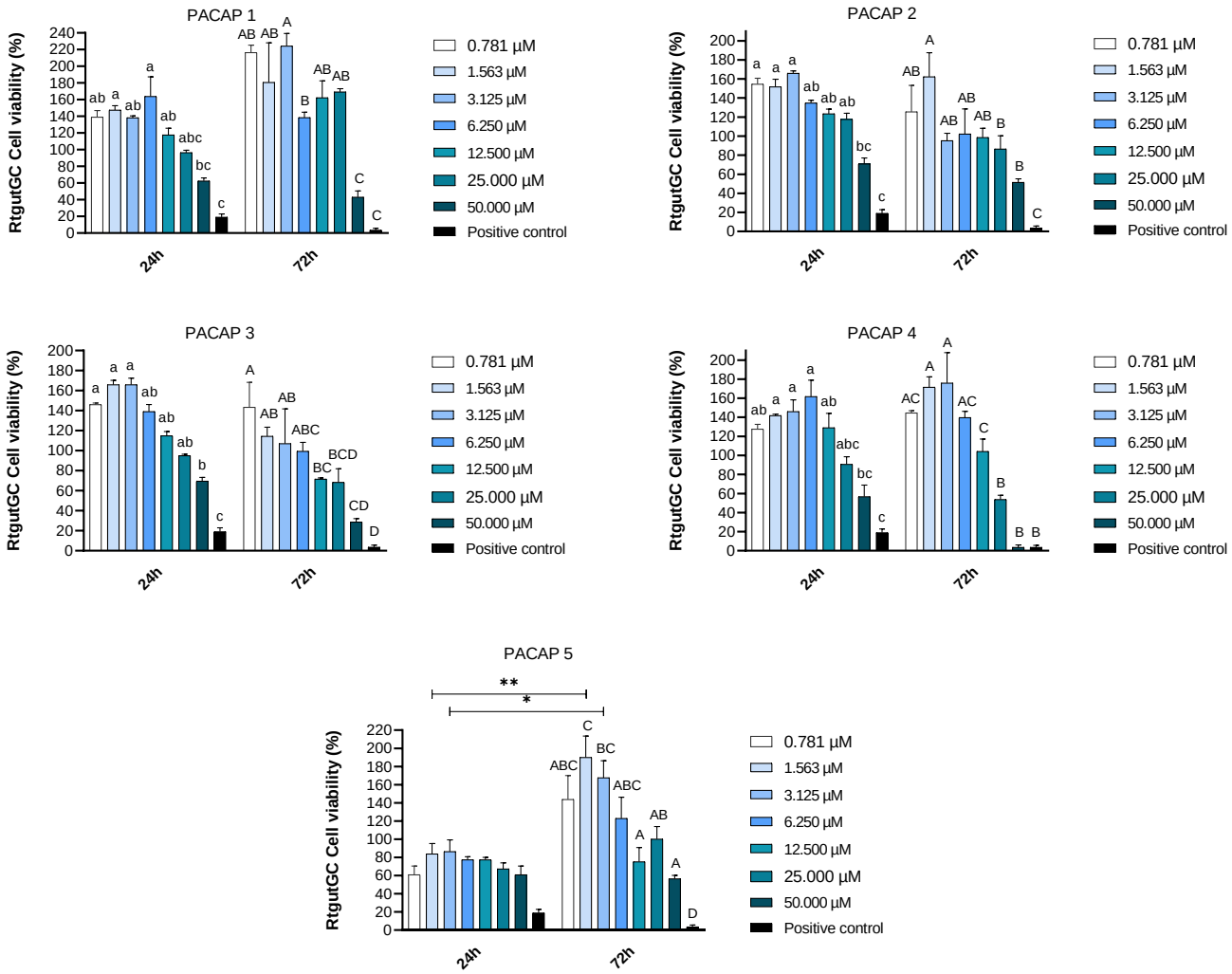

Figure 1.2. PACAP toxicity at 24h and 72h of treatment. Cell viability of RTgutGC in the presence of concentrations from 0.78 µM to 50.00 µM of PACAP 1, 2, 3, 4 and 5. Bars indicate mean and standard error of the mean (SEM) (n=4). Statistical analysis with Two-way ANOVA and Sidak test were developed by using GraphPad Prism 8.0.1. Different letters indicate significant differences between treatment groups while asterisks indicate significant differences in the same experimental group at different time points.

The next experiment was carried out using the two methods in parallel, MTT and Trypan blue assays (figure2). Cells were exposed to concentrations from 10 µM to 45 µM of the five variants of PACAP for 48h. PACAP 1 and 5 caused less than a 50% reduction in viability at the highest concentration evaluated, 45 µM. Moreover, PACAP 2, 3 and 4 were the more toxic with a 50% effect on RTgutGC viability at 25 µM or higher concentrations. In almost all cases it was possible to observe significant differences between both experimental methods.

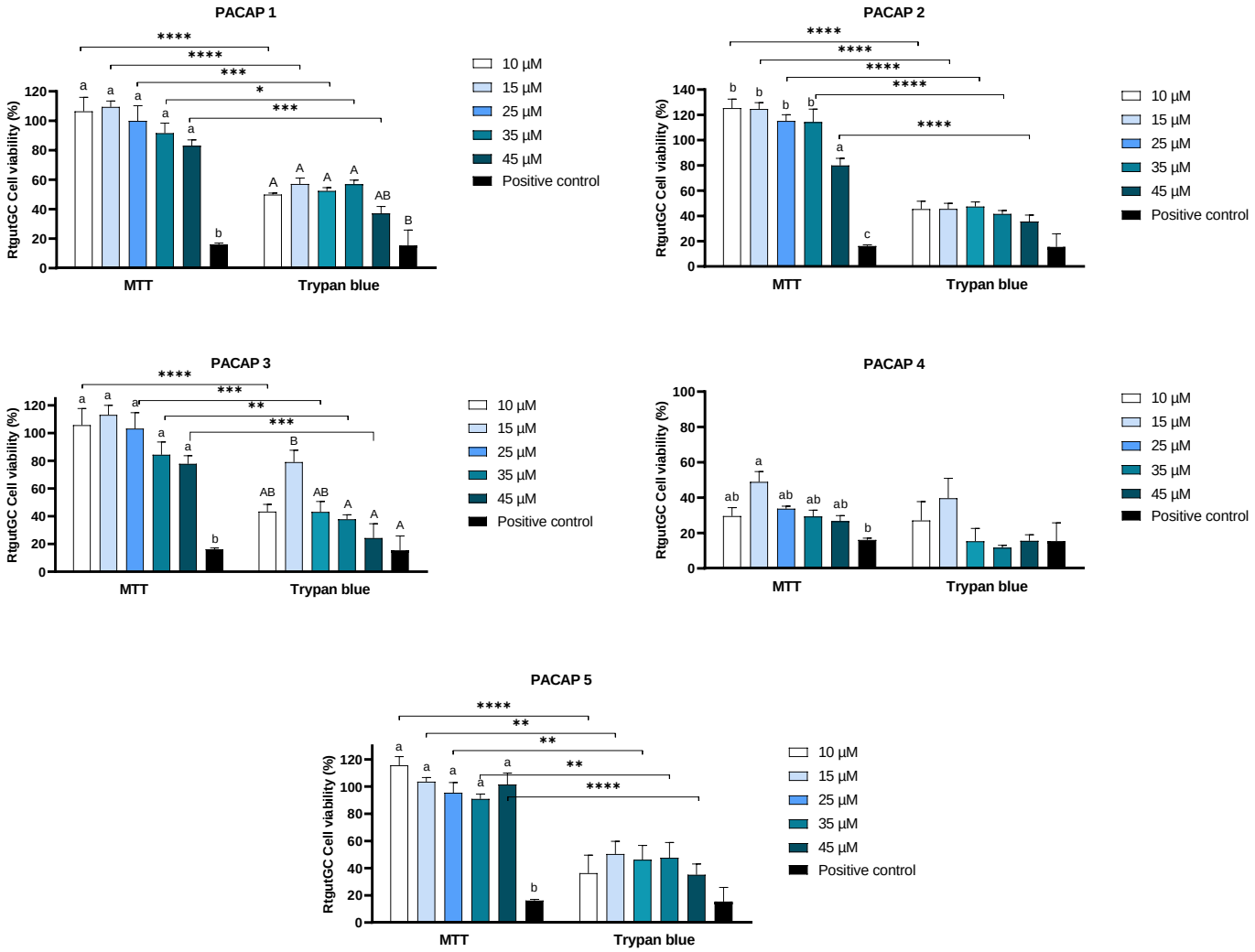

Figure 1.3. PACAP toxicity at 48h of treatment. Cell viability of RTgutGC cell line in the presence of 48h of exposure to concentrations from 10 µM to 45 µM of PACAP 1, 2, 3, 4 and 5. Metanalysis of four different experiments using two different techniques, MTT and Trypan blue. Bars indicate mean and SEM (n=4). Statistical analysis with Two-way ANOVA and Sidak test were developed by using GraphPad Prism 8.0.1. Different letters indicate significant differences between treatments groups while asterisks indicate significant differences in the same experimental group at different time points, $p < 0.05$.

2. Antimicrobial effect of PACAPs in *A. hydrophila*, *A. salmonicida*, and *Y. ruckeri* cultures

After evaluating PACAP toxicity to host cells, the subsequent step was to test for functionality. In order to test the inhibition of bacterial growth due to the direct action of PACAP, *A. hydrophila*, *A. salmonicida*, *Yersinia ruckeri* cultures were incubated with the five variants of PACAP (1 to 50 µM) for 24hours. Figure 1.4 shows the percentage growth inhibition for each bacteria culture. *A. salmonicida* growth was affected by concentrations of PACAP 4 higher than 15 µM with an 80% inhibition of bacterial growth, while for *A. hydrophila* similar results were observed for concentrations higher than 45 µM of PACAP 4. Furthermore, *Y. ruckeri* strain 11 showed 50% growth inhibition with 10 µM of PACAP 2 and 50 µM of PACAP 3 and an 80% growth inhibition at 50 µM PACAP 2. The other variants did not significantly inhibit growth of these bacterial strains and none of the PACAP variants showed any inhibition of *Y. ruckeri* strain 14.

In addition to these results other researchers in the Dixon laboratory have identified higher percentages of growth inhibition for *A. salmonicida*, by using Cytophaga Media Broth (CM) instead of the using Mueller Hinton Broth used here. More than 90% inhibition of bacterial growth was obtained by using CM, with 2.5 µM of PACAP 2, 3 and 4, and 5 µM for PACAP 1 (Rodriguez Cornejo, 2021). Therefore, another experiment was developed to identify factors underlaying these differences.

In general, Cytophaga Media composition is tryptone 0.5g, yeast extract 0.5g, sodium acetate 0.2g, beef extract 0.2g, and distilled water 1.0L, pH 7.2 (ATCC); while Mueller Hinton Broth is composed of beef infusion solids 2g, starch 1.5g, casein hydrolysate 17.5g, and distilled water 1.0L, pH 7.4 (SigmaAldrich, 2018). Also, proteases are virulence factors of bacteria (Ruiz-Perez & Nataro, 2014; Wandersman, 1989). One of the possible explanations for this difference could be the presence of a potential protease inhibitor component in CM, so that the higher activity of PACAP

molecules in CM might be because of an increased half-life of the peptide, due to a lower effect of proteases.

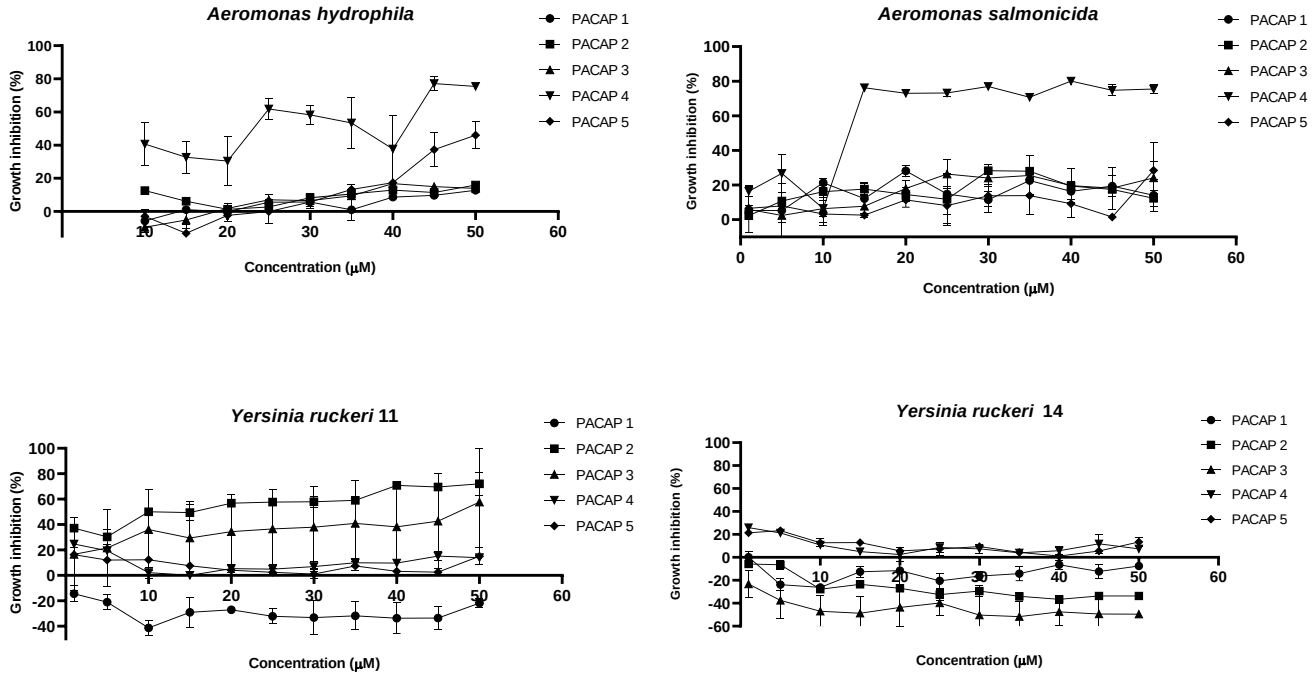

Figure 1.4. Growth inhibition assays. *A. hydrophila*, *A. salmonicida* and two different strains of *Y.ruckeri* (11 and 14) were incubated for 24 hours at room temperature with concentrations of PACAP 1, 2, 3 ,4 and 5 from 1 to 50 µM and the percentage of growth inhibition was evaluated. Mean and SEM of each concentration were graphed. 10mg/mL ampicillin was used as a positive control with a growth inhibition of 85.07 ± 0.2 % for *A.hydrophila*, 82.40 ± 0.4 % for *A.salmonicida,* 86.06 ± 0.2 % for *Y.ruckeri* 11 and 86.31 ± 0.1 % for *Y.ruckeri* 14.

Figure 1.5 shows the bacterial growth of *A. salmonicida* in the presence of two enzymatic inhibitors, Val boroPro and Sitagliptin, a general serine protease inhibitor and a specific inhibitor of DPPIV, respectively. Inhibitors were used at the IC_{50} value reported by Rea *et al.* (2017) as well as at a concentration 3 times higher. While significant differences were observed in the effect of 50 µM of PACAP1 in TSB or CM, no significant differences were determined because of the inhibitors.

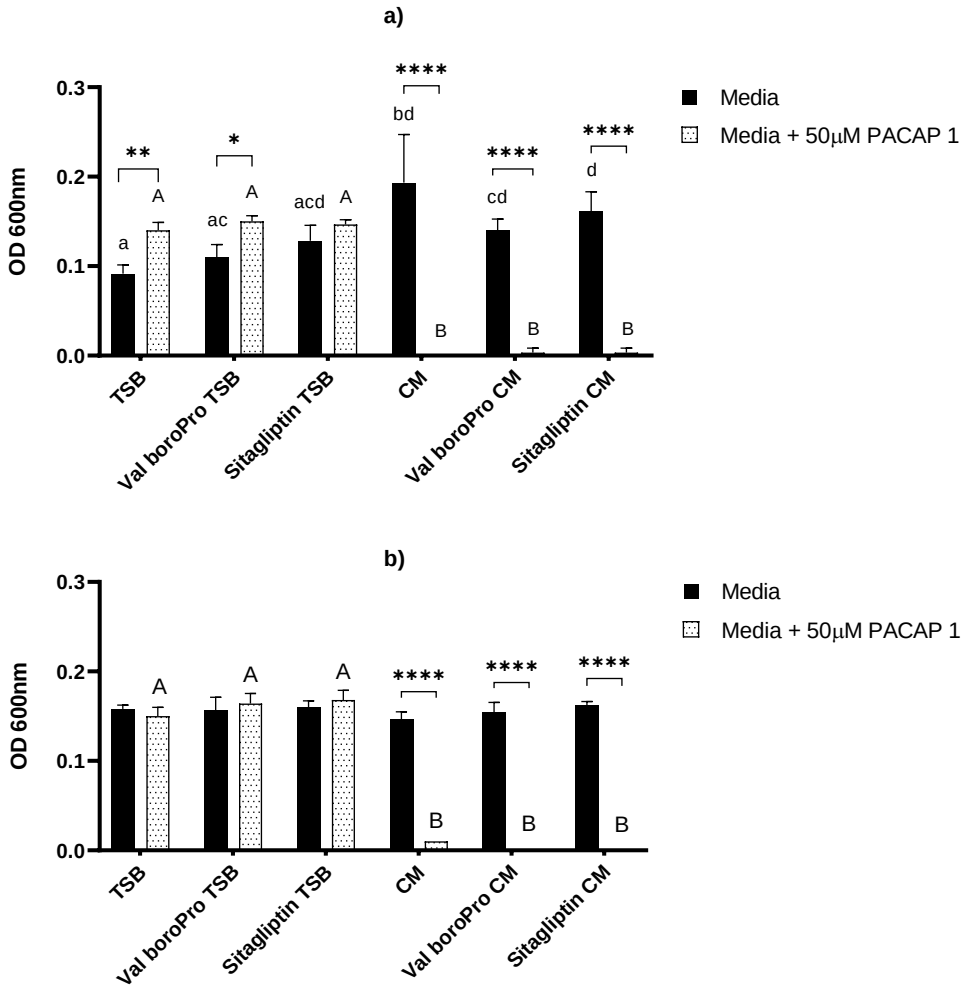

Figure 1.5. Bacterial growth of A. salmonicida in the presence of 0.14 µM Val boroPro and 18 µM Sitagliptin (a) and 0.42 µM Val boroPro and 54 µM Sitagliptin (b). TSB and CM refers to the use of Tryptic Soy Broth and Cytophaga Media in the experiment. Two independent experiments were conducted each one with n=3, mean and SEM were graphed. Two-way ANOVA and Sidak test were carried out using GraphPad Prism 8.0.1. Different letters indicate significant differences between treatments groups while asterisks indicate significant differences in the same experimental group at different time points, $p < 0.05$.

3. Permeabilizing effect of PACAP on the membrane of *Aeromonas* cultures

After testing the direct effect of PACAP on bacteria growth and considering effects on activity by the media, an experiment was designed to find out the possible underlying mechanism of the effect of PACAP on the growth of bacteria. First, bacterial cell wall permeability was tested in *A. hydrophila* and *A. salmonicida* cultures incubated for 24h with the five variants of PACAP and using

both TSB and CM. One low (0.1 µM) and one high concentration (20 µM) of each PACAP variant were evaluated (Figure 1.6).

Membrane integrity was affected in both *Aeromonas* species at the high concentration. Significant differences with the negative control (non-treated cultures / live bacteria) were observed for all five PACAP variants at 20 µM. In addition, in most cases no significant differences were found between PACAPs 20 µM and the positive control (heat - killed bacteria). On the other hand, the lower concentration (0.1 µM) was not able to induce an increase in membrane permeability of *Aeromonas*, with no significant differences with the negative control in the majority of cases.

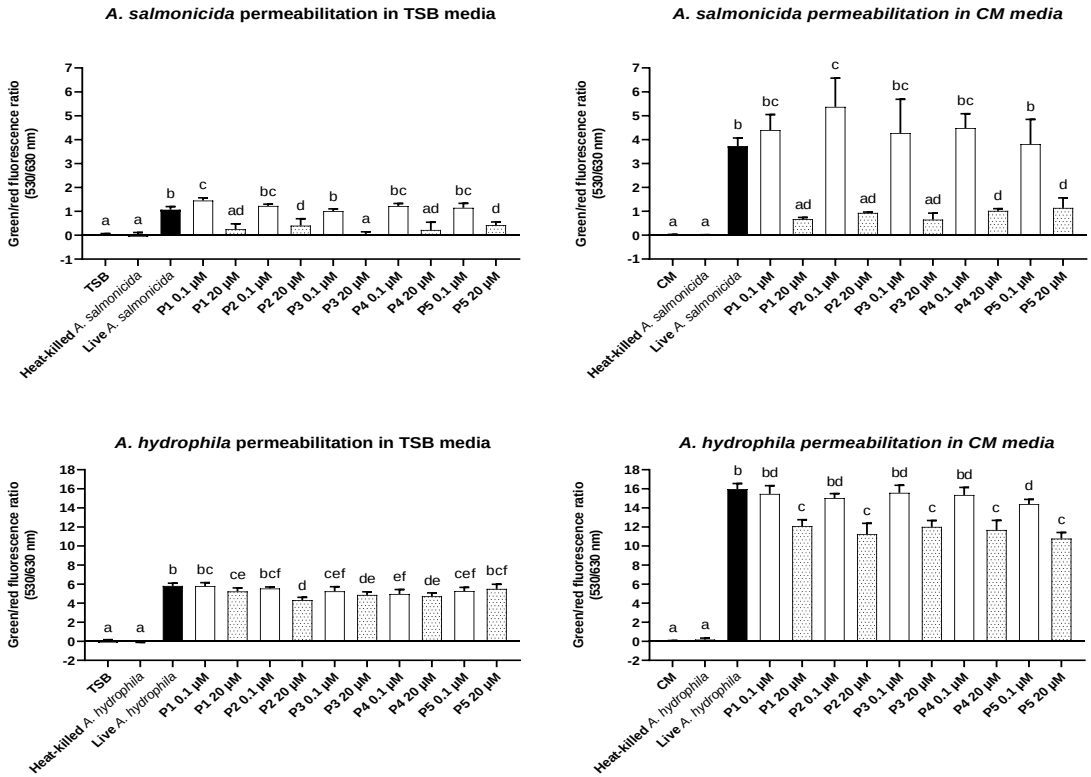

Figure 1.6. Membrane permeabilizing effect of PACAP 1, 2, 3, 4 and 5 (0.1 and 20 uM) on *A. hydrophila* and *A. salmonicida* cultures after 24h of treatment in TSB and CM. Two experiments were developed with n=6. Bacterial membrane permeability was calculated as a ratio of 530/630 fluorescence intensities and presented as the green/red fluorescence ratio. Mean and SEM were graphed. One-way ANOVA and Tukey test were developed by using GraphPad Prism 8.0.1. Different letters indicate significant differences, $p < 0.05$.

D. Discussion

1. PACAP analogs toxicity to the RTgutGC cell line

Antimicrobial peptides usually have short halflives, poor oral bioavailability and can potentially be toxic. In fact, it has been reported that natural or synthetic AMP can disrupt the membranes of different cell types despite differences in composition (Greco *et al.*, 2020). PACAP toxicity was evaluated in RTgutGC cell line by testing cell viability through both MTT cytotoxicity and Trypan blue assays. No toxicity was observed at concentrations from 0.003 µM - 0.2 µM. In addition, at 48 hours of treatment with concentrations of PACAP 1 and 5 lower than 45 µM, the reduction of cell viability percentage was never higher than 50%. On the other side, PACAP 2, 3 and 4 were slightly more toxic with a 50% decrease of RTgutGC viability at concentrations equal or higher than 35µM. These results are in agreement with previous publications in which PACAP toxicity was tested for RTS11 with the same Trypan blue method and the percentage of mortality never reached 50% for concentrations from 0.002 µM to 20 µM (Semple *et al.*, 2019).

The dose or concentration required to kill 50% of the organisms (LC_{50}/LD_{50}) is useful to determine acute toxicity values. Fish feed additives can be classified as category 1 acute or chronic hazard to aquatic environment, when presenting $IC_{50,\ 96h}$ values of lower than 1mg/L, category 2 when presenting $IC_{50,\ 96h}$ values between 1 and 10 mg/L and category 3 when concentrations greater than 10 and lower than 100 mg/L are needed to achieve the IC_{50} effect. Unless there is some reason for concern with the use of the product (category 4), the substance can be classified as safe if it is not included in any of the previous groups (EC: 1272/2008 of the European Parliament and of the Council, 2008).

The concentration intended for use of the additive, or the useful treatment dose are therefore important. For instance, antibiotic treatments are usually at least one order of magnitude below the LC_{50} value for the specific antibiotic. Florfenicol is usually used at 10 mg/Kg, and its LC_{50} value is

higher than 780 mg/Kg for Rainbow trout. Also, oxytetracycline, a broad spectrum tetracycline like antibiotic, is usually administered at a dose between 50-125 mg/Kg while its reported IC_{50} value is more than 4000 mg/Kg (DFO Can. Sci. Advis. Sec. Res. Doc. 2010/017, 2011).

Going further, other authors suggested that one of the described toxic side effects of AMP is hemolytic activity (Greco *et al.*, 2020) and as long as the AMP effective concentration is significantly lower than the one that causes 50% hemolysis in host red cells, the peptide can be considered non-toxic (Semple *et al.*, 2019). It has been reported that concentrations of AMP with hemolytic activity are significantly higher than concentrations necessary to kill bacteria (Matsuzaki, 2009). In addition, hemolytic activity of PACAPs was tested on rainbow trout red blood cells by Rodriguez *et al*. (2021). In this study only 50 µM PACAP 4 induced 20% hemolysis and 50 µM PACAP 1, 2, 3 and 5 caused a 6% of hemolysis, in addition lower concentrations induced no hemolysis (Rodriguez Cornejo, 2021).

Therefore, our results combined with results of other researchers validate the non-toxicity *in vitro* for concentrations lower than 25 µM of all PACAP variants. This is reinforced by the fact that AMP exhibit target selectivity in interactions with prokaryotic cells due to their more negatively charged membrane compared to eukaryotic cell membranes (Matsuzaki, 2009). As suggested by Greco *et al.*, (2020), *in vivo* assays are also important before the use of PACAP in the feeding of animals. However, recent unpublished results of our research team have demonstrated that *in vivo* administration of PACAP 1 fed to 20-70g Atlantic salmon, for 28 days, showed no associated mortality or physical symptoms of toxicity (Mark Fast, personal communication).

Another important role of these experiments was to notice a higher toxicity of the chemical modified PACAP (PACAP 2, 3 and 4). In addition, these experiments were also useful to clarify the importance of using cytotoxicity assays that do not include a metabolic component because of the role of PACAP in cell metabolism. PACAP viability can be overestimated by using assays like MTT, as

was demonstrated, because in almost all cases it was possible to observe significant differences between both experimental methods (MTT and Trypan blue).

2. Antimicrobial effect of PACAPs in *A. hydrophila, A. salmonicida*, and *Y. ruckeri* cultures

C. gariepinus PACAP-38 primary structure, which is the base amino acid sequence for the five PACAP variants used, is a strongly basic peptide with a pI of 11.03 and a highly cationic nature, conferring a positive net charge of 9 at physiological pH (Lugo *et al.*, 2019). PACAP direct antimicrobial activity has been widely reported and is based on a mix of different and synergic mechanisms, including membrane permeabilization, disruption of cellular energetics, and activation of regulated cell death pathways (E. Y. Lee *et al.*, 2021). According to results presented here *A. salmonicida* and *A. hydrophila* growth was affected mainly by PACAP 4, while strain 11 of *Y. ruckeri* cultures were more susceptible to PACAP 2 and PACAP 3.

Previous work from our research group also showed a higher effect of the PACAP variants used here, in the inhibition of *A. salmonicida* and *Y. ruckeri.* They found a 90% or higher inhibition of bacterial growth by using 2.5 µM for PACAP 2, 3 and 4, and 5 µM for PACAP 1. They also observed *Y. ruckeri* growth inhibition higher than 90% by using 50 µM of PACAP 2 or 4 (Rodriguez Cornejo, 2021). Differences in the previous experimental procedure included the use of a different media for the evaluation of the bacterial growth inhibition (Cytophaga media). Other experiments conducted with Tryptic Soy Broth (TSB) showed similar results to those obtained here using Mueller Hinton Broth (MHB). MHB is the media recommended for determining minimal inhibitory concentrations (MICs), specially when working with antimicrobial peptides due to the possible inactivation of the peptide with salts (Otvos & Cudic, 2007b). However, similar results were also obtained by Lugo *et al*., (2019), as they observed that 300 µM PACAP is active against both Gram-negative and Gram-positive bacteria. Lugo *et al.* also mentioned *A. salmonicida* was sensitive to PACAP (the variant 1

peptide in this thesis). They also observed susceptibility in *Y. ruckeri* and *A. hydrophila*, cultures with IC_{50} values of 3.125, 7.2 and 0.023 µM for *A. salmonicida*, *A. hydrophila* and *Y. ruckeri*, respectively (Lugo *et al.*, 2019). Differences in results could be due to the use of different bacteria strains but could also be related to the media selected for the determinations. Trying to understand these differences another experiment was developed, with the hypothesis: the differential effectiveness of PACAP relies on its half-life, due to the presence of possible different protease inhibitors in the CM compared to TSB/MHB.

Pathogenic gram-negative bacteria can secrete enzymes into the periplasm, outer membrane, or external environment through different secretion pathways (Ruiz-Perez & Nataro, 2014). Some of these extracellular secreted proteases are toxins or factors involved in virulence, while others exhibit low specificity and degrade proteins to produce small peptides or amino acids which can be utilized by several bacteria (Wandersman, 1989). Serine proteases are the most abundant and functionally diverse proteolytic enzymes usually secreted by gram-negative bacteria and these are implicated in virulence (Ruiz-Perez & Nataro, 2014). Serine protease secretion has been reported in *A. salmonicida* and *A. hydrophila* and *Yersinia pestis* among other proteases secreted (Nieto & Ellis, 1986; Pemberton *et al.*, 1997; Tobback *et al.*, 2007). After testing bacterial growth of *A. salmonicida* in the presence of two serine inhibitors (Val boroPro and Sitagliptin) at their IC_{50} values Rea *et al.* (2017) and at a concentration 3 times higher, significant differences in the bacterial growth using 50 µM of PACAP1 in TSB or CM were seen. However, no significant differences were determined after the use of the inhibitors. This suggests that serine protease secretion may not occur in *A. salmonicida* cultures under these experimental conditions or that serine proteases do not affect PACAP 1 halflife. Similar experiments were used to test the effect of the presence of sodium acetate (NaAc) in CM but no significant differences were observed after adding NaAc to TSB or

removing NaAc from CM. These results suggest that PACAP 1 functional differences are not due to the presence of NaAc in the media.

3. Permeabilizing effect of PACAPs on the membrane of *Aeromonas* cultures

Bioinformatic and structural analyzes have shown possible membrane permeabilizing properties of PACAP. Induction of negative Gaussian curvature in bacterial membranes is a requirement for membrane-penetrating antimicrobial processes, like pore formation, blebbing, and most of others membrane perturbing events. Synchrotron X-ray scattering analysis indicated that PACAP can induce negative Gaussian curvature (E. Y. Lee *et al.*, 2021). PACAPs permeabilizing effect on *A. hydrophila* and *A. salmonicida* cultures showed significant decrease in green/red (SYTO 9 /propidium iodide) fluorescence ratio for bacteria cultures treated with of 20 µM of PACAPs compared to non-treated cultures. Both fluorophores are able to emit when bound DNA and RNA but different to SYTO9, PI only permeates dead or damaged cells. Therefore, higher ratios green/red indicate poor permeabilizing effect (low propidium iodide bound to DNA or RNA) while lower ratios are indicative of a strong permeabilizing effect (Deng *et al*., 2020). PACAPs at concentrations of 20 µM and higher exert a permeabilizing effect for both bacteria. However, 0.1 µM was not enough to induce membrane permeability. Other researchers also found that 0.1 µM of PACAP 1, was not sufficient to cause a permeabilizing effect in *Flavobacterium psychrophilum* cultures and that higher doses such as 30 or 50 µM can induce a level of membrane permeabilization comparable to that observed for the heat-killed bacteria using *F. psychrophilum* (Semple *et al.*, 2019).

Also, there seems to be a slightly higher basal permeability of *A. salmonicida* to propidium iodide than *A. hydrophila*. In addition, this study confirms that minimal growth medium or media with lower salt content like CM (0.85% saline solution) are better to use than rich media like TSB. TSB contains components that fluoresce following staining with Syto9 and PI. TSB especially

increases Syto9 basal fluorescence by almost two orders of magnitude (Robertson *et al.*, 2019). Therefore, an attenuated green/red signal was observed after binding of the dyes in the results obtained using TSB. There were also ratios lower than one for heat killed bacteria, positive control of permeability. That is due to the slightly higher affinity of PI for nucleic acids than Syto9, which displaces the latter and therefore decreases the signal (Stiefel *et al.*, 2015). The signal of green/red ratio can also be decreased due to the quenching phenomenon of SYTO9 emissions caused by fluorescence resonance energy transfer to PI (Stocks, 2004)

Overall, these results show that membrane permeability is one of the mechanisms of direct antimicrobial activity of PACAPs. In addition, there were significant differences between culture media employed; PACAPs seems to have a higher permeabilizing effect in CM than in TSB.

E. Conclusions

The five variants of PACAP evaluated showed no toxicity at concentrations lower than 25 µM *in vitro* and can be considered save for use in aquaculture, especially PACAP 1 and 5. Experiments in this chapter also proved that PACAP toxicity and the toxicity of other AMP with actions on cell metabolism can be misestimated by using MTT assays. In addition, it was observed that some of the PACAP variants are able to inhibit *Aeromonas* and strain 11 of *Y. ruckeri* growth by direct antimicrobial action, but certain conditions can modulate this activity. For instance, the direct antimicrobial effects of PACAPs are dependent on the culture broth used, PACAP 1 functionality did not differ due to the presence or absence of NaAc in the broth, and serine protease secretion does not occur in *A. salmonicida* cultures, or it does not affect PACAP 1 half-life. Furthermore, findings of the experiments in this chapter also clarified the mechanism of action of PACAP peptides, demonstrating that membrane permeability is one of the mechanisms underlying the direct antimicrobial activity of PACAPs.

II. Chapter 2: PACAP analogs with potential immunostimulant application in aquaculture.

A. Introduction

1. Antibiotics and resistant bacterial strains generation

In the early 20th century, the use of antibiotics represented a significant shift for society, with an increase in the quality and length of life (Rocha-Granados *et al.*, 2020). The clinical use of antibiotics has allowed human life expectancy to be extended from an average of 56.4 years to an average of nearly 80 years in the Unites States, with similar effects worldwide (Ventola, 2015). However, many factors, such as the prolonged use of antibiotic treatments, have led to the emergence of antibiotic resistance in pathogenic bacteria for both fish and humans (Rocha-Granados *et al.*, 2020). Clinically re-emergent infections and treatment failures due to antibiotic resistance of pathogenic bacteria are a huge problem in different sectors. Human recurrent infections are reported to cost about $20 billion USD per year in United States with an estimated of 10 million annual deaths predicted by 2050 (O'Neill, 2014; Rocha-Granados *et al.*, 2020). In addition, global loses in aquaculture are in the order of billions each year due to disease outbreaks (Akazawa *et al.*, 2014).

In general, the specific mechanism of action of the specific antibiotic or bacterial genetic plasticity are responsible for antibiotic resistance. Antibiotics can lead to bacterial resistance by inducing the selection of specific mutations or increasing the mutation rate. Resistance-conferring alleles can be inherited vertically and usually facilitate bacterial replication in the presence of antibiotics (Rocha-Granados *et al.*, 2020). In addition to vertical gene transfer, horizontal gene transfer is also possible. Antibiotic-resistant genetic determinants can be found on mobile genetic elements easily exchangeable among bacteria sharing the same ecological niche (Riesenfeld *et al.*, 2004). Therefore, it is very important to consider the host immune system as a target to avoid or overcome this problem. The innate immune response is the first line of defense in solving infections.

2. Immune system fighting bacterial infections

Macrophages are innate immune cells that conduct phagocytosis and antigen-presentation (Linnerz & Hall, 2020). These cells help in the clearance of pathogens and maintain tissue homeostasis by triggering inflammatory responses and engulfing dead cells (Wu & Lu, 2019). Phagocytosis is the cellular process of engulfing particles of more than 0.5 µm of diameter and these particles usually refers to microbes or cellular debris. (Jain *et al.*, 2019). Phagocytosis can be initiated by opsonins or by the direct recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Y. Zhang *et al.*, 2022). Pathogens are captured by membrane evaginations of the phagocyte, called pseudopodia, and are internalized in a phagosome. Once bacteria are phagocytosed, lysosomal enzymes, antimicrobial peptides, and a flood of ROS kill most of the ingested pathogens (Hommes & Surewaard, 2022) as this phagosome fuses with a lysosome becoming a phagolysosome with a low internal pH. The low pH activates some lysosomal enzymes able to kill and digest bacterium. Finally, the products of the enzymatic digestion are released to the extracellular space (Punt *et al.*, 2007). Professional phagocytes, as macrophages, have an important role in eliminating infections (Hommes & Surewaard, 2022).

There are also other very important low molecular weight glycoproteins, called cytokines, involved on pathogen clearance and in general, on the defence mounted by the immune system. They are secreted by cells of both the innate and acquired immune systems (Sakai *et al.*, 2021). Cytokines regulate the interaction and communication between cells (J.-M. Zhang & An, 2007) through specific receptors on the surface of the target cells (Sakai *et al.*, 2021). They are pleiotropic, meaning that different cell types can secrete the same cytokine and, one cytokine can act on different cell types (J.-M. Zhang & An, 2007). Cytokine function can also be redundant. Different cytokines can stimulate similar function and they can also stimulate their target cell for a positive regulation of its own secretion (J.-M. Zhang & An, 2007).

Hundreds of types of cytokines have been found in humans and most of them have been also identified in fish (Sakai *et al.*, 2021; Zou & Secombes, 2016). The main five cytokine families described are β-Trefoil Cytokines (interleukin (IL)-1 family), type I α Helical Cytokines (IL-2 family, IL-6 family), type II α-helical cytokines (IL-10 family, interferon family), Cysteine Knot Cytokines (IL-17 family) and B-Jellyroll Cytokines (TNF family) (Sakai *et al*., 2021; Zou & Secombes, 2016). Members of all these families have been found in fish (Savan & Sakai, 2006).

The interleukin (IL)-1 family, also known as β-Trefoil Cytokines, play a critical role in regulating inflammation (Schmitz *et al.*, 2005; Sims *et al.*, 2001), and comprises 11 cytokines: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-36 receptor antagonist (IL-36Ra), IL-36α, IL-37, IL-36β, IL-36γ, IL-38 and IL-33. The family can be subdivided according to its function as suppressors of inflammation: IL-1Ra, IL-36Ra, IL-37 and IL-38, or promotors of inflammation, which is the rest of them (Zou & Secombes, 2016).

In addition, B-Jellyroll Cytokines, also called tumour necrosis factor (TNF) superfamily are usually type II membrane proteins with a short N-terminal intracellular domain, a transmembrane domain, and a C-terminal extracellular domain with a conserved family motif. However, some of them can be released as soluble forms following enzymatic cleavage. The main members of this family are TNF-α, lymphotoxin (LT) -α (also called TNF-β) and LT-β (Zou & Secombes, 2016).

Cysteine Knot superfamily cytokines include the IL-17 and Transforming Growth Factor (TGF-β) subfamilies. This superfamily owes its name to the presence a characteristic beta core domain rich in disulphide bonds (Zou & Secombes, 2016). In addition, Type I α Helical Cytokines superfamily include IL-2, IL-6 and IL-12 subfamilies. The type II α-helical cytokines include the IL-10 and interferon subfamilies (Sakai *et al.*, 2021; Zou & Secombes, 2016).

3. PACAP as immunostimulant

Pituitary adenylate cyclase activating polypeptide (PACAP) has three main receptors: vasoactive intestinal polypeptide receptor 1 (VPAC1), vasoactive intestinal polypeptide receptor 2 (VPAC2) and pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1). The broad tissue distribution of these receptors suggests that PACAP is involved in many functions like the regulation of antimicrobial activity, growth, neural development, anti-tumor activity, metabolism, immunomodulation, among others (Velázquez *et al.*, 2020a). As for the immunomodulatory role of PACAP, this peptide has shown pro-inflammatory and anti-inflammatory properties in mammals through the modulation of T helper type 1 (TH1) and type 2 (TH2) cytokine production (Rodríguez *et al*., 2021).

Interaction of PACAP with its receptors has been strongly correlated with its immunological activity (Campbell *et al.,* 2023). All three receptors have similar pathways of activation. The slight differences are only related to the conformational changes, involving TM helix deformations, that take place for G-protein binding (macroswitches), and ligand-receptor binding (microswitches) (Campbell *et al.*, 2023; Langer *et al.*, 2022). Lugo *et al.* (2011) studied PACAP receptors distribution in trout tissues and found that PAC1 and VPAC1 are mainly constitutive receptors of macrophages, while VPAC 2 expression is dependent of LPS stimulation and specific of tissues with a role as first barrier of defense, like skin and gills in fish, where VPAC1 was not detected. Accordingly, the RTS11 cell line was observed to express PAC1 and VPAC1 but not VPAC2 expression (Lugo *et al*., 2011). Aside, even when there are no records of the expression of these receptors in RTgutGC cell line, there are reports of the presence of VPAC1, VPAC2 and PAC1 in gut (Lugo *et al*., 2011; Montpetit *et al.*, 2003).

Therefore, this chapter addresses the immunological role of PACAP in trout. With this aim two *hypotheses* were generated. First hypothesis is that *if PACPA1 and 5 modulate the expression of*

cytokine genes and proteins in rainbow trout cell lines, then PACAP is an immunomodulator of trout immune system. Going further, this immunomodulation should be useful also in the *in vivo* response of trout to bacterial infections. The second hypothesis generated is that *if there are specific amino acid residues of PACAP peptide that led PACAP-receptor interactions then these residues should be related with its immunological function.* This second hypothesis was accomplished trough *in-silico* modelling.

For accomplishing these hypotheses, three main objectives were generated. Objective number one is to measure, via RT-qPCR, cytokines genes relative expressions in RTgut (rainbow trout intestinal epithelial cell line) and RTS11 (rainbow trout monocyte / macrophage - like cell line) cells pre-treated with PACAP 1 and 5, and exposed and non-exposed to *A.salmonicida*. The second objective is to quantify IL1-β and IFNγ protein concentrations in the conditioned media of RTgut and RTS11 cells pre-treated with PACAP1 and 5 and exposed and non-exposed to *A. salmonicida*, using ELISA assays. Objective number three is to identify residues of PACAP specifics for receptors interaction via *in silico* modelling of the PACAP-receptor binding.

B. Materials and methods

1. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

a) Experimental design, RNA extraction and cDNA synthesis

RTgutGC and RTS11 adherent cells were plated on six 12-well plates per cell line (3×10^5 cells/well) using L15 media supplemented with 10% FBS and 1% polymyxin / streptomycin. Cells were kept at room temperature for 24h – 48h allowing cells to attach to the plate. After that, the supernatant was removed and PACAP 1 and 5 were added in L15 supplemented with 2%FBS, for a final concentration of 200 nM. HSP (200 nM) and an equal volume of 1X PBS was added as control treatment (a non-related peptide and a no peptide group). HSP in this thesis refers to HSP70 peptide (inducible heat shock protein 70), and it was used as a non-related peptide control.

After 24 h of treatment three of the six plates were infected with *A. salmonicida*, for a multiplicity of infection (MOI) of 0.1. Cells were kept at room temperature for 24h, 48h and 96h. At those time-points supernatant from two plates (infected and non-infected) was collected, and cells were lysed for RNA extraction and cDNA synthesis. RNA was extracted using an RNeasy RNA Extraction Kit (Qiagen) as described by the manufacturer (RNeasy Kits, n.d.). In addition, any genomic DNA contamination was removed by treating all RNA samples with DNase I (Thermo-Fisher Scientific). 250 ng of RNA, quantified by using Take3 Trio^{TM} Micro-Volume Plate (BioTek), were used per sample for complementary DNA (cDNA) synthesis using the qScript cDNA Supermix (Quanta Biosciences) in a final volume of 10µL. The program used for cDNA synthesis was: 25˚C for 5 min followed by 42˚C for 30 min, then 80˚C for 10min and finally a cooling step at 4˚C, and the program was run in a Thermal Cycler (C 1000 Touch, BioRad). Then, cDNA was stored at -80˚C for further use in qPCR reactions.

b) qPCR reactions

IL-1β, TNFα, IL-6, MyD88, TGFb and PACAP genetic expression were assessed by RT-qPCR (Green & Sambrook, 2018; Soto-Dávila *et al*., 2020). 12.5 ng of cDNA (2.5 µL) from each sample was added to the reaction mix which contained 0.5µM of each primer (2.5µL) (Sigma Aldrich) and 5µL of master mix (Wisent-1), for a final volume of 10µL per well. All qPCR reactions were run on a LightCycler® 480 II (Roche), following the program settings of: pre-incubation at 95˚C for 2 min followed by 40 cycles of 43 sec (denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 8 sec), then 1 cycle melt of around 1 min (95˚C for 1 sec, 65˚C for 1 min) to continue with the last cycle of cooling at 60 ˚C for 2 min. Primer sequences are outlined in Table 2.1. Water controls were added to each plate in triplicate and samples were also run in triplicates. Primer product specificity was determined through single peaks on PCR melting curves. qRT-PCR data was analyzed with the method of Riedel *et al.* (Riedel *et al.*, 2014) for analyzing three housekeeping genes (*β-actin*, *18S* and *its*). This methodology is an extension of the usual ΔCT calculation method, to include the analysis of multiple housekeeping genes (reference genes). It allows the reduction of variability and stabilisation of reference, due to the lower probability that all reference genes are co-regulated in the same experimental design. This procedure is based on the calculation of the arithmetic mean of the CTs of multiple reference genes, three in this case. Therefore, it leads to more accurate determinations of the expression level of the target genes. Data is presented as the average of $\log_2$ (Fold Change) of three experimental replicates of two experiments. The efficiency of reference genes was 96.70%, 99.1% and 92.3% for β-actin, 18S and ITS, respectively.

Table 2.1. Primers used for RT-qPCR studies

	Nucleotide sequence		
	FW	**RV**	**Reference**
β-actin	TGGACTTTGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG	(Ma *et al.*, 2019)
18S	CGTCGTAGTTCCGACCATAAA	CCACCCACAGAATCGAGAAA	(Giroux *et al.*, 2019)
its	TCATCAATCGGAACCTCTGG	AAGGAAGAGCGCACGGG	(Eder *et al*., 2009)
tgfb	TGTGGGGAGACAACACAAGG	AAACCAGCGCCATCAAAAAGG	Soto-Dávila (unpublished)
il1b	CCACAAAGTGCATTTGAAC	GCAACCTCCTCTAGGTGC	(Semple *et al.*, 2018, 2019)
tnfa	CGGACTCCATCGGGGTTAAT	GACTCAGCATCACCGTAGTTTTG	Soto-Dávila (unpublished)
myd88	GACAAAGTTTGCCCTCAGTCTCT	CCGTCAGGAACCTCAGGATACT	(Skjesol *et al.*, 2011; Wang *et al.*, 2020)
il8	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	(Hynes *et al.*, 2011)
pacap	AAATTGCTATAAGAAGTCCCCCATC	GTATTTCTTGACTGCCATTTGCTTT	(Lugo *et al.*, 2011)
ifny	GAAGGCTCTGTCCGAGTTCA	TGTGTGATTTGAGCCTCTGG	(Chaves-Pozo *et al.*, 2010)

2. Enzyme-Linked Immunosorbent Assays (ELISA)

Filtered culture supernatant was used for quantifying IL-1β and IFN-γ cytokines through quantitative sandwich ELISA (Abram *et al.*, 2019; Dixon *et al.*, 2018). 100 µL of capture antibody against the specific rainbow trout cytokine, diluted in carbonate/bicarbonate coating buffer (pH 7.4) to a final concentration of 2µg/mL, was added to each well of a 96 well plate (Immulon 4HBX, Thermo Fisher Scientific) and plate was incubated 4h at room temperature. Then, the plate was washed three times with 300 µL of TBS-Tween (0.1%) (the same wash procedure was repeated after each following incubation step in this protocol, otherwise is specified). 300µL per well of a blocking solution (5% skim milk in TBS) was added and plate was incubated for 1h at 37˚C. After that, 100 µL of samples and standard curve with the recombinant protein (IL-1β or IFN-γ) was added to the corresponding wells and incubated overnight at 4˚C (Standard curves were made with dilutions of the corresponding recombinant protein in 2% FBS L15 to reach a final concentration in a range from 6.25 - 400 pg/mL and each dilution was plated in triplicate).

Subsequently, 100 µL of the detection antibody (primary Ab) against rainbow trout cytokines IL-1β or IFN-γ were added, diluted in 5% skim milk TBS-Tween solution to a final concentration of

1µg/mL and plate was incubated for 3h at room temperature. 100µL of the corresponding secondary antibody conjugated with biotin was added in a 5% skim milk TBS-Tween solution to a final concentration of 0.75 µg/mL and was incubated for one hour. Then, 100 µL of horseradish peroxidase-conjugated streptavidin, Streptavidin-HRP (BioLegend), diluted in 5% skim milk TBS-Tween up to a final concentration specified for each case in the Table 2.2 below, were added to each well and pate was incubated for 1h in the dark. After that, 100 µL of the substrate, TMB (Plus2® ECO-Tek) were added to the plate, and it was incubated at room temperature for 30 minutes in the dark. Without any washing step, 100 µl of 0.3M of H_2SO_4 was added to stop the reaction and absorbance at 450 nm was immediately measured by using the Synergy H1 plate reader (BioTek Instruments).

Table 2.2. ELISA antibodies

Cytokne to detect	**IL1β**	**IFN-γ**
Capture antibody	Chicken anti-IL-1β antibody (Cedarlane)	Rabbit anti - IFNγ antibody (Cedarlane)
Primary antibodies	Rabbit anti-IL-1β antibody (Cedarlane)	Chicken anti - IFNγ antibody (Cedarlane)
Secondary antibodies	Biotin-conjugated goat anti-rabbit antibody (BioRad). Final assay concentration - 0.5 µg/mL	Biotin-conjugated goat anti-chicken antibody (BioRad). Final assay concentration - 0.25 µg/mL

3. *In silico* study of PACAP – receptor interactions

An analysis of PACAP – receptor interaction was developed. PACAP sequences of *Clarias gariepinus* and *Oncorhynchus mykiss* as well as Alpha Fold prediction of Vasoactive intestinal polypeptide receptor type 1 (VPAC1), Vasoactive intestinal polypeptide receptor type 2 (VPAC2) and Pituitary adenylate cyclase activating polypeptide receptor (PAC1) were obtained from UniProt server (*UniProt*, n.d.). Table 2.3 summarizes sequences used in the analysis.

Table 2.3. Sequences used for the analysis.

	UniProt ID or sequence used
PACAP *Clarias gariepinus*	HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
PACAP *Oncorhynchus mykiss*	Q75W87
PAC1 receptor	Q64FL5
VPAC1 receptor	Q64FL3
VPAC2 receptor	Q64FL4

ChimeraX program (developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases) was employed for the modeling of interactions of both PACAP and the three different PACAP receptors (Goddard *et al.*, 2018; Pettersen *et al.*, 2021) following the methodology described by Mirdita *et al.* (2022) (Mirdita *et al.*, 2022). AlphaFold modeling was employed for the 3D prediction of receptors and PACAP.

C. Results

After considering the direct antimicrobial effects of PACAP, its effect on the fish immune system must still be examined. In this chapter experiments tested PACAP as an immunostimulant. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) assays were developed to measure the expression of immune genes in RTgutGC and RTS11 cultures. For both cell lines analyzed the amount of RNA extracted from the cells was decreased after 24h of infection with *A.salmonicida*. Enzyme-Linked Immunosorbent Assays (ELISA) were also used to test IL-1b and IFNγ protein expression. Additionally, a PACAP – receptor in-silico study was conducted to compare the interaction of PACAP with its three cellular receptors to find which amino acids have high probability of participating in the immunostimulatory function of PACAP.

1. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) on RTgutGC

Gut functionality is not restricted to digestion, but also includes nutrient absorption, sensing, electrolyte balance, hormone secretion and immune response (Cain & Swan, 2010; P.-T. Lee *et al.*, 2021). In addition, prophylactic oral administration of immunostimulants in finfish aquaculture has been shown to increase in the growth of fish and strengthen gut immunity (P.-T. Lee *et al.,* 2021). Therefore, experiments were conducted to test the expression of immune genes in the RTgutGC cell line (Figures 2.1 and 2.2).

After 24h of treatment with 200 nM PACAP 1 and 5, *il1b* transcript expression was significantly downregulated after PACAP 1 treatment compared to the control groups (PBS, HSP). Also, *il8* expression was downregulated by PACAP 1 treatment, and *tgfb* expression upregulated by PACAP 5 treatment (figure 2.1).

After 96h of treatment with 200 nM of PACAP 1 and 5 followed by 72h of bacterial exposure, significant differences were observed between PACAP 1 treatment with and without bacterial exposure for the *il8* and *pacap* genes.

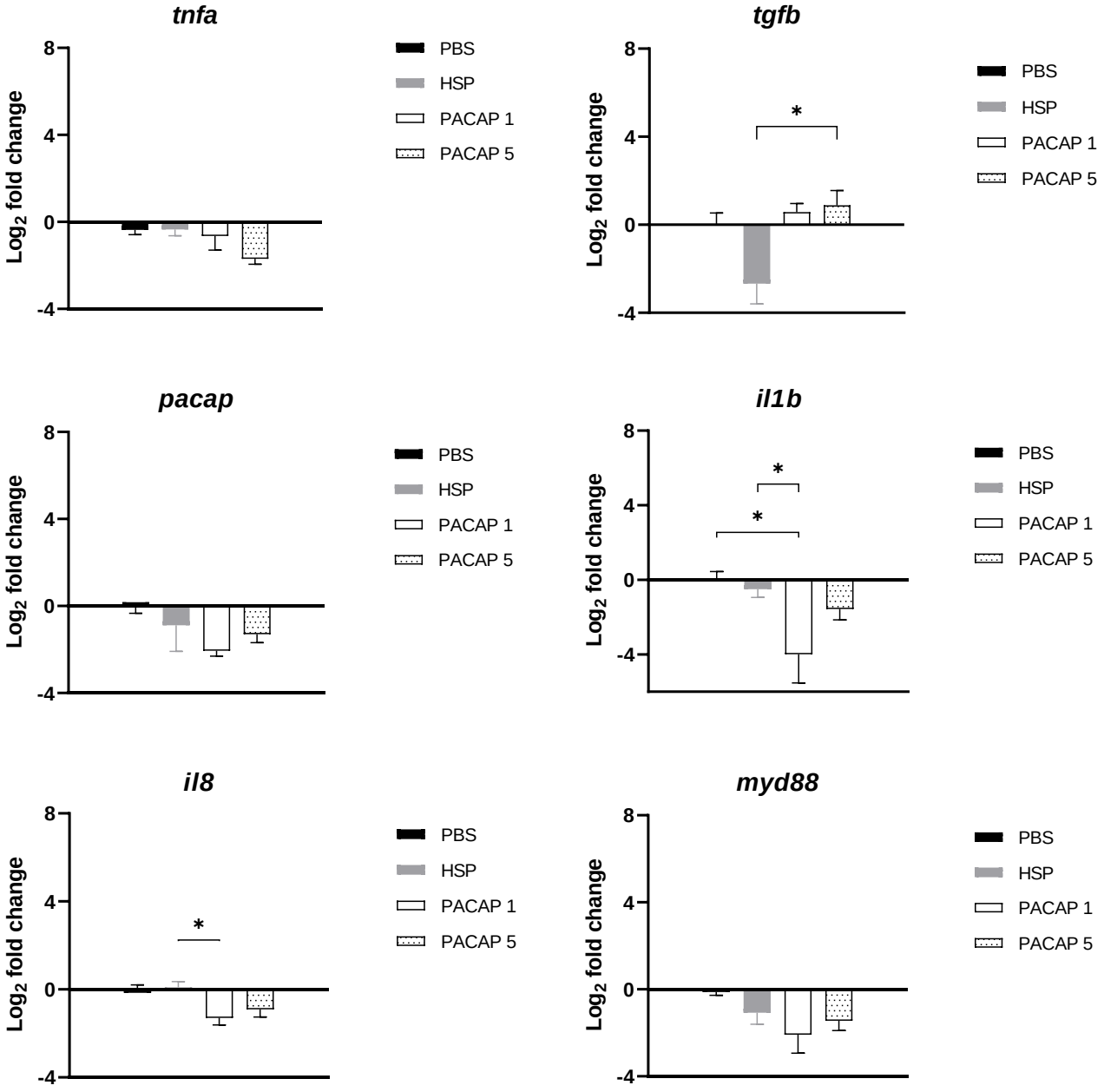

Figure 2.1. qPCR graphs showing the relative expression of each gene in RTgutGC after 24h of treatment with PACAP (200 nM), HSP or PBS. Bars show the mean and standard error of the mean (SEM) of two different experiments with n=3. Statistical analysis with One-way ANOVA and Sidak test were performed using GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$.

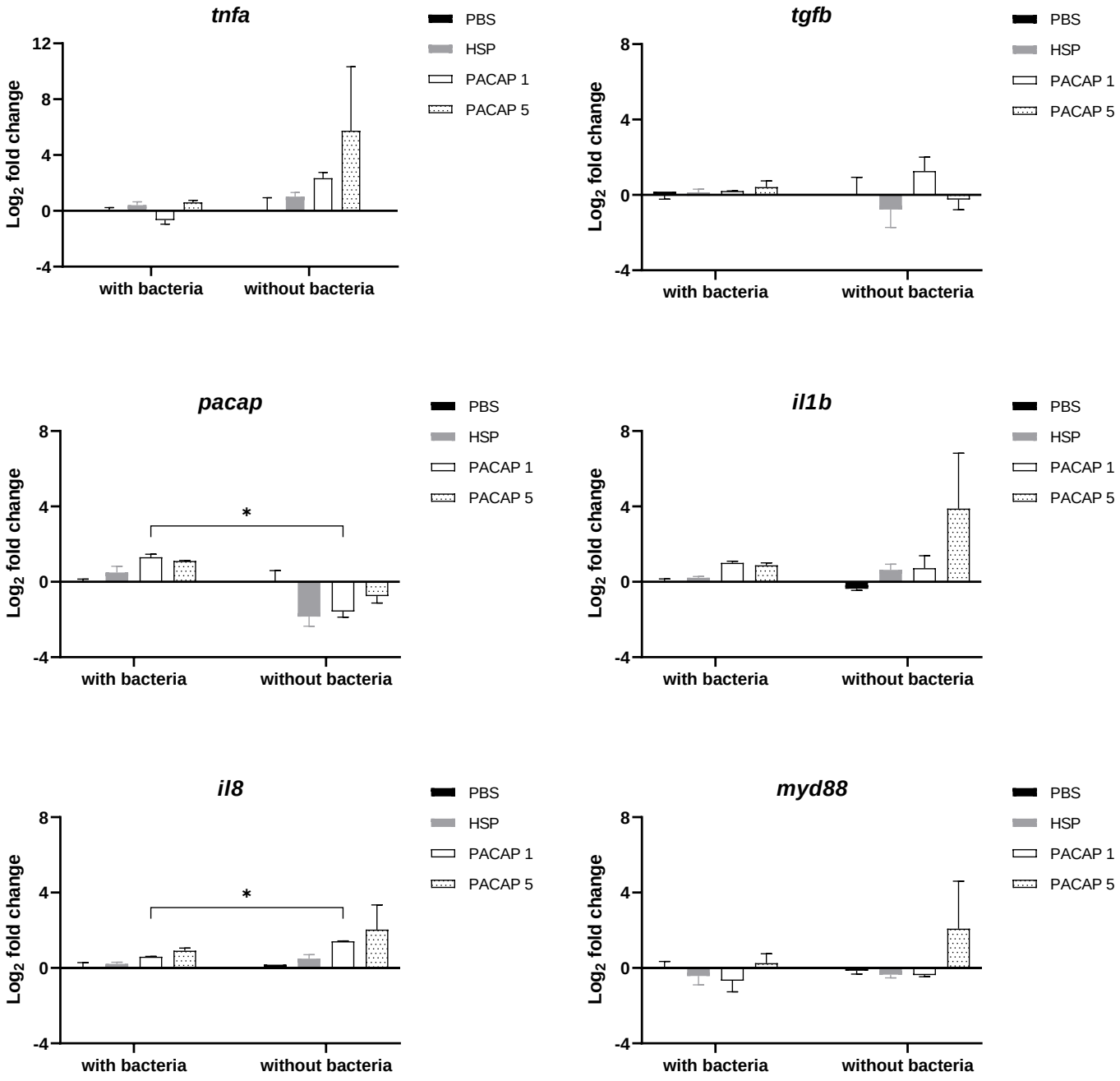

Figure 2.2. qPCR graphs showing the relative expression of each gene in RtgutGC cell line after 96h of treatment with PACAP 1 and 5 (200 nM), HSP or PBS, and 72h with *A. salmonicida* (MOI:0.1). Bars show the mean and SEM of two different experiments with n=3. Statistical analysis with Two-way ANOVA and Sidak test were done using GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$.

2. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) on RTS11

Thymus, kidney and spleen are the major lymphoid organs of teleost fish (Zapata *et al.*, 2006). In addition, modulation of immune function by feeding is not specifically intestine-localized, it can also act systemically, especially in active immune organs such as the spleen. Also, spleen has been referred as a good target for characterizing immunomodulatory effects of immunostimulants in feed (Morales-Lange *et al.*, 2021). That is why after testing immune modulation at the gut level another experiment was carried out to evaluate the immunomodulatory effect of PACAP 1 and 5 in RTS11 cell line (Figures 2.3 and 2.4).

Figure 2.3 depicts the log2 Fold Change, of the transcripts for the evaluated genes (*tnfa*, *il-1b*, *Il8*, *ifny*, *myd88*, *tgfb* and *pacap*) after 24h of incubation with 200 nM PACAP 1 and 5. No significant difference was determined between the control group (PBS) and PACAP treatments (figure 2.3). After a 96h incubation with 200 nM PACAP1 and 5, and 72h of bacterial exposure, significant differences were observed only between PACAP 1 treatments with and without bacterial exposure for the *il1b* gene (figure 2.4).

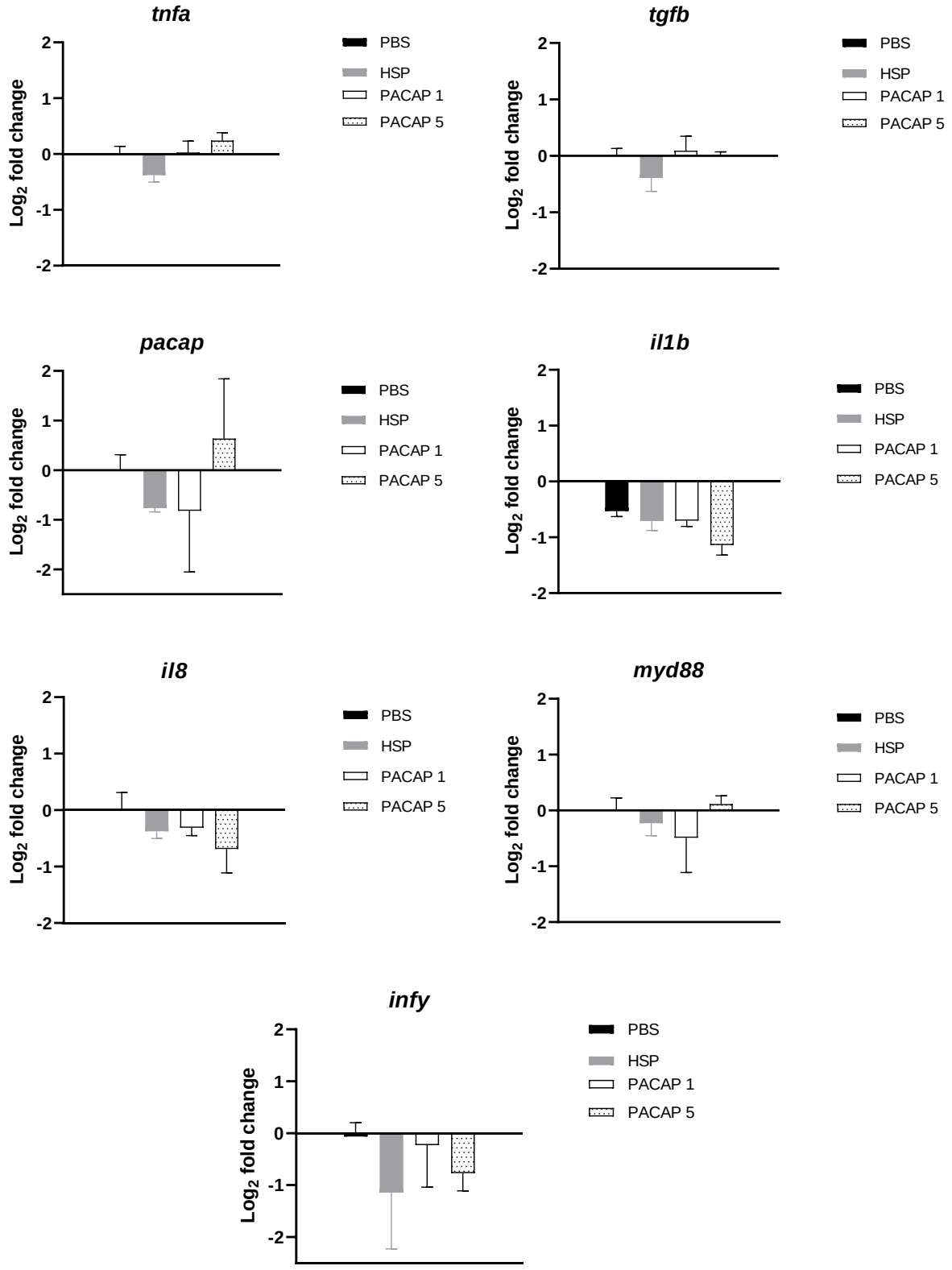

Figure 2.3. qPCR graphs demonstrating the relative expression of each gene in RTS11after 24h of treatment with PACAP 1 and 5 (200 nM), HSP or PBS. Bars show the mean and SEM of two different experiments with n=3. Statistical analysis with One-way ANOVA and Sidak test were done using GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$

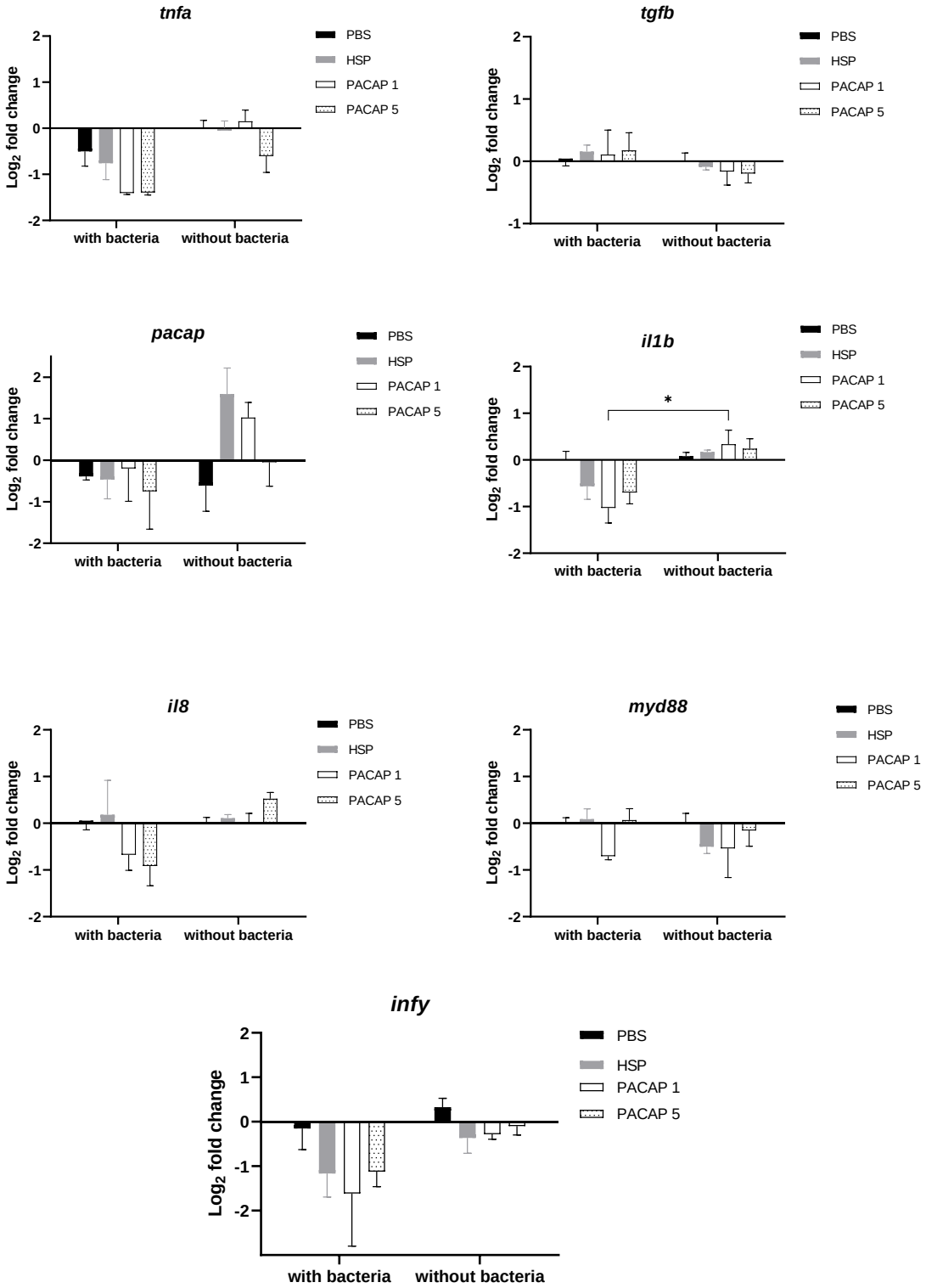

Figure 2.4. qPCR graphs showing the relative expression of each gene in RTS11 after 96h of treatment with PACAP 1 and 5 (200 nM), HSP or PBS and 72h with *A. salmonicida* (MOI:0.1). Bars show the mean and SEM of two different experiments with n=3. Statistical analysis with Two-way ANOVA and Sidak test were done in GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$.

3. Enzyme-Linked Immunosorbent Assays (ELISA)

Even when the number of gene transcripts is related with the concentration of protein, the relationship is not always one to one. A low correlation between transcript and protein expression has been reported because it depends on many factors like mRNA processing and translation processes (Bauernfeind & Babbitt, 2017). Therefore, experiments were conducted to test protein levels in the supernatant of RTS11 cell cultures, treated with PACAP 1 and 5 (200 µM) at 24 hours (Figures 2.5 and 2.6, a). Protein levels were also tested at 48 and 96 hours of PACAP, and 24 and 72 hours of *A. salmonicida* exposure, respectively (Figures 2.5 and 2.6, b). The amount of IFNγ increased in the conditioned media of RTS11 cells treated with PACAP 1 for 48 hours and exposed to bacteria for 24 hours, showing significant differences from the control (PBS) (Figure 2.5, b).

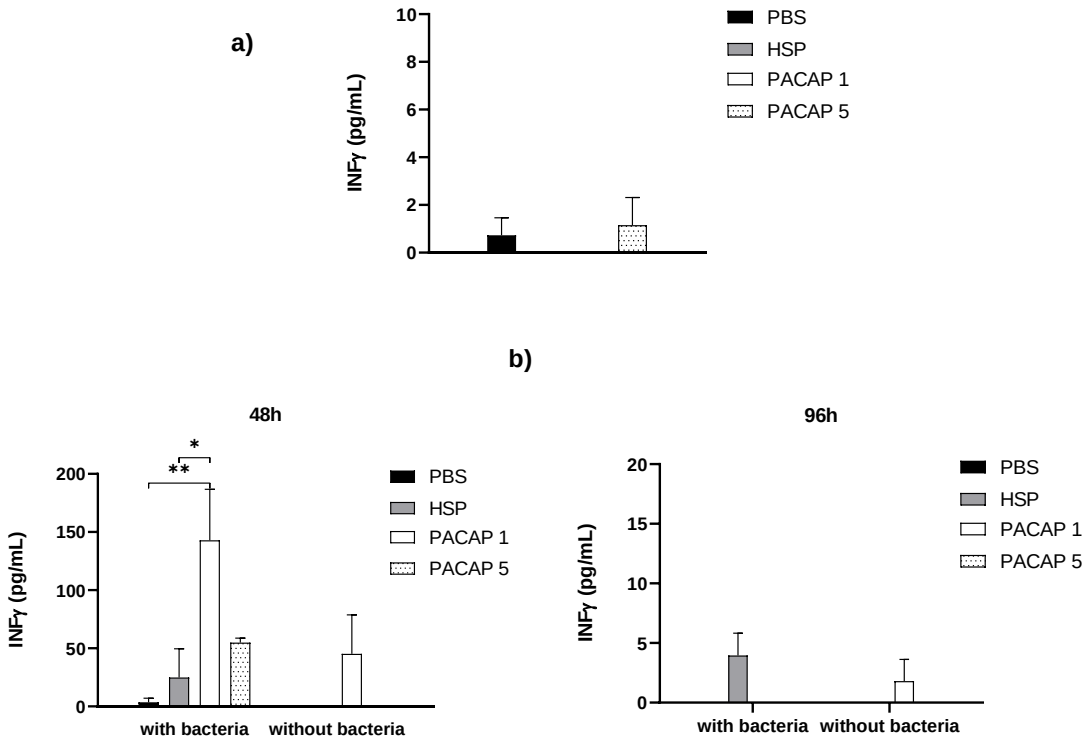

Figure 2.5. IFNγ protein concentration in the conditioned media of RTS11 after 24h of PACAPs(200nM), HSP or PBS treatments (a) as well as 48h and 96h of treatment, with exposure to *A. salmonicida* for 24 and 72h, respectively (b). Bars represent the mean and SEM. Statistical analysis with Two-way ANOVA and Sidak test were done using GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$.

Also, IL-1β protein concentration was increased in the conditioned media of RTS11 cells treated with PACAP 1 for 24 hours, showing significant differences with the controls (PBS, HSP) (Figure 2.6, a). Also, an increase in IL-1β concentrations was observed when RTS11 cells were treated with PACAP 1 and 5 for 48 hours and exposed to bacteria for 24 hours with significant differences with HSP control. In addition, both treatments (PACAP 1 and 5) showed significant differences in the amount of protein between the conditioned media of cells exposed to and not exposed to bacteria (Figure 2.6, b). Moreover, in the conditioned media of cells treated with PACAPs for 96 hours showed an increase of IL-1β with significant differences with both controls (PBS, HSP). Finally, differences were found between the concentration of IL-1b, regardless the treatment, in te conditioned media of cells that were exposed to bacteria for 72h respect that cells that were not (Figure 2.6, b).

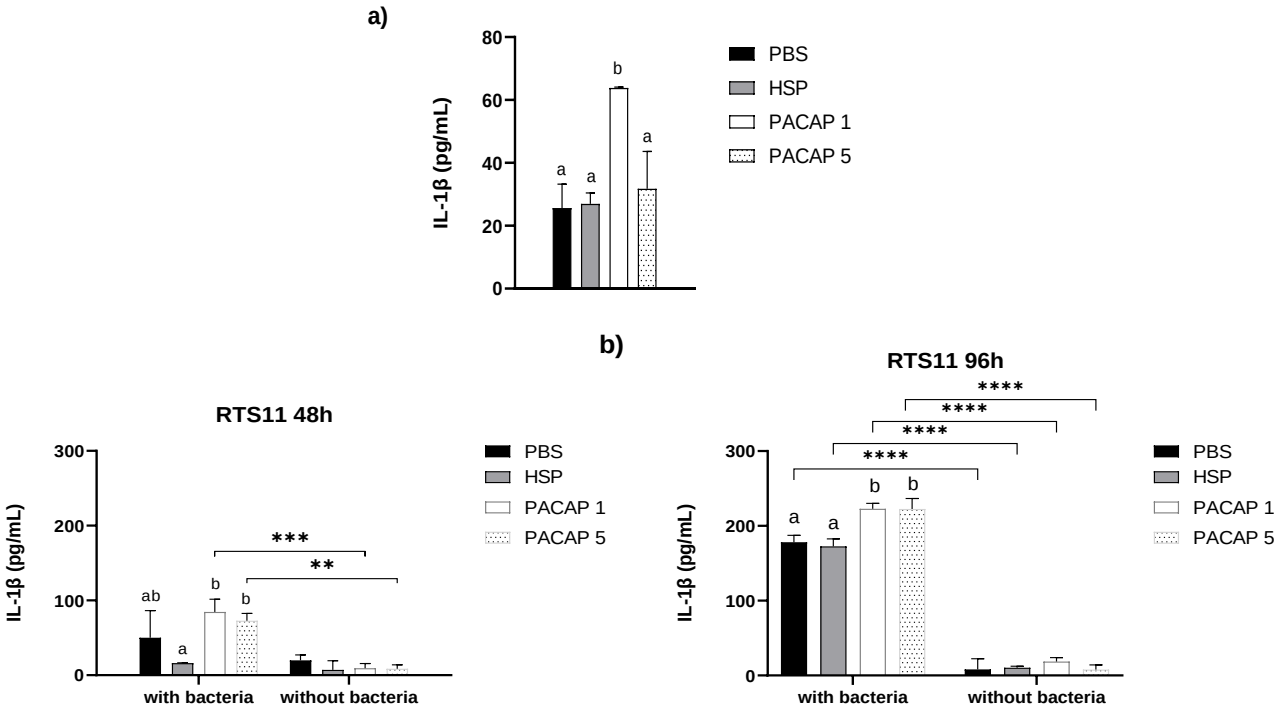

Figure 2.6. IL-1β protein concentration in the conditioned media of RTS11 after 24h (a) of PACAPs (200nM), HSP and PBS treatments, as well as 48h and 96h (b) of treatments and 24 and 72h of bacterial exposure respectively. Bars represent the mean and SEM. Statistical analysis with Two-way ANOVA and Sidak test were developed by using GraphPad Prism 8.0.1, asterisks represent significant differences, $p < 0.05$.

4. *In silico* study of PACAP – receptor interactions

It is well known that PACAP interacts with three main receptors in the cell: PAC1, vasoactive intestinal peptide (PAC1), vasoactive intestinal peptide receptor type 1 (VPAC1) and vasoactive intestinal peptide receptor type 2 (VPAC 2). Also two of these three main receptors (PAC1 and VPAC2) are expressed in RTS11 (Lugo *et al.*, 2011). Therefore, after evaluating the direct antimicrobial effect, as well as the immunomodulatory function of PACAP in cells, *in silico* studies were conducted to compare the interactions of PACAP38 (named as PACAP1 variant in previous experiments) with these three receptors.

For analysing immunomodulatory segments of PACAP, AlphaFold predictions of PACAP from *Clarias gariepinus* and *Oncoryhnchus mykiss* were used for predicting interactions with AlphaFold structures of the *O. mykiss* PAC1, VPAC1, and VPAC2 receptors from the UniProt server. Interactions predicted by Chimera X program using the best model prediction are shown in figures 2.7, 2.8 and 2.9. According to these models, accommodation of PACAP38 peptide with PAC receptor seems to involve a turn of the peptide starting around TYR22 residue (Figure 2.7). While peptide remains straight for interaction with receptors VPAC1 and VPAC2 (Figures 2.8 and 2.9).

In addition, interactions with a confidence rate from 4 to 5 are summarized in table 2.4, 2.5 and 2.6. PACAP displays a higher number of interactions with PAC1 receptor. 14 interactions were observed as more probable interactions with *C. gariepinus* and 16 with *O.mykiss.* Both PACAPs share interactions with SER2, THR7, ASP8, SER11, ARG12, ARG14, LYS15 and TYR22. While HIS1, PHE6, ARG30, ARG34, as well as an interaction with ARG12, are only present in *O.mykiss* peptide bind prediction. Also, ASP3, TYR10, TYR13 and LYS15 were only predicted to interact with high probability in *C.gariepinus* peptide (Table 2.4).

On the other hand, VPAC1 show 8 and 9 interactions with PACAP *C.gariepinus* and PACAP *O.mykiss*, both interacting with residues HIS1, THR7, ASP8, SER11, TYR13, ARG14, GLN16. While the

interaction with ASP3 was specific to *C.gariepinus* PACAP38 (Table 2.5). Aside, VPAC2 have 10 interactions with both PACAPs. HIS1, SER2, THR7, ARG12, ARG14, LYS15, LEU23, VAL26 residues were common interactions with VPAC2 receptor, while residue TYR13 of *C.gariepinus* and TYR22 of *O.mykiss* were peptide specific interactions (Table 2.6)

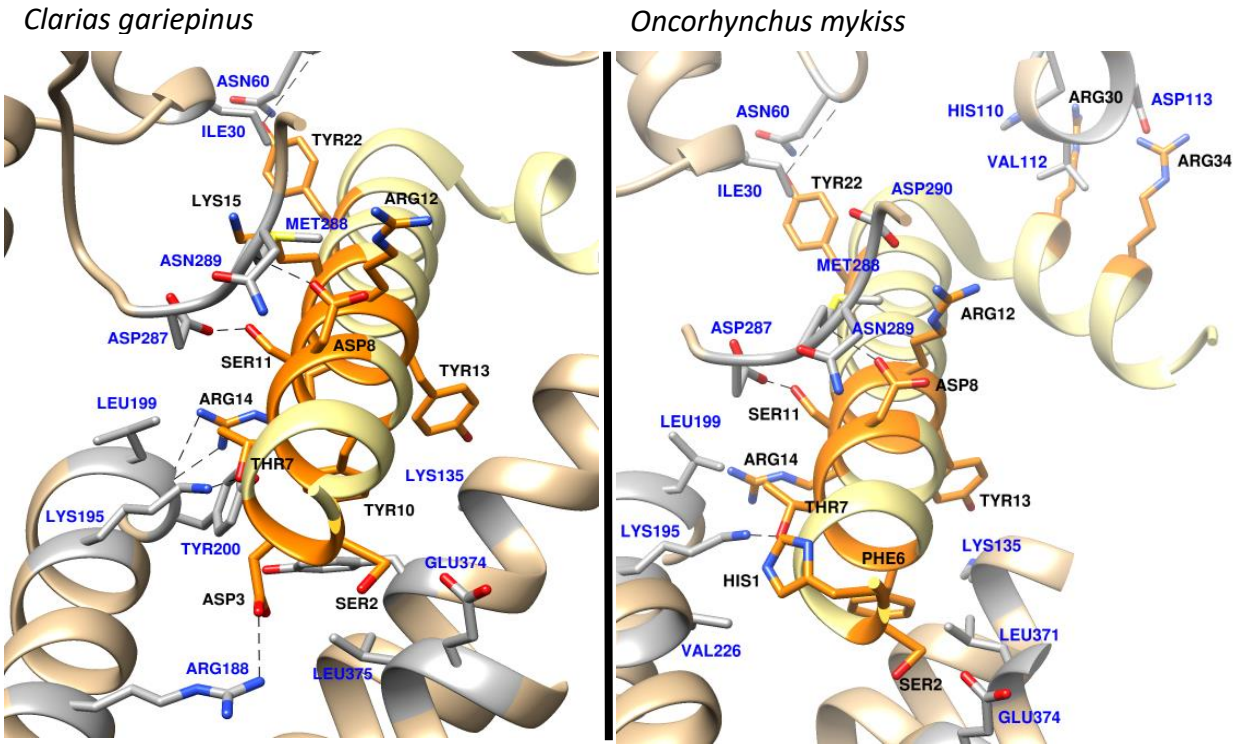

Figure 2.7. PAC1 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences predicted by ChimeraX program and visualized through Swiss-Pdb Viewer software. PACAP residues are presented in black and receptor residues in blue.

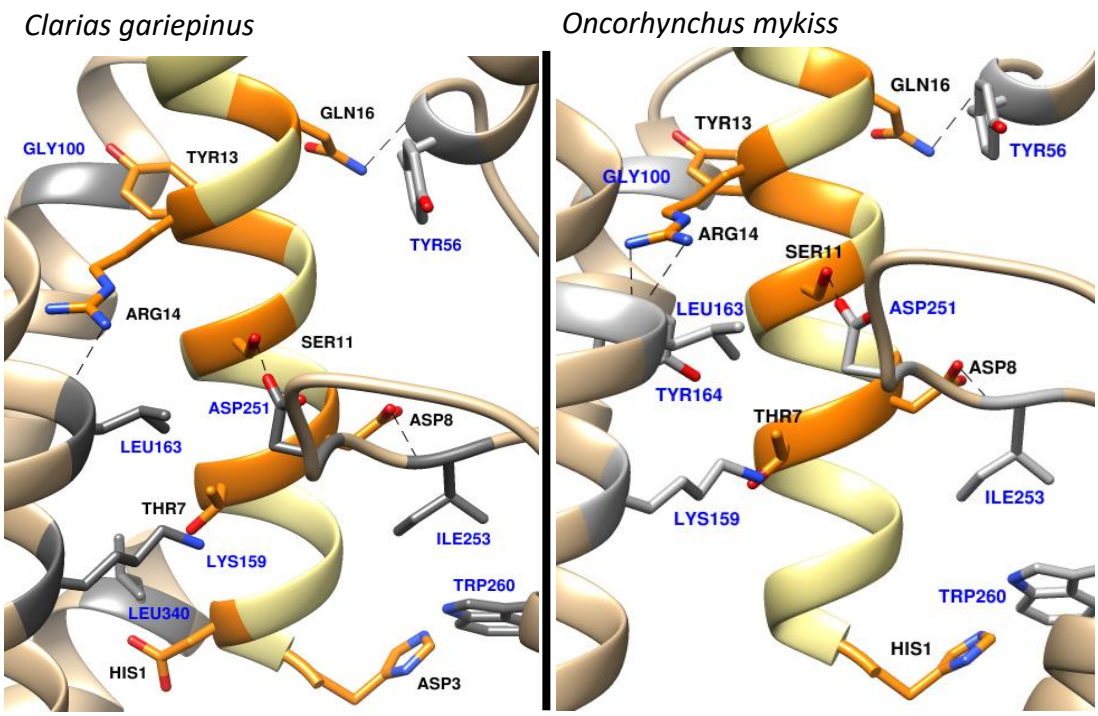

Figure 2.8. VPAC1 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences predicted by ChimeraX program and visualized through Swiss-Pdb Viewer software. PACAP residues are presented in black and receptor residues in blue.

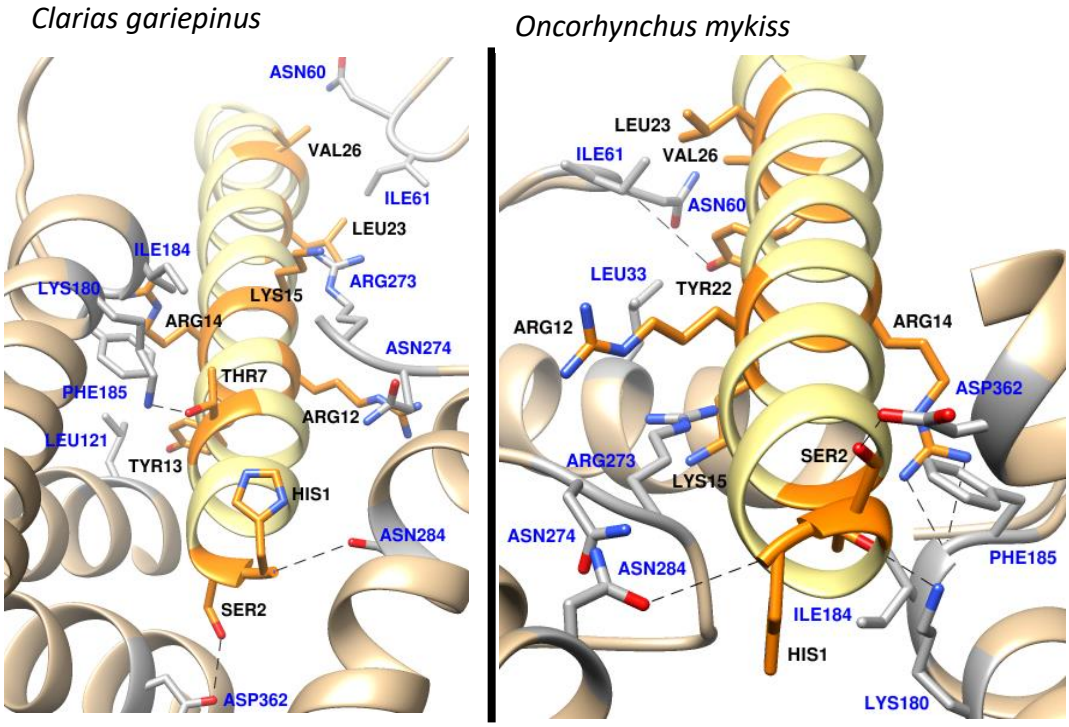

Figure 2.9. VPAC2 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences predicted by ChimeraX program and visualized through Swiss-Pdb Viewer software. PACAP residues are presented in black and receptor residues in blue.

Table 2.4. PAC1 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences.

PAC 1	PACAP *C. gariepinus*	Confidence (1-5)	Interaction	PAC 1	PACAP *O. mykiss*	Confidence (1-5)	Interaction
GLU374	SER2	5		VAL226	HIS1	5	
LEU375	ASP3	5		GLU374	SER2	5	
ARG188	ASP3	5	H-bond (2.9Å)	LEU371	PHE6	5	
LYS195	THR7	5	H-bond (1.4Å)	TYR142	PHE6	3	
ASN289	ASP8	5	H-bond (2.7Å)	LYS195	THR7	5	H-bond (1.4Å)
TYR200	TYR10	5		ASN289	ASP8	5	H-bond (2.6Å)
TYR138	TYR10	4		ASP287	SER11	5	H-bond (1.7Å)
ASP287	SER11	5	H-bond (1.8Å)	MET288	ARG12	5	
MET288	ARG12	5		ASP290	ARG12	5	
LYS135	TYR13	4		LYS135	TYR13	4	
LEU199	ARG14	5	H-bond (2.8Å/2.2Å)	LEU199	ARG14	5	
MET288	LYS15	5		ILE30	TYR22	4	
ILE30	TYR22	4		ASN60	TYR22	5	
ASN60	TYR22	5		VAL112	ARG34	4	
				ASP113	ARG34	4	
				HIS110	ARG30	4	

Table 2.5. VPAC1 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences.

VPAC 1	PACAP *C. gariepinus*	Confidence (1-5)	Interaction	VPAC 1	PACAP *O. mykiss*	Confidence (1-5)	Interaction
TRP260	HIS1	4		TRP260	HIS1	5	
LEU340	ASP3	5		LYS159	THR7	5	
LYS159	THR7	5		ILE253	ASP8	5	H-bond (2.6Å)
ILE253	ASP8	4	H-bond (2.8Å)	ASP251	SER11	5	H-bond (2.1Å)
ASP251	SER11	4	H-bond (2.1Å)	GLY100	TYR13	4	
GLY100	TYR13	4		LEU163	ARG14	5	H-bond (2.9Å/2.4Å)
TYR164	ARG14	5		TYR164	ARG14	5	
LEU163	ARG14	5		TYR56	GLN16	5	H-bond (2.0Å)

TYR56	GLN16	4	H-bond (2.1Å)				

Table 2.6. VPAC2 receptor interactions with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences.

VPAC 2	**PACAP *C. gariepinus***	**Confidence (1-5)**	**Interaction**	**VPAC 2**	**PACAP *O. mykiss***	**Confidence (1-5)**	**Interaction**
ASN284	HIS1	5	H-bond (2.2Å)	ASN284	HIS1	5	H-bond (2.2Å)
ASP362	SER2	5	H-bond (2.9Å)	ASP362	SER2	5	H-bond (2.9Å)
LYS180	THR7	5	H-bond (2.4Å)	LYS180	THR7	5	H-bond (2.5Å)
ASN274	ARG12	4		ASN274	ARG12	4	
LEU121	TYR13	5		PHE185	ARG14	5	
ILE184	ARG14	5	H-bond (2.4Å/2.9Å)	ILE184	ARG14	5	H-bond (2.3Å/2.9Å)
PHE185	ARG14	5		ARG273	LYS15	4	
ARG273	LYS15	4		LEU33	TYR22	4	
ILE61	LEU23	4		ILE61	LEU23	4	
ASN60	VAL26	4		ASN60	VAL26	4	

D. Discussion

1. Relative cytokine expression in RTgutGC: qRT-PCR

PACAP has immunomodulatory properties and these effects have been reported in *in vivo* experiments as well as in fish cell lines (Lugo *et al.*, 2019; Semple *et al.*, 2019; Velázquez *et al.*, 2020b). In addition, the vertebrate immune system is regulated by cytokines, which are secreted by cells from both the innate and acquired immune systems. These molecules can modulate signal transmission between cells via specific receptors on the target cell (Sakai *et al.*, 2021), and therefore, they are important elements of immune responses.

Expression of selected cytokines genes, *tnfa, il-1b, il8, myd88, tgfb* and *pacap,* was tested in RTgutGC cultures treated with either PACAP 1 or 5 (200 nM) for 24 and 96 hours. *il1b* and *il8* transcript expression in RTgutGC was significantly downregulated after to 24h of 200 nM PACAP 1 treatment. Alternatively, *tgfb* expression was upregulated by PACAP 5 treatment (figure 2.1). This suggests that these PACAP isoforms cause a downregulation of inflammatory cytokines (IL-1b and IL-8) and an upregulation of the mainly anti-inflammatory *tgfb.* A downregulation of *il-1b* transcript was also reported for RTgill-W1 cells exposed to PACAP and *F. psychrophilum* (Rodriguez Cornejo, 2021).

In addition, when RTgutGC cells were infected with *A. salmonicida* altered transcript expression was observed between infected, PACAP treated cells and cells treated only with PACAP. Also, after 96h of treatment with PACAP1 and 72h of bacterial exposure, significant differences in *il8* and *pacap* expression patterns were observed between cells exposed and non-exposed to bacteria. These results agree with Semple *et al.* (2019), who described modified cytokine expression levels not only by PACAP but also after exposure to a specific bacterial pathogen.

2. Relative cytokine expression in RTS11: RT-qPCR and ELISA.

Thymus, kidney and spleen are the main lymphoid organs of teleost fishes (Zapata *et al.*, 2006). The spleen is considered a secondary lymphoid organ of high importance in fish, with a key role in the antigen presentation processes, lymphocyte activation and promoting humoral immunity (Morales-Lange *et al.*, 2021).

Transcript expression of *tnfa*, *il-1b*, *Il8*, *ifny*, *myd88*, *tgfb* and *pacap* genes did not seem to change in RTS11 cells exposed to PACAP 1 or 5 for 24 hours, while a treatment with PACAP1 for 96h, and 72h of *A. salmonicida* exposure, seems to upregulate *il-1b* gene. Other researchers have evaluated the effect of PACAP treatments on RTS11 cells infected with *F. psychrophilum* and they also observed that transcript expression is different when RTS11 were only incubated for 24h with PACAP without bacterial exposure. However, these authors also observed an upregulation *of il6, il1b* and *tnfa* at 48 hours of PACAP (100 nm) treatment, with and without 24h exposure to *F. psychrophilum*. However, their results obtained after 96h of PACAP treatment and 72h of *F. psychrophilum* infection showed a much lower upregulation (Semple *et al.*, 2019). Therefore, comparing with our findings, it is possible to say that RTS11 cells infected with bacteria and pre-treated with PACAP (concentrations in the order of 10^2 nM), upregulate these proinflammatory cytokines after 24h, with a high peak at 48h, and then a decline with much less upregulation at 96h, which is probably completely lost after that time point.

Due to the importance of the spleen for fish immunity, cytokine modulation by PACAP treatment and *A. salmonicida* exposure was tested not only at the transcript level but also at the protein level for IL-1b and IFNγ, two inflammatory cytokines. RTS11 cells treated for 24h with PACAP 1 increased IL-1β protein concentration secreted into the conditioned media. In addition, RTS11 cells exposed to PACAP 1 and 5 for 48 h and 96 h, and then infected with *A. salmonicida*, also showed an increase in IL-1β protein concentration. As this increase in IL-1β concentration was not observed at

transcript level, it is possible that PACAP protein is following an increase in transcript earlier than the first measurement point, or there are intracellular stores of protein released prior to an induced transcription increase, as seen by Frenette *et al.* (2023).

It is also possible that there is more than one transcript encoding for IL1-β protein. In agreement with this hypothesis, Zou *et al.* sequenced the complete rainbow trout genome and found that trout IL-1b gene produces at least three transcript variants (Zou *et al.*, 2001). Another option is that the transcript level is not increasing, but the translation rate of this basal level of accumulated transcripts increases.

For example, Liang *et al.* reported a constitutive relatively high level of IL-1β transcripts in peripheral blood, intestine, spleen, lung and liver of *Pelodiscus sinensis*, also highly sensitive to *A. hydrophila* infections (Liang *et al.*, 2016). This finding, in addition to the dissociation of transcription from translation, described from Dinarello *et al.* for IL1b, support this hypothesis. Dinarello *et al.* (1996) explained that different stimulants such as C5a complement component or hypoxia, can increase IL-1β transcripts in monocytic cells without significant translation into protein. Nonetheless, these transcripts could rapidly decrease due to the synthesis of transcriptional repressors, like miRNA. These researchers also stated that the addition of certain stimuli like bacterial endotoxin can lead to high levels of steady state IL-1β mRNA and enhanced translation in cells, also arguing for the stabilization of the mRNA AU-rich 3' untranslated region in cells treated with LPS. This supports the higher variations in protein level and lower variations in transcript levels founded in this study. However, more experiments are necessary to corroborate these ideas.

Other authors also found a low correlation between transcript and protein expression (Bauernfeind & Babbitt, 2017). For example in mammalian cells, this correlation is 9% for human monocytes and 40% for mouse fibroblasts (Guo *et al.*, 2008; Schwanhäusser *et al.*, 2011). However, cells exposed and not exposed to bacteria with both treatments (PACAP 1 and 5) showed significant

differences in the amount of IL-1β in conditioned media in agreement with significant differences observed at transcript level, suggesting that the synthesized protein is mostly secreted. Moreover, IFNγ protein levels significantly increased in the conditioned media of RTS11 cells incubated 48h with PACAP 1 and exposed to *A. salmonicida*, while cells that were not exposed to the bacteria did not show such a large increase. Similar to Il-1β, protein levels were also not correlated to the timing and magnitude of transcript changes.

3. *In silico* study of PACAP – receptor interactions

PACAP, as described in the previous section, is considered to have both antimicrobial and immunomodulatory properties (Velázquez *et al.*, 2020b). Antimicrobial properties have been more related with the N-terminal (Lugo *et al.*, 2019) of the peptide. While immunostimulatory section has not been properly identified, but seems to be related with PAC1, VPAC1 and VPAC2 cell receptor interaction (Lugo *et al.*, 2011).

PACAP-38 residues Val19, Leu23, Val26 and Leu27 belong to a highly conserved region on the hydrophobic surface of the α-helix and were described by Lugo *et al.* (2019) as involved in antimicrobial activity by multiple sequence alignment with peptides from different antimicrobial databases, which also conferred high antimicrobial scores for PACAP (CAMPr3 score: 0.67; AntiBP2 score: 1.08; iAMPpred score: 0.79, criteria of inclusion: score > 0.5). Other conserved residues found using antimicrobial and anticancer peptide databases are located in the N-terminal of the peptide, His1, Gly4, Phe6, Asp8. Single mutations of this residues were calculated by 'Design-Peptide' module of AntiCP as favorable mutations for increasing anticancer activity (Lugo *et al.*, 2019). Considering this information as well as the fact that the residues of PACAP showed similar conservation to antimicrobial or anticancer databases, it is possible to think that these suggested

mutations (His1: F, I, K, R; Gly4: A, C, L, M, V, W; Phe6: E, R and Asp8: E) may have also positive effect on PACAP antimicrobial activity.

The *in-silico* experiment for determining immunomodulatory segments of PACAP (figures 2.7, 2.8 and 2.9) showed a turn of the peptide characteristic of PAC 1 interaction. This finding suggests that this turn ensures a higher number of interactions of PACAP with this specific receptor. As described in the results there is an average of 15 interactions of PACAP38 peptide with PAC1, 8.5 with VPAC1 and 10 with VPAC2 (tables 2.4, 2.5, 2.6). This different positioning between receptors lead to less and different interactions with VPAC2, and a lack of this C-terminal interaction with VPAC1. These results are in agreement with the fact that PAC1 affinity for PACAP38 is higher than for VIP (vasoactive intestinal polypeptide), a peptide that is able to bind to the other two receptors (VPAC1, VPAC2) with similar affinities (Liao *et al.*, 2021; Sureshkumar *et al.*, 2022).

Table 2.7 was made for an easier interpretation of the results shown in tables 2.4, 2.5 and 2.6, which shown the interactions with a confidence equal or higher than 4 of *C. gariepinus* and *O. mykiss* PACAPs, with *O. mykiss* receptors (PAC1, VPAC1 and VPAC2). Then, by using table 2.7 it was possible to deduce the amino acids with higher probability to participate in the immunological role of PACAP.

Table 2.7. Summary of receptor interactions (confidence higher than 4) with *Clarias gariepinus* and *Oncorhynchus mykiss* PACAP sequences. Amino acids labeled in red means that are able to interact with the three receptors, orange amino acids interact just with two of the receptors, grey interacts with one of them and green labeled amino acids do not interact with any of the receptors.

***O. mykiss* PACAP**	**Receptors**	***C. gariepinus* PACAP**	**Receptors**
HIS1		**HIS1**	VPAC1/VPAC2
SER2	PAC1 / VPAC2	**SER2**	PAC1 / VPAC2
PHE6	PAC1	**ASP3**	PAC1 / VPAC1
THR7		**PHE6**	
ASP8	PAC1 / VPAC1	**THR7**	
SER11	PAC1 / VPAC1	**ASP8**	PAC1
ARG12	PAC1 / VPAC2	**TYR10**	PAC1
TYR13	PAC1 / VPAC1	**SER11**	PAC1 / VPAC1

ARG14		**ARG12**	PAC1 / VPAC2
LYS15	VPAC2	**TYR13**	
GLN16	VPAC1	**ARG14**	
TYR22	PAC1 / VPAC2	**LYS15**	PAC1 / VPAC2
LEU23	VPAC2	**GLN16**	VPAC1
VAL26	VPAC2	**TYR22**	PAC1
ARG34	PAC1	**LEU23**	VPAC2
ARG30	PAC1	**VAL26**	VPAC2
		ARG30	
		ARG34	

Amino acids that are interacting with at least one of the receptors for both PACAP peptides were included in the analysis. Then nine amino acids were selected for their interaction with at least two of the receptors (HIS1, SER2, THR7, ASP8, SER11, ARG12, TYR13, ARG14, TYR22). These amino acids seem to be the most related with the immunological function or at least with the receptor associated PACAP functionality. In addition, it would be important to highlight THR7 and ARG14 as amino acids in both peptides that interacted with all of the three receptors.

The observed differences in cytokine expression between PACAP 1 (PACAP38) and PACAP 5 treatments could be related to the absence of HIS and SER at positions 1 and 2 of PACAP 5. The rest of the seven suggested amino acids were conserved in an alignment of the linear sequence of PACAP 1 and 5. This suggests an important role of HIS1 and SER2 in the functionality mediated by PACAP-receptor interaction. Three-dimensional alignments for more specific analyzes are suggested for future studies.

In addition, other interesting amino acid interactions seem to be receptor specific, like the ones formed with LEU23 and VAL26 that appear to be VPAC2 specifics and GLN16 was VPAC1 specific. However more experiments with PACAP from other species and receptors from other species would be interesting to confirm these observations.

E. Conclusions

tnfa, il-1b, il8, myd88, tgfb and *pacap* gene regulation was evaluated in RTgutGC and *il1b* and *il8* transcript expression was significantly downregulated after 24h of PACAP 1 treatment. Conversely, *tgfb* expression was upregulated after 24h of PACAP 5 treatment. suggesting a downregulation of inflammatory cytokines and upregulation of the anti-inflammatory cytokines due to PACAP treatments in RTgutGC cell line. In addition, for both cell lines evaluated (RTgutGC, RTS11) it was possible to prove that exposure to *A. salmonicida* affect cytokine transcripts expression patterns. Findings of this chapter showed that IFNγ protein concentration was increased in the conditioned media of RTS11 cells treated for 48h with PACAP 1 and exposed to *A. salmonicida*. Likewise, RTS11 cells incubated with PACAP 1 for 24h, increase IL-1β protein concentration in conditioned media, and this increase was even higher for those cells exposed to *A. salmonicida* after 24h of PACAP pre-treatment. This chapter also discussed the differences between protein and transcript expressions, reaching the conclusion that PACAP and *A.salmonicida* treatments can possibly stimulate the translation of IL1-β protein from previously accumulated transcripts (mRNA), corroborating a low correlation of transcription and translation found by many other authors. Finally, thanks to the *in-silico* study, it was possible to suggest that HIS1, SER2, THR7, ASP8, SER11, ARG14, ARG12, TYR13, TYR22 residues of PACAP38 seems to be the most related with the immunological function or at least with the receptor associated PACAP functionality.

III. Chapter 3. General Discussion and Future Outlook

In vitro experiments are a good first approach for addressing research hypotheses, especially when the phenomenon or effect under study has not been validated enough to justify *in vivo* experiments. It will also tentatively reduce the number of hypotheses to test *in vivo* saving time, animals and resources.

The intended use of PACAP in Canadian aquaculture, particularly in salmonids, triggered the *in vitro* testing of PACAP effects in fish. Results of this thesis confirms the direct antimicrobial effect expected for PACAP-38 on *Aeromonas* cultures. It also identified the dependence of PACAP antimicrobial effect to the culture broth employed**.** Furthermore, it validated that this effect on *Aeromonas* cultures relies on the membrane permeabilizing effect of PACAP, similar to what was observed in *Flavobacterium psychrophilum* (Semple *et al.*, 2019). It was also verified a dose dependant behaviour of PACAP with no visible effect at concentrations in the sub-micromolar order and significant effects on bacterial membrane integrity at concentrations of 20 µM and higher. Altogether these results support the idea of PACAP as an antimicrobial agent.

For future applications, such as fish feed additive, a validation of its cytotoxicity *in vivo* is mandatory. Therefore, an important outcome was to determine that PACAP is not toxic to RTgutGC at concentrations lower than 25 µM and similar results were obtained for RTS11 (Semple *et al.,* 2019). This thesis is in agreement with other studies where even higher (50µM) concentrations of PACAP caused only 6% of hemolysis on rainbow trout red blood cells, and no hemolytic activity was reported at lower concentrations (Rodriguez Cornejo, 2021). Furthermore, PACAP, as an AMP has the advantage of target selectivity, due to the more negatively charged prokaryotic membrane compared to eukaryotic cell membranes (Matsuzaki, 2009). This translates into lower concentrations needed to cause the same effect on bacteria, because the selectivity for bacterial

membrane increases the effective concentration, reducing the possible harmful effects of PACAP even more.

In vivo assays are necessary before the inclusion of PACAP in the feed of animals, as suggested by Greco *et al.* (2020), but recent unpublished experiments suggested that 250 µg of PACAP/Kg of feed in formulations described by Herrera *et al.* (2021), provoke no associated mortality or physical symptoms of toxicity when fed to 20-70 g trout for a period of around one month (Mark Fast, personal communication). Future experiments intended to certify the application of PACAP as safe food additive should include studies with 3–6-month-old fish, between 0.5-5g, considering that the largest fish should not be more than twice the size of the smallest one as regulated (Canada, 2009). Assurance of non-acute toxicity of PACAP requires that concentration producing 50% mortality of fish should be not lower than 5mg/Kg (20 times the concentration used in the referred study) and not lower that 1mg/kg to be considered a non-aquatic hazard (EC: 1272/2008 of the European Parliament and of the Council, 2008)

Going further, treatments in aquaculture, which mostly include the bio-dispersion of the product in the ecosystem, especially in open-net pens or pond aquaculture, need an environmentally safe product. Therefore, the regulations for safety of feed additives include the requirement that the product is fully metabolised in the target animal into common metabolites in the excreta without biological activity of environmental concern, like H_2O, CO_2, and salts. Otherwise, calculation of "predicted environmental concentrations (PEC) in surface water from aquaculture (PECswaq) in raceway/pond/tanks and recirculation systems is needed. For PACAP formulations of 250µg of PACAP/Kg of feed, this estimation is $3.5 * 10^{-4}$ µg/L, lower than the 0.1 µg/L advised for environmentally friendly feed additives (Bampidis *et al.*, 2019).

In addition to its direct antimicrobial effect PACAP has immunostimulatory activity. The problematic lack of quick diagnostics for fish disease outbreaks, makes it difficult to treat infections.

In several cases, once the signs are visible it is already too hard to control the occurrence. Therefore, immunostimulants like PACAP are very interesting tools for fish farming. The immunomodulatory activity of PACAP was also assessed through *in vitro* experiments in chapter two of this thesis. The modulation of different cytokines expression after 24 hours of PACAP treatment in RTgutGC showed that *il1b* and *il8* transcript expression was significantly downregulated, while *tgfb* expression was upregulated, suggesting a role of PACAP in the downregulation of inflammatory cytokines (IL-1β and IL-8) and upregulation of the mainly anti-inflammatory TGF-β**.** However, at 96 hours of treatment with PACAP and exposure to *A. salmonicida* no significant differences from the controls were found. RTS11 cells treated with PACAP for 96 hours and exposed to *A. salmonicida*, modulated the *il-1b* gene expression differently than in the absence of bacteria, but showed no significant differences with the controls**.** In addition, IFNγ and iL-1β protein levels significantly increased in the conditioned media of RTS11 cells incubated with PACAP and exposed to *A. salmonicida*, while cells not exposed to the bacteria did not show such a large increase.

Altogether**,** results obtained for IL-1β transcript amounts and protein concentration in conditioned media of cells treated with PACAP and *A.salmonicida* showed a poor correlation**.** Thus this study also corroborated the low correlation between transcript and protein expression, found by other authors (Bauernfeind & Babbitt, 2017; Frenette *et al.*, 2023), and suggest that PACAP and *A. salmonicida* treatments can possibly stimulate the translation of IL1-β protein from previously accumulated transcripts or the release of stored protein (Frenette *et al.*, 2023).

Even though PACAP peptide structure has been studied and certain amino acids have been described as essential for PACAP antimicrobial activity (Lugo *et al.*, 2011), amino acids involved in the immunological role of PACAP in trout have not been properly identified. In this regard, *in-silico* experiments conducted showed a bend of the peptide, characteristic of PACAP-PAC 1 interaction,

correlated with the higher number of interactions observed with this specific receptor. It was determined that there are a higher number of interactions of PACAP38 peptide with PAC1, less interactions with VPAC2 than PAC1 and a lack of C-terminal interactions with VPAC1. These results agreed with the higher PAC1 specificity for PACAP38 compared to the lower specificity of the other two receptors (VPAC1, VPAC2), broadly exposed by other authors (Liao *et al.*, 2021; Sureshkumar *et al.*, 2022). The *in-silico* studies not only corroborated the differences in PAC1 -, VPAC1 -, and VPAC2 – PACAP binding, but also determined the nine amino acids that interact with the three receptors with high probability and therefore, should most be related with the immunological function or at least with the receptor associated PACAP functionality (HIS1, SER2, THR7, ASP8, SER11, ARG12, TYR13, ARG14, TYR22).

For PACAP receptors' role in fish immune responses, recent investigations of Campbell *et al.*, (2023) show that cAMP accumulation led to mitigation of PACAP immune modulation (stopping the upregulation of IL-1β, TNFα and IL10 transcripts). These findings suggest that when there are conditions of high energy in the cell, causing natural or induced cAMP accumulation, the immunomodulatory role of PACAP is mitigated, perhaps through an attenuated interaction with its main receptor PAC1. Multiple outcomes of PACAP signaling pathways include increasing cell metabolism rate (Campbell *et al.*, 2023; Langer *et al.*, 2022). It is not metabolically possible to increase metabolic rate in conditions with internal high energy levels.

Accordingly, Campbell *et al.*, (2023) also found that inhibition of the VPAC1 receptor led to an increase in PACAP activity (increased IL1-β and IL-10 transcripts), probably because of a reduction of PACAP binding to receptor VPAC1 there is an increase in bio-available PACAP (effective concentration) to bind to PAC1. In the same line of thinking, it was observed that this accumulated cAMP also caused increases of VPAC1 receptor expression, suggesting a feedback regulation of inhibition by cAMP, controlling energetic balance of the cell by increasing levels a receptor with

lower affinity for PACAP (VPAC1), decreasing the effect of PACAP in the big picture and keeping the cell in equilibrium. Campbell *et al.,* (2023) also found that inhibition downstream in the signaling pathway (inhibitors of phospholipase C) did not change PACAP receptor expression. This suggests that metabolites downstream in the signaling pathway of PACAP did not participate in the feedback regulation of receptor expression. All these results support the idea that the immunological role of PACAP involves receptor signaling and VPAC1-PAC1 receptor balance participates in the response. Therefore, other inhibitors that block PACAP38 binding to VPAC1, and 2 receptors should potentially increase PACAP functionality.

In summary, PACAP is a peptide with very low toxicity to fish, with direct antimicrobial activity based on the permeabilization of bacteria membranes. This peptide also plays immunological roles such as the observed induction of secretion of IL1-β and IFN-γ proteins. This immunological function is related to its interaction with PAC1, VPAC1 and VPAC2, presumably through nine specific residues. All these properties make PACAP a very good alternative to antibiotics for applications in salmonids aquaculture that is easily transferable to other fish species.

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