



**In vitro effects of different innate-immune
stimulants on Chinook salmon embryo cell
line (CHSE-214): transcriptional and
translational studies.**

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Sciences School of Engineering and
Natural Science
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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in Biology

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Abstract

The innate immune response in fish is of particular relevance for infections as it forms the first line of defense against bacteria and viruses. Antibiotics and vaccination are the most common method used to control the infectious diseases in aquaculture. The disadvantages with these approaches are the selection of resistant bacteria and poor protection. Defining inducers for the innate immunity in fish could provide alternative approach against infections.

The expression of innate effectors: cathelicidin, hepcidin, iNOS, MDA5, RIG-I and ISG25 mRNA was assessed by qPCR in Chinook salmon cell line (CHSE-214) before and after treatment with potential inducers: β -hydroxy- β -methylbutyrate, 4-phenylbutyrate, 1,25(OH)₂ D3, omega-3 and omega-3 ethyl ester from fish oil and cod liver oil. Cathelicidin was found to be up-regulated by all the inducers, while the other innate immune effectors were found up-regulated by specific inducers. Antimicrobial activity was also measured and indicated enhancement at the protein level.

In addition, we tried to acquire an antibody for Atlantic salmon cathelicidin by immunization of rabbit with synthetic peptides derived from cathelicidin. Specific cathelicidin antibodies were not obtained in this experiment.

The findings might contribute to the development of alternatives to the classical prophylactic methods, antibiotics and vaccination in aquaculture improving the fish immune response and consequently the health of fish in aquaculture.

Útdráttur

Náttúrulega ónæmiskerfið skiptir fiska miklu máli enda verða þeir fyrir stöðugu áreiti frá bakteríum og veirum sem reynir á fyrstu varnarlínuna. Sýklalyf og bólusetningar hafa verið mest notuð til varnar fiskum í eldi. Gallarnir eru val á ónæmum bakteríustofnum og ófullkomin vörn. Skilgreining á efnum sem örva náttúrulegt ónæmi í fiskum gæti nýst gegn sýkingum eða í forvarnarskyni og dregið úr sýklalyfjanotkun.

Tjáning á virkni sameindum náttúrulegs ónæmis í fiskum: cathelicidin, hepcidin, iNOS, MDA5, RIG-I og ISG25 var metin með qPCR mRNA mælingu í Chinook salmon frumu línu (CHSE-214) fyrir og eftir meðhöndlun með ónæmis örvandi efnum: β -hydroxy- β -methylbutyrate, 4-phenylbutyrate, 1,25(OH)₂ D3, omega-3, omega-3 ethyl ester úr lýsi eða þorskalýsi beint. Cathelicidin tjáning var örvuð af öllum efnunum en hin ónæmispróteinin voru einungis aukin af sumum efnanna. Bakteríudrepandi virkni í seyti frá frumum var einnig mæld eftir örvun sem gefur mynd af aukningu próteinanna.

Í öðrum hluta verkefnisins var reynt að mynda mótefni gegn cathelicidinum fiska. Peptíð voru útbúin eftir amínósýruröð cathelicidins fiska og sprautað í kanínur. Ekki tókst í þessari tilraun að mynda sérhæft mótefni gegn cathelicidinum.

Samantekið gæti örvun náttúrulegs ónæmis nýst sem forvörn gegn sýkingum eða meðhöndlun gegn sýkingum í fiskeldi.

The true science teaches, above all, to doubt and to be ignorant.

- Miguel de Unamuno -

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Abbreviations

AA	Arachidonic acid
ACN	Acetonitrile
AMPs	Antimicrobial peptides
ASA	<i>Aeromonas salmonicida</i> ssp. <i>Achromogenes</i>
<i>A.Salmonicida</i>	<i>Aeromonas salmonicida</i>
BCAA	Branched chain amino acids
Bf	Complement factor B
BH4	Tetrahydrobiopterin
Bm11	<i>Bacillus megaterium</i>
BSA	Bovine serum albumin
C3	Complement component 3
CAMP	Cathelicidin antimicrobial peptides in humans
CARDs	Caspase recruitment domains
CATH1	Cathelicidin 1
CATH2	Cathelicidin 2
cDNA	Complementary DNA
CLO	Cod liver oil
DAMPs	Damage-associated molecular patterns
DHA	Docosahexaenoic acid
ds	Double strand
dsRNA	Double stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
eNOS	Endothelial NOS
EPA	Eicosapentaenoic acid
<i>F. rubripes</i>	<i>Fugu rubripes</i>
FAD	Flavin adenine dinucleotide
FAO	Food and agriculture organization of united nations
FBS	Foetal bovine serum
HDAC	Histones deacetylase
HMB	β -hydroxy- β -methylbutyrate
IFNs	Interferons
IHNV	Infectious hematopoietic necrosis virus
IL	Interleukine
INF	Interferon
iNOS	Inducible NOS
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISAV	Infectious salmon anemia virus
ISGs	IFN stimulated genes
ISP	Isopropanol
KIC	Ketoisocaproate
LB	Luria-Bertani medium
LCFAs	Long-chain fatty acids

LGP2	Laboratory of genetics and physiology 2
LL-37	Mature human peptide of CAMP
LPS	Lipopolysaccharide
LT	Leukotrienes
LTA	Lipoteichoic acids
MAPK	Mitogen activated protein kinase
MAS	Motile aeromonas septicaemia
MCFAs	Medium-chain fatty acids
MDA5	Melanoma differentiation-associated gene 5
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	NO synthase
OM3EE	Omega-3 ethyl ester
OM3FO	Omega-3 from fish oil
ONOO-	Peroxynitrite
PAMPs	Pathogen associated molecular patterns
PBA	4-Phenylbutyrate
PG	Prostaglandins
PMSF	Phenylmethanesulfonylfluoride
PRPs	Pattern recognition proteins
PRRs	Pattern recognition receptors
<i>P. salmonis</i>	<i>Piscirickettsia salmonis</i>
PUFAs	Poly-unsaturated fatty acids
<i>R. salmoninarum</i>	<i>Renibacterium salmoninarum</i>
RIG-I	Retinoic acid-inducible gene I
RIPA	Radio-Immunoprecipitation Assay
RLRs	RIG-I like receptors
RQ	Relative quantification
SCFAs	Short-chain fatty acids
SPDV	Salmon pancreas disease virus
ss	Single strand
TA	Teichoic acids
TFA	Trifluoroacetic acid
TLRs	Toll-like receptors
TNF- α	Tumour necrosis factor α
VDREs	Vitamin D response elements
VDRs	Vitamin D receptors
Vit D3	Vitamin D3

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1 Introduction

1.1 Aquaculture and diseases

During the last decades catching wild fish has been in a deep crisis due to overfishing, indiscriminate exploitation or pollution and cannot supply the demand of seafood, including fish, in the world. Aquaculture has become an important alternative to wild fish and nowadays, fish farming produces around 50% of the world's total supply of seafood. Farmed salmon which is one of the most successfully raised aquaculture species can provide around 60% of the salmon consumed (FAO 2014). In Iceland Atlantic salmon aquaculture production reaches almost 50% of the total aquaculture production (Figure 1) and is an important source of income. Developing a sustainable and profitable fish farming industry is a challenge for government and entrepreneurs.

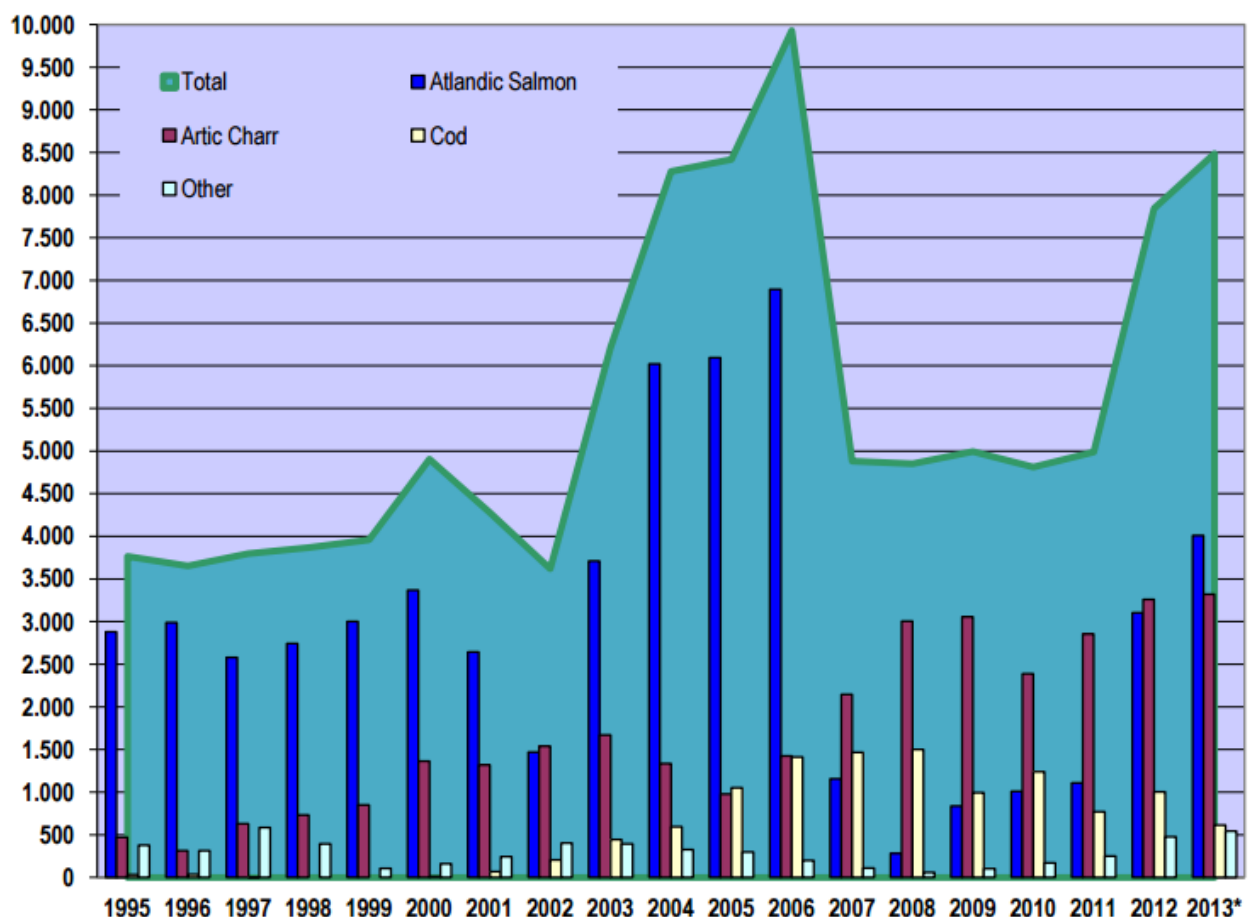


Figure 1: Production in aquaculture in Iceland in metric tonnes. *Forecast. Source: The Icelandic Aquaculture Association.

The main reason for economic losses in aquaculture is infectious diseases, caused by pathogens which have significant impact on global aquaculture production and account for 10% of the mortality (Noga et al. 2011). Antibiotics and vaccines are the main solutions used in aquaculture to control diseases, but there are several problems associated to their application. The overuse of antibiotics causes environmental damages, threat to public health, development of antibiotic-resistant pathogens and they are therefore banned in aquaculture in some countries. Vaccination, despite having been the main preventive method in fish farming in the last decades and being available against many of the serious bacterial diseases in the fish farming industries, are not effective against all the pathogens. So far there are no parasite vaccines commercially available. Viral vaccines, against infectious salmon anemia virus (ISAV), salmon pancreas disease virus (SPDV) or infectious hematopoietic necrosis virus (IHNV) if available, are less efficient than bacterial vaccines. Therefore viruses are often responsible for the high mortality rate observed in farming fish. Important limiting factors for vaccine efficacy are the delayed adaptive immune response and poor immunological memory. (Magnadóttir 2006; Whyte 2007).

Taking together these considerations, it is important to develop new prophylactic alternatives to vaccines and antibiotics.

In recent years, several studies have focused on studying antimicrobial peptides (AMPs) as antimicrobial agents to be use as substitute for vaccines and antibiotics. AMPs are an important part of the innate immune system and they play a critical role in the defense against pathogens. Fish AMPs gain in importance due to the fact that fish are in close contact with their environment thereby exposed to a large amount of potential pathogens and their adaptive immune system has limited repertoire of antibodies and a short-lived secondary response (Magnadóttir, 2006; Whyte, 2007).

Fish peptides have features that make them especially interesting to be investigated, including a broad-spectrum antimicrobial activity, immune modulatory functions and ability to act at high salt concentrations (Masso-Silva & Diamond 2014). Another factor that make AMPs interesting is that their expression can be regulated and they can be used as prophylactic for diseases and stress.

Finding new inducers to up-regulate fish AMPs expression could be the key to prevent infectious diseases in aquaculture and therefore to improve the efficiency of the farm fish industry

1.2 The immune system in fish

The function of the immune system is to fight against diseases by identifying and eliminating bacteria, viruses, fungi or parasites. Another role of the immune system is to modulate and control biological conditions during development and growth and to mediate inflammatory reactions and in tissue damage (Magnadóttir 2010). Classical division of the immune system is into the adaptive and innate immune response. This study is focused on the innate immune response.

1.2.1 Innate immune system in fish

The innate immune system, of prime importance in the immune defence of fish, is the first line of defence against pathogens and it is also important in activating the adaptive immune response. It is normally divided into 3 parts: the epithelial/mucosal barrier, the humoral parameters and the cellular components. Skin, gills and alimentary tract constitute the epithelial and mucosal barrier, an important disease barrier, due to the fact that fish are constantly immersed in media containing potentially harmful agents. Fish mucus provides a physical and chemical protection barrier rich in several immune defence components like lectins, pentraxins, lysozymes, antimicrobial peptides, complement factors and immunoglobulins (Alvarez-Pellitero 2008). The humoral parameters can be classified as cell associated parameters or soluble molecules including growth and protease inhibitors, lytic enzymes, lysozymes, agglutinins and poly-peptides. Finally, the cell components in fish include non-specific cytotoxic cells (similar to natural killer cells in mammals) and phagocytic cells, mostly neutrophils and macrophages (Secombes & Fletcher 1992). In teleost fish, several of these innate parameters are more active and with higher diversity than comparable components of mammals, for example complement components like C3 and Bf that play an important role in the activation of the complement system (Magnadóttir 2006).

All these components together with the pattern recognition receptors (PRRs) make the innate immune system an impressive weapon to fight against pathogens.

1.2.2 Pattern recognition receptors (PRRs)

Immune response is a complex system composed by a large amount of components working together to immune modulate the response and to link the innate and the acquire immune system (Akira et al. 2001). Activation of the innate immune system by pathogens and the subsequent activation of signaling cascades is orchestrated by germline-encoded pattern recognition receptors (PRRs), or pattern recognition proteins (PRPs). These receptors can recognize pathogen associated molecular patterns (PAMPs) associated with microbes and the damage-associated molecular patterns (DAMPs) an inherent danger signals from malignant tissues or apoptotic cells that trigger the innate immune response (Matzinger 2002). Typical PAMPs are various polysaccharides and glycoproteins, like bacterial lipopolysaccharide (LPS), flagellins, teichoic acid and peptidoglycan, DNA CpG motifs and virus associated double stranded RNA (dsRNA) (Janeway & Medzhitov 2002).

The recognition receptors of the innate system are a reflection of the evolution and adaptation of the species to specific environmental conditions. Environmental factors such as temperature or salinity as well as pathogenic associations are decisive for the innate immune response which has a non-specific recognition parameters (Magnadóttir 2006).

1.2.2.1 Toll-like receptors (TLRs) and RIG-I like receptors (RLRs).

The best characterized PRRs are the Toll-like receptors (TLRs), transmembrane proteins expressed by cells of the innate immune system. Members of the TLR family contribute both to microbe–cell interaction and to signaling. They are key components of the innate immune system, crucially involved in pathogen defense. Different TLRs, each one specific

for a broad range of PAMPs and DAMPs, are common for all vertebrate and even invertebrate species.

The evolutionary conservation of the key components of TLR signaling in vertebrates has been demonstrated by the study of several TLR genes in some teleost species (Roach et al. 2005). TLR activation pathways, features, immune response and the factors involved in downstream signaling in fish are similar to the mammals (Rebl et al. 2010). The following TLR families were identified in all vertebrates: TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11 (Roach et al. 2005). Respect to fish, the TLR arsenal differs among species and it is also reflected in their role as recognition receptors, for example TLR2 shows great structural diversity and genomic TLR2 sequences are intronless in channel catfish (*Ictalurus punctatus*) and zebrafish, but in pufferfish TLR2 has ten introns. TLR4 is another example. The role of TLR4 in LPS recognition is still unclear in fish and has been called into question in some fish species. TLR4 was not been detected in *F. rubripes* (Oshiumi et al. 2003), while two TLR4 were found in zebrafish genome (Rebl et al. 2010).

TLRs are an important key in the innate immune response against bacterial or viral infections (Figure 2) and TLR gene expression is summarized in the Pietretti review (Pietretti & Wiegertjes 2014). Larger-scale transcriptomic studies show clear changes in TLR gene expression after infection. In fish TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR9 are supposed to be important to recognize bacterial ligands while TLR3, TLR8, TLR9 and TLR22 seem to play an important role in the recognition of viruses (Langevin et al. 2013; Pietretti & Wiegertjes 2014).

			Up	Down	No change
B a c t e r i a		<i>L.crocea</i>	TLR1, 3 ¹	TLR2, 22 ¹	
		<i>S.aurata</i>	TLR2 ²		
	<i>A.hydrophila</i>	<i>S.salar</i>	TLR5 ³		TLR1, 3, 5, 20 ⁴
	<i>A.salmonicida</i>	<i>D.rerio</i>	TLR2, 4, 5 ⁵		
	<i>E.tarda</i>	<i>O.mykiss</i>			TLR2, 3, 5, 9, 22 ⁶
	<i>F.psychrophilum</i>	<i>D.rerio</i>	TLR5 ⁷	TLR8, 18 ⁸	
	<i>S.typhimurium</i>	<i>L.japonicus</i>		TLR1, 3, 18 ⁸	
	<i>V.harveyi</i>	<i>D.rerio</i>	TLR5, 21 ⁹		
v i r u s	HRV (dsRNA)	<i>P.olivaceus</i>	TLR2 ¹⁰		
	IPNV (dsRNA)	<i>S.salar</i>	TLR8, 9, 22 ¹²		
	SGIV (dsDNA)	<i>E.coioides</i>	TLR2, 5 ¹¹		

Figure 2: Summary of transcriptome studies in fish indicating the detection of clear changes in TLR gene expression following bacterial or viral challenge. Abbreviations: HRV = hirame rhabdovirus; IPNV = infectious pancreatic necrosis virus; SGIV = singapore grouper irido virus. Source: (Pietretti & Wiegertjes, 2014).

The antiviral strategies mediated by the innate immunity, include recognizing viral infection, as well as direct killing of infected cells. The main receptors for viruses are the Toll-like receptors (TLRs) and RIG-I like receptors (RLRs), both recognize pathogen-associated molecular patterns (PAMPs) and induce expression of interferons (IFNs), chemokines and pro-inflammatory cytokines (Figure 3) (Aoki et al. 2013). IFNs can act on infected cells by inducing expression of proteins with antiviral activity like MX proteins (Zou & Secombes 2011). RLRs are a cytosolic family of recognition receptors essential in the activation of type I IFN signaling and viral RNA-induced antiviral response (Takeuchi & Akira 2008). The RLR family is encoded by three genes: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), all sharing a functional helicase domain which contain a DEAD

domain, a C-terminal repression domain responsible of the antiviral activity in teleost and two tandem caspase recruitment domains (CARDs) present only in RI-I and MDA5, but not in LGP2 (Aoki et al. 2013). The RLRs family has been well characterized in mammals, but little is known about them in fish. MDA5 and LGP2 were found in many teleost fish genomes, but RIG-I has only been reported in cyprinids and salmonids (Biacchesi et al. 2009).

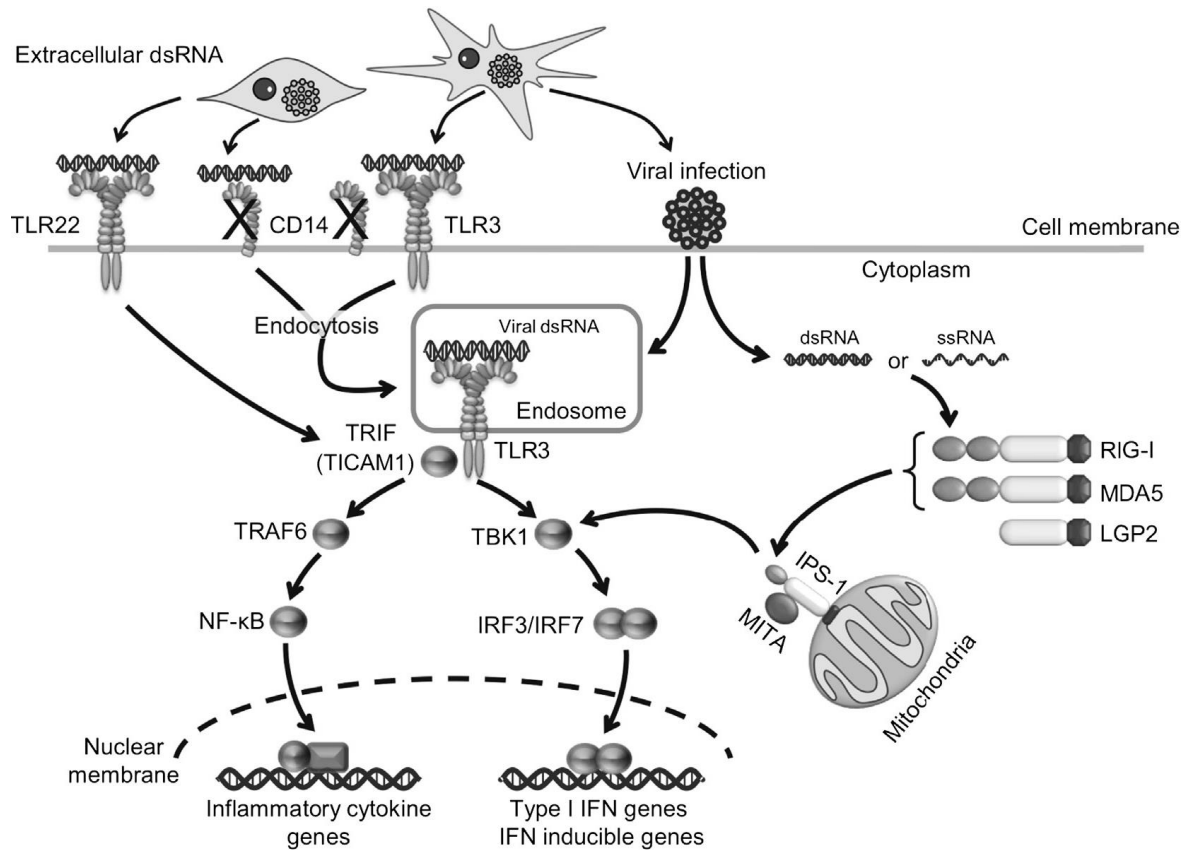


Figure 3: Schematic overview of TLR- and RLR-mediated viral dsRNA recognition in teleosts. Source (Aoki et al., 2013).

1.3 Antimicrobial peptides (AMPs)

Important components of the innate immune system to fight against pathogens are the antimicrobial peptides (AMPs), a conserved component of the innate immune system in all organisms, including plants and animals (Kościuczuk et al. 2012). AMPs represents a broad category of different families of highly conserved peptides, which exhibit broad-spectrum antimicrobial activity by killing bacteria, fungi, viruses or protozoa (Rivas et al. 2009; Zasloff 2002). While vertebrate antimicrobial peptides were initially characterized in rabbits (Selsted et al. 1983) and in humans during the mid-1980s (Ganz et al. 1985), the antimicrobial activity of fish peptides was not described until the 90s (Oren & Shai 1996).

Most of these peptides maintain certain common features such as containing positive charge and an amphipathic structure, but they can vary in sequence, structure and size, with

a range between 12 and 80 amino acid residues (Tomasinsig & Zanetti 2005). Based on their amino acid composition, size and conformational structures, AMPs can be divided into several categories, such as peptides with α -helix structures, peptides with β -sheet structures and linear peptides with a predominance of certain amino acids

The antimicrobial peptides act as endogenous antibiotics. Due to their cationic and amphipathic character they attack the microbial membranes and inhibit multiple internal targets like DNA/RNA synthesis, protein synthesis, cell division or different process regarding translation and protein folding (Zasloff 2002). AMPs can disrupt lipid bilayers through a variety of mechanisms, but generally AMPs are attracted by electrostatic forces to the negative phospholipid head groups on the membrane surface of the bacteria provided by capsular polysaccharides: lipopolysaccharides (LPS) in Gram-negative bacteria and teichoic acids (TA), lipoteichoic acids (LTA) and lysylphosphatidylglycerol in Gram-positive bacteria.

AMPs have more functions apart from direct antimicrobial action, like mobilizing and amplifying innate and adaptive immune responses of the host as 'immune regulators'. Upon exposure to danger, AMPs keep an overall balance by inhibiting microbial growth and inflammatory responses and they also play a protective and regenerative role after injury (Lai & Gallo 2009).

Multicellular organism tends to have a variety of different antimicrobial peptides in their cells and tissues (Zasloff 2002). Due to the fact that most of the AMPs are encoded as groups in the genome and their expression is regulated at transcriptional and post-transcriptional levels, they can be co-expressed and act together at a single site (Lai & Gallo 2009).

Focusing on fish, AMPs exhibit many if not all of the same characteristics as other vertebrate AMPs, like broad-spectrum antimicrobial activities and also immunomodulatory functions (Bridle et al. 2011). There are however interesting differences specific to fish due to the unique aquatic environments, for example piscidins show antibacterial activity even at high salt concentrations (Masso-Silva & Diamond 2014). Different AMPs were found in fish: i) cathelicidins, with cationic and amphipathic characteristic; ii) piscidins and pleurocidins which comprise a family of linear and amphipathic AMPs; iii) β -defensin-like proteins including the conserved 6-cysteine motif; iv) hepcidins that are cysteine-rich peptides or v) histone-derived peptides that have been identified in a number of fish species to be expressed and secreted in fish skin, gill, spleen and gut (Noga et al. 2011).

1.3.1 Cathelicidins

One important class of effector molecules of the innate immune system are the cathelicidins, a class of antibacterial peptides restricted to vertebrates which are produced as pre- pro-peptides with a conserved cathelin pro-part at the N-terminal and a varied C-terminal peptide region which is cleaved off upon activation (Zanetti et al. 2002). The cathelin domain is relatively well conserved in distant vertebrates and gives the name to the family. It has two disulphide bonds between 4 cysteine residues and, although still unclear, it seems to act as cysteine protease inhibitor with the capacity to inhibit cathepsin-L (Lai & Gallo 2009) and assist in the biogenesis of the mature peptide (Bals & Wilson 2003). The

C-terminal region shows a considerable sequence diversity and it is the result of an enzymatic process giving a relative small (less than 100 amino acid residue) and diverse C-terminal region. This region corresponds, for most of the AMPs, to the active antimicrobial peptide being usually cationic and amphipathic and aids in bacterial cell membrane disruption (Chang et al. 2006).

A conserved four-exon/three-intron organization characterizes the mammalian cathelicidins. Exons 1 to 3 contain the signal peptide and the cathelin domain, whilst the region coding for the predicted mature peptide, the processing site and the 3'UTR are specified in the exon 4 (Gudmundsson et al. 1995; Tomasinsig & Zanetti 2005) which exhibit a hypervariable sequence (Figure 4).

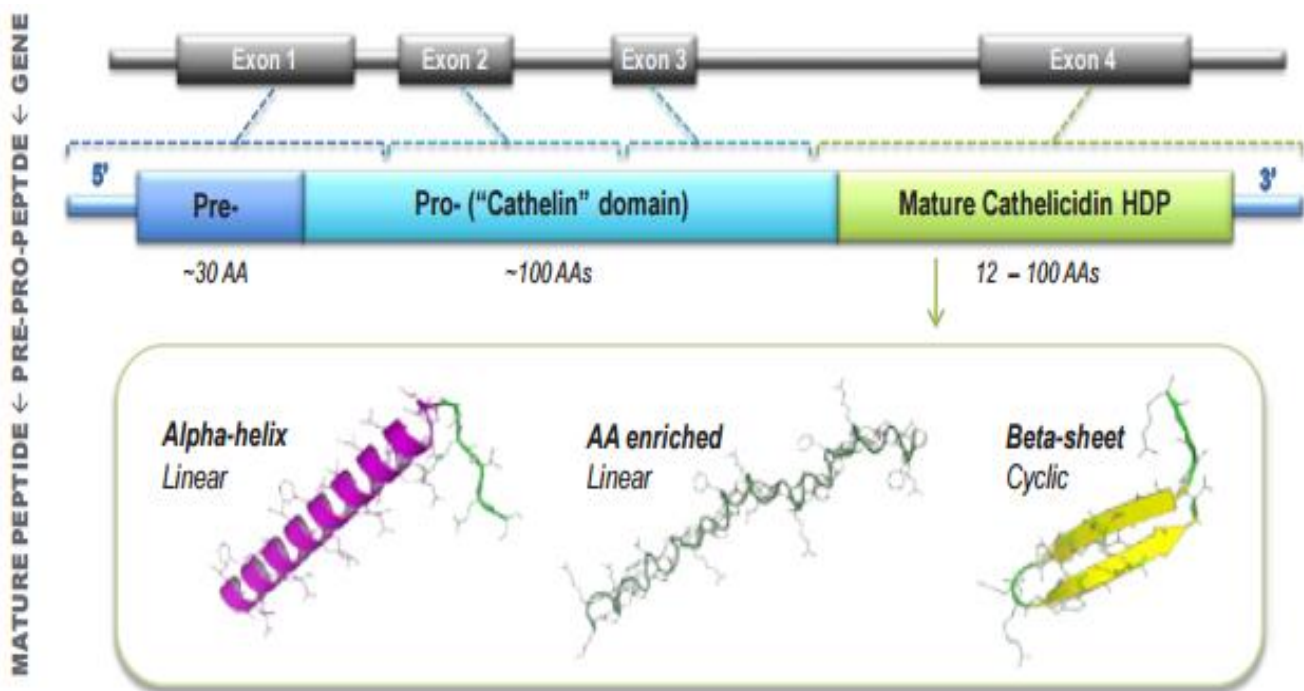


Figure 4: Gene organization and major structural classes of Cathelicidins. Source (Linde A. et al., 2013).

In the early 90s, cathelicidins were identified in bovine neutrophils (Zanetti et al. 1993) and subsequently they have been found in many vertebrates. A cathelicidin-like protein has been detected in the primitive vertebrate Atlantic hagfish (Uzzell et al. 2003), in birds (Xiao et al. 2006), chickens (Mukhopadhyay et al. 2010) and reptiles (van Hoek 2014). In mammals, cathelicidins are the best studied and have been reported in many different species (Dürr et al. 2006). The different members of the mammalian cathelicidins were found with a noticeable variability in the number of genes between different species (Tomasinsig & Zanetti 2005). In humans, the unique cathelicidin gene present (LL-37) is induced by inflammation, phagocytosis and microbial products (Frohm et al. 1997).

In fish several cathelicidins have been identified, for example rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), both have two different cathelicidins named CATH1 and CATH2 (Chang et al. 2006). Two years later cathelicidin

genes were identified in salmonid Arctic charr and Atlantic cod as well as a CATH2 orthologue in Chinook salmon (Maier et al. 2008a, b).

The fish and mammalian cathelicidins share several features, fish cathelicidin genes have the conserved four-exon/three-intron organization with the cleavage site and the antimicrobial domain in the fourth exon and the signal peptide and the cathelin domain through the 1-3 exons, with the exception that fish cathelicidins have a shorter signal peptide and a longer cathelin-like domain (Chang et al. 2006). An exon deletion in the cathelin coding region has been found in the cathelicidin genes in Arctic char (*Salvelinus alpinus*) and Brook trout (*Salvelinus fontinalis*) (Maier et al. 2008b).

Little is known about the function of cathelicidins in fish. Although recent studies have demonstrated their antimicrobial and potential haemolytic activity (Bridle et al. 2011) only a few studies investigated expression changes after bacterial infection. Up-regulation in the expression of Arctic charr and Atlantic cod cathelicidin as well as in Atlantic salmon after bacterial infection could suggest a role of cathelicidins in the innate immune response of fish (Maier et al. 2008a; Bridle et al. 2011)

Recently the immunomodulatory activity of cathelicidin was demonstrated in Atlantic salmon, the two Atlantic salmon cathelicidins induced the expression of IL-8 in peripheral blood leukocytes, suggesting that cathelicidins may be an evolutionarily conserved mechanism of innate immune regulation (Bridle et al. 2011).

1.3.2 Hepcidins

Together with cathelicidins and defensins, hepcidins are one of the major AMP families in fish (Rajanbabu & Chen 2011). Hepcidin is a small peptide hormone, cysteine-rich, that functions both as a homeostatic regulator of iron metabolism and as a mediator of host defence and inflammation (Masso-Silva & Diamond 2014). Hepcidins were first discovered in humans (Park et al. 2001) and since then they have been identified in many other vertebrates including reptiles (van Hoek 2014), amphibians and fish (Shi & Camus 2006). Hepcidins have been identified in near 40 fish species (Hilton & Lambert 2008; Álvarez et al. 2014), suggesting that the hepcidins are widespread among fish.

Fish hepcidin genes encode a pre-pro-protein, with a signal peptide, a prodomain and the mature peptide. Hepcidin mature peptide is cysteine-rich and 20-25 amino acid residues in length, the cysteine residues being highly conserved from mammals to teleost. The variants of teleost hepcidins are based on the number of cysteine residues, thus to the date there are three variants: 4, 6 or 8 cysteine residues (Xu et al. 2008). The disulphide bridges between the cysteine residue in hepcidin have been evolutionarily conserved and may form a hepcidin-like pattern (Douglas et al. 2003; Rodrigues et al. 2006). It was demonstrated that the number of disulfide bonds has an important role in their antimicrobial activity (Álvarez et al. 2014).

Similar to other AMP genes, fish hepcidins can be induced by exposure to both Gram-positive and Gram-negative bacteria (Gong et al. 2014) and also by viruses (Nicolas et al. 2002).

Fish hepcidins, similar to mammalian hepcidins, have dual functions: iron regulatory and host defence (Shi & Camus 2006). Iron regulation in fish is mediated by hepcidin that controls the serum iron levels during inflammation and infection by negatively regulating intestinal iron absorption and macrophage iron release (Falzacappa & Muckenthaler 2005). Little is known regarding the antimicrobial activity of hepcidin, nevertheless to control the iron levels could be the hepcidin way to indirectly interfere with pathogen survival, since low iron availability is a limiting factor for the bacterial growth (Ong et al. 2006).

1.4 Inducible nitric oxide synthase (iNOS)

Different strategies of host defence have evolved to protect the organism against pathogens and the nitric oxide (NO) system is one of them. Nitric oxide (NO), produced by NO synthase (NOS), an oxidative enzyme homologous to cytochrome P-450 reductase (Øyan et al. 2000) is an important ubiquitous gaseous signaling molecule. NO biosynthesis takes place in many cell types such as neurons, astrocytes, immunocytes or endothelial cells using L-arginine and L-citrulline as substrates through the action of nitric oxide synthases (NOS) and cofactor as NADPH, FAD, BH₄, calcium and calmodulin (Gorren & Mayer 2007). NO biosynthesis is dynamically modulated at the transcriptional and post-transcriptional level (Reviewed in Aktan 2004).

NO has different roles. In the immune response it is involved in reducing the infection response and, due to its high oxidative capacity, has an important role in the fight against pathogens (Steinert et al. 2010) while in physiological processes it is implicated in vascular regulation (Aktan 2004) or neural transmission (Steinert et al. 2010). In aquatic invertebrates, NO has been found to play an important role not only in immune defence, but also in biological functions like feeding, metamorphosis, regulation of blood pressure or environmental stress (Palumbo, 2005). There are two pathways to NO eliminates pathogens, indirectly generating the powerful oxidant peroxynitrite (ONOO-) (Thomas et al. 2008) or directly interacting with tyrosyl radicals to form nitrotyrosine, a marker of cell damage and inflammation (Davis et al. 2001) (Figure 5).

Direct Effects

([NO] < 200 nM)

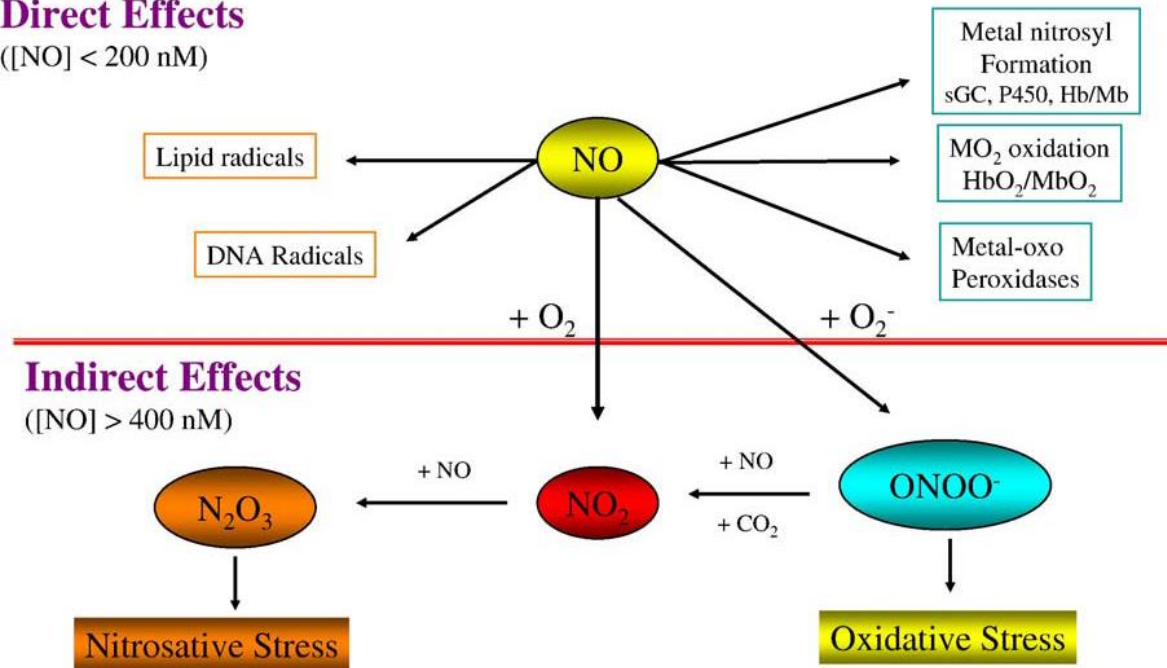


Figure 5: Direct and indirect effects of NO. Source (Thomas et al., 2008).

In vertebrates, three different isoforms of NOS are known: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), which are expressed by three different genes. iNOS (independent of Ca²⁺) is not constitutively expressed in resting cells, but can be activated in phagocytes, mainly macrophages through pro-inflammatory cytokines like TNF- α or INF- γ . Activation is through the mitogen activated protein kinase (MAPK) and Jak-Stat pathways, respectively, but other agents such as bacterial products (LPS), can also cause iNOS expression (Nathan & Xie 1994). iNOS mediates the ability of macrophages to kill or inhibit the growth of bacteria, viruses and fungi (Yang et al. 2013), however iNOS regulation is species-specific (Jungi et al. 1996) and depends on the type of stimulants. For example iNOS expression is directly or indirectly regulated by different amino acids such as arginine, asparagine, aspartate or phenylalanine, but not by leucine (Li et al. 2007).

Schoor and Plumb in 1994 reported for the first time the possible presence of NOS activity in fish tissues (Schoor & Plumb 1994) and later the partial sequence for iNOS in rainbow trout was cloned using cDNA from head kidney macrophages previously stimulated by the Gram-positive bacteria *Renibacterium salmoninarum* (Grabowski et al. 1996). iNOS has also been identified in salmon (Øyan et al. 2000) and characterized at the cell and molecular level in head kidney and liver in many fish tissues (Barroso et al. 2000).

1.5 Viral receptors

1.5.1 MDA5 and RIG1

Recognition of viral RNAs is one of the challenges for the innate immune system. Two different mechanisms have been developed to detect virus. On one hand, the TLRs (transmembrane receptors) recognizing viral dsRNA released by infected cells and on the other hand through RNA helicase proteins belonging to the RIG-I like receptors (RLRs) family: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), which function as cytoplasmic viral RNA sensors (Kang et al. 2002; Yoneyama et al. 2005).

A previous study has shown that the RLR family is well conserved among vertebrates (Zou et al. 2009). In teleost, the RIG-I, MDA5, and LGP2 proteins share a number of structural similarities, including their organization into three distinct domains (except for lack of caspase recruitment domains (CARDs) in LGP2).

Different studies showed that MDA5 emerged before RIG-I and the domain arrangements of the two genes seems to be the result of domain grafting and not because of a simple gene duplication followed by divergent evolution (Sarkar et al. 2008). MDA5 and RIG-I show similarities in their structure and signaling pathways converging both at the adaptor level, however the identity between them at the gene level is not very high, for example the identity between MDA5 and RIG-I in grass carp is 27.4% (Aoki et al. 2013) or in catfish is 25% (Rajendran et al. 2012). Teleost homologues of the family of RLRs have been identified and characterized in different fish species: RIG-I in salmon, trout and cyprinids, MDA5 and LGP2 in trout, cyprinids and flounder (Lauksund 2014; Chang et al. 2011).

The three proteins belonging to the RLRs family are not excluding one another but it seems that they may act at the same time to detect viral RNA in fish cells (Chang et al. 2011). The RLRs family is considered RNA virus receptors, although some studies shown that LPS can induce the MDA5 mRNA expression levels in Japanese flounder (Ohtani et al. 2010) or the Gram-negative bacteria *Edwardsiella ictaluri* can induce MDA5 and RIG-I in channel catfish liver suggesting that both proteins have a broad sensing range and can respond to both viral dsRNA and bacterial components displaying an important role not only in the defence against virus, but also against bacteria (Rajendran et al. 2012).

1.5.2 Interferon stimulated genes (ISG15)

IFNs type I play an important role in the innate immune system to fight against viruses and are present in all vertebrates, including mammals and fish (Kileng et al. 2007).

In mammals, the family of IFNs includes type I IFN, type II IFN and type III IFN also called IFN- λ and the differences between them are at the gene and protein structure level as well as in functional properties. Type I IFN family has a major role in the first line of the antiviral response. Two families of IFN genes have been found in teleost, (type I and II). In fish, type II IFN is divided in two groups : IFN- γ and a teleost specific IFN- γ -related molecule while type I IFN is divided in two subfamilies (Zou & Secombes 2011). Little is known about the IFN system in fish, but it seems that both type I and II IFNs can bind to

surface receptors and trigger the immune response via the Jak/Stat pathway (Samuel 2001). Type I IFNs induce the expression of many interferon stimulated genes (ISG), including ISG15 (Røkenes et al. 2007) and Mx (an antiviral protein) (Altmann et al. 2003), but type II IFN can also induce the expression of ISG15 and Mx, suggesting a cross-activation of the innate antiviral response for both type I and II IFNs (Zou & Secombes 2011).

IFN stimulated genes (ISGs), are a group of genes whose expression is directly induced by IFN. Recently, several kinds of ISGs were identified in fish such as Mx, ISGs, PKR, PKZ, Gig, viperin, Drel, and TRIMs (Langevin et al. 2013) (Figure 6). One of the earliest and most predominant proteins induced after IFN stimulation is ISG15 which suggests an important function of this protein in the IFN system and therefore in the response against viruses (Briolat et al. 2014).

ISG15 homologues have been discovered in different fish species and one copy of the gene was found in Atlantic salmon (Røkenes et al. 2007), three copies in Atlantic cod (Furnes et al. 2009) and two in crucian carp (Zhang et al. 2007).

The function of ISG15 is not clear, but it seems to be similar to ubiquitin. ISG15 contains two ubiquitin-like domains and a LRGG motif in the C-terminus, necessary for the covalent conjugation in ISGylation. Unlike ubiquitination, ISGylation does not target proteins for proteasome degradation, but modifies using activation or suppression of target proteins, enzymatic degradation, subcellular localization and half-life of proteins (Herrmann et al. 2007). Therefore, ISG15 has an important role mediating in the activation or attraction of cells implicated in antiviral defence and is induced by either RNA or DNA viruses (Furnes et al. 2009).

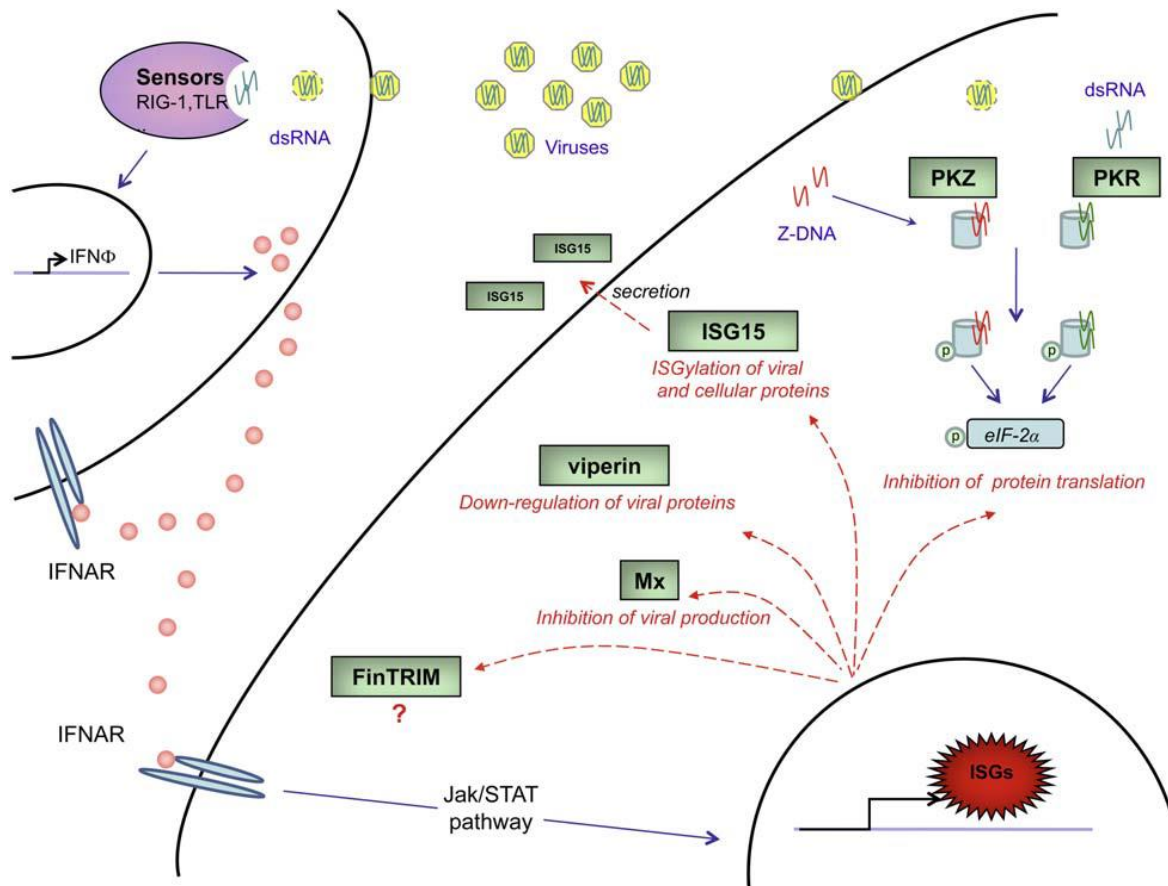


Figure 6: Fish virus induced genes in the context of interferon-signaling. Following virus entry in the cell, viral nucleic acids bind different sensors as RIG-1 and TLRs. Activation of these sensors leads to the induction and secretion of interferons. This cytokine has auto- and paracrine activity mediated through interactions with a transmembrane receptor (IFNAR) that activates Jak/Stat pathway. It then leads to the induction of a set of interferon stimulated genes (ISGs) such as *Isg15*, *pkz*, *pkz*, *Mx*, *vig1/viperin* or *finTRIMs*. The entities inducing the IFN and effector responses are indicated in blue and the putative antiviral functions of the different genes are in red. Source (Verrier et al. 2011).

1.6 INDUCERS

Stress and diseases caused by pathogens can be responsible for losses in aquaculture. The expansion and profitability of the sector is therefore limited by the efficiency of antibiotics and vaccines, but at the same time aquaculture is under pressure to limit the use of drugs, due to their risk to humans and the environment. The key to solve this problem could be the use of compounds that induce the immune system, specifically the innate immune response. The use of natural compounds has several advantages such as biocompatibility, biodegradability, safe by human health and cost-effectiveness. In this project, different inducers were assessed as immunostimulants and their effectiveness was measured in terms of immune genes expression.

1.6.1 Butyrates

Short chain fatty acids are an important source of energy and in the mammalian digestive tract they are produced through the anaerobic microbial fermentation of unabsorbed complex carbohydrates from dietary fiber or they derive from structured lipids like tributyrin (Wächtershäuser & Stein 2000). Fatty acids are a large group of carboxylic acids with an aliphatic hydrocarbon chain that can be saturated or unsaturated. They can be classified as short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), or long-chain fatty acids (LCFAs).

SCFAs are mainly absorbed and metabolized as an energy source, but a small part passes to the blood. Acetate, propionate and butyrate are the three major species of SCFAs, but butyrate is the major form of short-chain fatty acid. Butyrate is known for its ability to influence cell function by altering the regulation of gene expression via inhibition of histones deacetylase (HDAC), which leads to increase transcriptional activity by chromatin remodeling (Wächtershäuser & Stein 2000). Butyrate was found to not only induce expression of antimicrobial peptides in different species like humans (Schauber et al. 2003) or chicken (Sunkara 2003), but also reduce mucosal inflammation and enhance barrier defences thereby acting as modulator of the host immune response (Wilson & Gibson 1997).

The anti-inflammatory effect of butyrate is connected to the suppression of NF- κ B activation (Yin et al. 2001), the inhibition of the interferon- γ synthesis (Klampfer et al. 2003) and the vitamin D receptor (VDR). Expression of VDR is enhanced by butyrate at both mRNA and protein level and its role in anti-inflammatory regulation is well known (Gaschott et al. 2001).

1.6.1.1 β -hydroxy- β -methylbutyrate (HMB)

HMB (β -hydroxy- β -methylbutyrate) is a metabolite of the amino acid leucine that is produced naturally in animals, including humans. Leucine is an essential amino acid in muscle metabolism and also one of the three amino acids that are termed branched chain amino acids (BCAA). Leucine has two important roles at the metabolism level: activator of some signaling pathways, like the mammalian target of rapamycin (mTOR) and as precursor for protein synthesis (Li et al. 2007).

HMB is produced through the transamination of leucine to α -ketoisocaproate (KIC) and then metabolized to HMB by the action of KIC-dioxygenase in the liver (Sabourin & Bieber 1983). Around 5 % of leucine metabolism is through this pathway and was observed in both humans and animals. HMB is not only implicated in the protein and cholesterol synthesis (Adamson & Greenberg 1957), but also increases the cellular and humoral immune response in animals (Siwicki et al. 2003). Little is known about the effect of HMB on the expression of immune genes in humans and even less in fish, T-cell proliferative response was not modified after stimulation with HMB at physiological concentrations, but it modulated the T-cell-derived cytokine production and reduced TNF- α availability in peripheral blood mononuclear cells (Nunes et al. 2011).

Some studies have demonstrated the effectiveness HMB supplementation in the diet. In common carp for example the oral administration of HMB reduced the mortality induced by motile *Aeromonas septicaemia* (MAS) suggesting that HMB might be used as a dietary immunostimulant (Siwicki et al. 2011).

1.6.1.2 4-Phenylbutyrate (PBA)

PBA is an aromatic short chain fatty acid converted to phenylacetate in the mitochondria by β -oxidation. PBA has been reported to act on cultured tumour cells with cytotoxic effects (Carducci et al., 1996) and it is also known to regulate gene expression in the cells through inhibition of histone deacetylase (HDAC) modifying the accessibility of transcription factors to the nucleosomal DNA (Hamer et al. 2008). PBA has been reported to stimulate the transcription of antimicrobial peptides in humans (Steinmann et al. 2009) and fish (Broekman et al. 2013), but the mechanism of how PBA stimulates antimicrobial peptides transcription is still not clear. Previous results suggest that PBA modulate cathelicidin expression in humans (*CAMP*), indirectly promoting the expression of other genes encoding important factors for *CAMP* gene expression and this study also demonstrated an important role of the mitogen activated protein kinase (MAPK) signaling pathway in the *CAMP* gene expression mediated by phenylbutyrate (Steinmann et al. 2009).

The advantages of using PBA as therapy for different diseases have been demonstrated in different studies, PBA was approved for clinical use in urea cycle disorders in children (Maestri N.E., Brusilow S.W., Clissold D.B. 1996) and protects against ischemic brain injury exerting its anti-inflammatory ability (Qi et al. 2004). Taking together the role of PBA as innate immunostimulant, anti-inflammatory effector and protection against some diseases, makes PBA a powerful candidate as an inducer of the innate immune response in fish.

1.6.2 Vitamin D₃

The role of the vitamin D, a steroid hormone, in the immune system of humans has gained in relevance over the past few years. The function of vitamin D in the endocrine system is well known, however how the vitamin D exert its influence in the immune response is still unclear and in fish totally unknown

In fish vitamin D₃ is part of the dietary intake including phytoplankton and zooplankton (Rao & Raghuramulu 1996), while in humans is obtained by exposition of the skin to the UVB radiation and in a lesser extent from dietary intake. Vitamin D₃ becomes active by the hydroxylation of 25-hydroxy-vitamin D₃ (25(OH) D₃) which happens first in the liver, producing the primary circulating form of vitamin D (24, 25(OH)₂ D₃) (Ponchon et al. 1969) and secondly in the kidneys and immune cells (White 2008), to be converted into the active vitamin D metabolite (1,25(OH)₂ D₃; calcitriol). Vitamin D₃, as a steroid hormone is directly linked to the endocrine system, regulating the metabolism of calcium and phosphorous (Bronner & Pansu 1999; Finch et al. 1999). A functional vitamin D endocrine system as well as all the vitamin D metabolites, were found in Atlantic salmon (Lock et al. 2007), carp, cod and zebrafish (Swarup et al. 1984; Sundell et al. 1993; Lin et al. 2012) and appears to function in fish in a similar manner to mammals (Larsson et al. 2003). In humans the function of vitamin D in the immune system is related to specific

cells and tissues, modifying the expression of immune products like cathelicidin (Liu et al. 2006) and β -defensin 2 (Wang et al. 2004) and playing an important role as booster of the innate immune response. Vitamin D exerts its modulation role in the immune system through the vitamin D receptors (VDRs), a member of the nuclear receptor superfamily and a ligand-activated transcription factor, which in turn binds to the vitamin D response elements (VDREs) located in the promoters of the target genes thereby activating their expression. VDRs are expressed in most immune cells, including inflammatory cells and through the binding with vitamin D₃ modulates the T-cell proliferation and cytokine production (Provvedini et al. 1983). Little is known about function and role of vitamin D₃ and the vitamin D pathway in the fish immune system. VDRs have been found and sequenced in several fish species including Atlantic salmon (Lock et al. 2007) and the presence of VDRs in different fish species and the similarity of the vitamin D endocrine system between mammals and fish suggest that vitamin D₃ might also play a role in the immune response in fish.

1.6.3 Lipids from fish

In the past decades, the interest in the effect of consumption of fish oils and their role as enhancers of the immune system has increased, but the mechanism of action at the genetic level is still poorly understood.

Omega fatty acids are classified as omega-3 fatty acids (n-3), omega-6 fatty acids (n-6) or omega-9 fatty acids (n-9). Biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both omega-3 fatty acids, are the result of the desaturation and elongation of n-3 poly-unsaturated fatty acids (PUFAs) (Arkhipenko & Sazontova 1995). Humans cannot synthesize EPA and DHA, but they are essential nutrients in the diet for optimal health and can be absorbed directly through the consumption of fish. Oil from marine fish like mackerel, herring, sardines and salmon are rich in PUFAs, especially those belonging to the family of EPA and DHA (Wanasundara & Shahidi 1998). EPA and DHA can also be found in high concentrations in cod liver oil extract (Curtis et al. 2004) along with vitamin D and vitamin A, an antioxidant member of the lipid-soluble retinoid compounds with important roles in immunity (Hünkar et al. 2002).

The membrane of most immune cells is rich in certain fatty acids, especially arachidonic acid (AA). AA is the principal substrate for eicosanoid which is produced after release of AA from the membrane. Eicosanoid is metabolized to produce mediators of the immune system like prostaglandins (PG), thromboxanes or leukotrienes (LT). Among them, prostaglandins (especially 2-series PG: PGE₂) have the most important pro-inflammatory effects. The fatty acid composition of immune cells is very sensitive to changes in its fatty acids proportion, thus dietary fish oil rich in n-3 PUFAs increases the amount of EPA and DHA and decreases the amount of AA. n-3 PUFAs are precursors of immune mediators such as 3-series PGs, all of them biologically less active than the immune mediators derived from AA (Hudert et al. 2006). At the same time, the mediators derived from EPA/DHA compete directly with those derived from AA for the receptors in the target cells inhibiting the pro-inflammatory effects, suppressing the lymphocyte proliferation and NK cell activity or inhibiting the production of tumour necrosis factor- α (TNF- α), interleukine (IL)1, IL-6, IL-2 and interferon- γ (INF- γ). Several studies have reported that EPA and DHA have a clear anti-inflammatory and immunomodulatory role not only because of the

substitution of derived mediators but also by acting through pathways which involve cyclooxygenase and lipoxygenase enzymes that generate new mediators, like resolvins or docosanoids (Serhan et al. 2002). On the other hand, n-3 PUFAs were demonstrated to decrease the production of reactive oxygen species by neutrophils and monocytes or the T lymphocytes proliferation, but the dose-response came up with conflicting results (Calder 2001). All the effects described above are related to the fatty acid composition of the immune cell membranes, but some studies suggest that n-3 PUFAs act as immune modulators by eicosanoid-independent intracellular pathways and transcription factors that regulate the immune gene expression (Miles & Calder 1998). The effect of the dietary fish oil in the host defences against pathogens is in controversy, thus some authors suggest that n-3 PUFAs impair the cell-mediated immune response in mice after challenge with bacteria (Bonilla et al. 2010) while others found that the host immune response was complete (Dumlao et al. 2012). Therefore, there are many open questions regarding the effect of the dietary fish oil in the immune system and this project can shed light on these questions.

1.7 Possible applications for fish AMPs

Fish AMPs share the structural characteristics of mammalian, insect or amphibian homologues, however they have special features that make them interesting for therapeutics treatments and for pharmaceuticals or food industries. Among others, fish AMPs have a broad-spectrum activity against a wide variety of pathogens including fish and human pathogens (Sung et al. 2008). Importantly they have a potent antimicrobial activity under a wide range of conditions, such as low temperature or at high salt concentrations (Lauth et al. 2002; Lee et al. 2008). The identification and characterization of peptides from fish have provided a unique contribution to the therapeutic area due to their effectiveness as antimicrobial and anti-tumoural agents in humans (Chen et al. 2009) or immunomodulators of the immune system (Rajanbabu & Chen 2011).

The last few years, AMPs have gained in importance as vaccine adjuvants by their ability to induce the production of cytokines or IFNs which at the same time can induce the adaptive immune response, thus combining AMPs with adjuvants may enhance the cell-mediated response (Kovacs-Nolan et al. 2009). Another interesting use for the AMPs is as inactivated vaccines and is related to their ability to kill pathogens. some of those pathogens can be used to stimulate the cell-mediated immune system, avoiding the problems related to the conventional vaccines like the use of formalin, closely linked with allergic reactions (Rajanbabu & Chen 2011). With regards to the food industry, AMPs have proved to be a natural and safe alternative to the massive use of food preservatives, thus some AMPs like bacteriocin (Rydlo T. et al. 2006) or pleurocidin (Cole et al. 2000) have demonstrated advantageous features as natural preservatives, because their ability to kill bacteria at different temperatures and salt concentrations.

In summary, characterizing fish AMPs and inducing their expression can provide many advantages, not only for the fish farming industry as an alternative for the classical prophylactic methods, but also for the food and pharmaceuticals industries as preservative and therapeutic treatment, respectively

2 AIMS

The aim of this thesis was twofold; Firstly to define new inducers of the innate immune system of salmon, using CHSE-214 cell line as a model system. Our specific aim was to determine the effect of the inducers on the expression of cathelicidin, hepcidin and iNOS, genes mainly implicated in the host defence against bacteria, and MDA5, RIG-I and ISG15, which play a critical role on the defence against viruses. Further we wanted to determine if the stimulation of the innate immune system seen at the gene level is also observed at the protein level, reflected in an increase of the antimicrobial activity.

The second aim of this thesis was to acquire an antibody against asCath 2, which can be used as a fast and efficient method for quantification and qualification of cathelicidin in Atlantic salmon.

3 MATERIALS AND METHODS

3.1 CHSE-214 cell culture

The Chinook salmon (*Oncorhynchus tshawytscha*) cell line CHSE-214 (Lannan, Winton, & Fryer, 1984) was cultured in MEM (Earles medium containing GlutaMAXTM-1 and 25 mM HEPES) supplemented with 10% foetal bovine serum (FBS), 0.5% penicillin-streptomycin (25 u/ml and 25 mg/ml, respectively), 1% non-essential amino acids and 1% sodium bicarbonate (all from Gibco/Invitrogen). Cells were culture in 75 cm² flasks and split (with 0.05 % Trypsin) into 25 cm² flasks for experiments. Cells were grown and cultures at 19°C and passaged every 2-3 weeks and never more than 10 times.

3.2 CHSE-214 stimulation

To find new inducers CHSE-214 cells were grown in 25 cm² flasks until 100% confluence. Five milliliters of medium, with serum and antibiotic, were added to each flask. Fish cells were induced with the following stimulants: *E.coli* LPS (Fluka #62325), β -Hydroxi- β -methyl butyrate (HMB) (Maximuscle), 4-phenylbutyrate (PBA) (Tocris), 1,25-dihydroxyvitamin D₃ (Vit D3) (Tocris) and purified lipids from fish (Lysi). *E. coli* LPS was added to a final concentration of 50 μ g/ml, HMB was added to the concentrations of 2 and 20 mM, PBA to 2 and 4 mM and Vit D3 to 20 nM. Cells were stimulated with LPS for 24 hours (h), with butyrates and Vit D3 for 24 h, 48 h and 72 h and with lipids for 3 h. The control was untreated cells for butyrates and Vit D3 and mineral oil for lipids.

For the experiment with fish oils, cells were grown in 25 cm² flasks until 100% confluence. Five ml of fish oils were added to the flasks after the medium was removed. Cells were stimulated for 3 h and 5 ml of new medium with serum and antibiotic was added to the flasks giving a total volume of 10 ml. Cells were incubated for 24 h at 19°C. Fish oils used were omega-3 fish oil (O3FO), cod liver oil (CLO) and omega-3 ethyl ester (O3EE). Fish oils used were of commercial quality from Lysi hf. (Reykjavik, Iceland). The omega-3 fish oil composition is 18% eicosapentaenoic acid [EPA] and 12% docosahexaenoic acid [DHA]. Cod liver oil is rich in EPA, DHA and vitamin A and omega-3 ethyl ester (highly refined omega-e fatty acids) resulting from the trans-esterification of free fatty acids (FFA) with ethanol.

For antimicrobial activity assays, cells were induced with butyrates, Vit D3, omega-3 (O3FO) and cod liver oil (CLO) at the concentrations of 20 mM for HMB, 4 mM for PBA and 20 nM for Vit D3 in 12 ml of medium, serum-free and without Penicillin-streptomycin, at the time points of 12 h, 24 h, 48 h and 72 h or 3 h for O3FO and CLO.

3.3 mRNA extraction from CHSE-214 cells

Total RNA was extracted after 3, 24, 48 or 72 h induction with TRIzol reagent (Invitrogen), the protocol was modified to use half of all the reagents, therefore 0.5 ml of TRIzol reagent, 0.1 ml of 3-Bromo-3-chloropropane (BCP) and 0.25 ml of isopropanol (ISP) per 25 cm² flask were used followed by ethanol precipitation and the resulting RNA pellet was resuspended in 30 µl TE buffer. Genomic DNA was removed by digestion using DNase I (New England Biolabs) following the manufacturer's instructions. Quantity and quality of the resulting RNA was measured using NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). To assess the RNA quality agarose gel electrophoresis were also performed. Total RNA was adjusted to a final concentration of 100 ng/µl. One µg of RNA was reverse transcribed to cDNA in a final volume of 20 µl using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences). The absence of genomic DNA was confirmed by preparing several samples without reverse transcriptase (RT-), used as negative control.

3.4 qPCR for CHSE-214 genes expression and statistical analysis

Gene expression was estimated by qPCR using 1 µl of cDNA template in a final volume of 10 µl. Water and RT- were used as control. All samples were run in duplicates. Real-time PCR was performed in 96 well-PCR plates using Power SYBR green PCR Master Mix as recommended by the manufacturer (Applied Biosystems) with the exception of using 10 µl as final reaction volume. Thermal cycling parameters were 50°C for 2 minutes (min), followed by a 95°C hot start for 10 min, the amplification was performed by 40 cycles of 95°C for 15 seconds (sec) and 60°C for 1 min. qPCR primers were using in a final concentration of 0.25 µM. The Chinook salmon cathelicidin primers were designed with PerlPrimer, additional primers were selected from the literature (Table 1). The qPCR expression was normalized using RPS20 and EF-1α, with primers sequences published previously (Peña et al. 2010; Ingerslev et al. 2006). Primer efficiencies were calculated and showed a range between 89 and 112% suitable for the $2^{-\Delta\Delta C_t}$ method (Table 1), although ideally the range for the efficiency should be between 90% and 110% (Livak & Schmittgen 2001). In the $2^{-\Delta\Delta C_t}$ method, the cycle threshold (Ct) was defined as the cycle number at which the fluorescence passes the fixed threshold and relative quantity (RQ) represent the relative changes in gene expression. Data were presented as Fold change (log of relative quantity) of target mRNA, normalized by the endogenous controls (RPS20 and EF-1α) Ct expression, and calibrated by Ct value obtained from untreated cells.

To estimate the gene expression, untreated cells (control) were set as value 1 and treated cells values were compared to this value. Each stimulated sample was compared to its control at each time point. One way ANOVA for the controls were performed and did not significant differences between the three time points (24 h, 48 h, and 72 h). Statistical analysis were performed with One way ANOVA and Tukey-test in Rstudio and treated samples were compared against the mean of all the controls. One way ANOVA was used to analyze the significant difference between means and Tukey test was used to a multiple comparison of means due to it is a more robust test. *P values* ≤ 0.05 were considered significant.

Table 1: Sequence for qPCR primers and efficiency in CHSE-214 cell lane.

Target	Primers	Efficiency
csCath2	Forward: 5'-ATG GGA AAC GAA TGA TGT GC-3' Reverse: 5'-CGG TCA GTG TTG AGG GTA TT-3'	107 %
asCath2	Forward: 5'-TAC TGA GCA CTC AGA AGA TTC GGA-3' Reverse: 5'-TCT TTA CTA CCC ATC TTA GAG CCC-3'	103%
Hepcidin 1	Forward: 5'-GCT TCT GCT GCA AAT TCT GAG G-3' Reverse: 5'-GTA CAA GAT TGA GGT TGT GCA G-3'	94 %
iNOS	Forward: 5'-AAC GAG AGC CAA CAG GTG TC-3' Reverse: 5'-GGT GCA GCA TGT CTT TGA GA-3'	112 %
MDA5	Forward: 5'-CAG AGG TGG GGT TCA ATG AT-3' Reverse: 5'-AGC TCG CTC CAC TTG TTG AT-3'	90 %
RIG1	Forward: 5'-GAC GGT CAG CAG GGT GTA CT-3' Reverse: 5'-CCC GTG TCC TAA CGA ACA GT-3'	101 %
ISG15	Forward: 5'-AAG TGA TGG TGC TGA TTA CGG-3' Reverse: 5'-TTG GCT TTG AAC TGG GTT ACA-3'	89 %
EF1a	Forward: 5'-GCT GTG CGT GAC ATG AGG-3' Reverse: 5'-ACT TTG TGA CCT TGC CGC-3'	101 %
RPS20	Forward: 5'-AGC CGC AAC GTC AAG TCT-3' Reverse: 5'-GTC TTG GTG GGC ATA CGG-3'	99 %

3.5 Induction of innate immunity in Atlantic salmon

For this experiment 100 healthy fish were reared at the farm of Stofnfiskur, in Iceland. The fish were reared in circular tanks with a water volume of 100 L each, the water was oxygenated by an EHEIN air pump 400. The temperature was maintained stable at 4°C. The fish of approx. 150 gr were fed with 50 mg HMB/kg food at 4% body weight per day with a commercial salmon feed (Inicio 1.5 mm, BioMar, Denmark) using automatic feeders. The control group was fed with pellets without HMB. Fish were stimulated for 96 h until samples were collected.

3.6 mRNA extraction and qPCR gene expression for Atlantic salmon.

Total RNA was extracted from gill and placed in a tube with TRIzol reagent (Invitrogen) as explained in section 3.3, with the exception of use 0.15 ml of TRIzol reagent, 35 μ l BCP and 0.2 ml ISP. Samples were homogenized using stainless steel Beads (Berani Uster, CH) in a mixer Minibeadbeater (Biospec products). Gene expression was measured by qPCR as described in section 3.4 with the exception of the qPCR expression was normalized using only EF-1 α as housekeeping gene and we used specific primers for Atlantic salmon cathelicidin (Table 1).

3.7 Bacterial culture.

For the antimicrobial assays, two different bacteria were chosen, the Gram-positive and non-pathogenic bacteria *Bacillus megaterium* (strain Bm11) and the Gram-negative *Aeromonas salmonicida* ssp. *Achromogenes* (ASA) that cause atypical furunculosis or ulcerative inflammations of skin and muscles in fish (Gudmundsdottir et al. 1990). Bm11 was culture in 5 ml of Luria-Bertani (LB) medium containing streptomycin, cultured shaking at 30°C for 16 h. The overnight culture was diluted 1:100 in new LB medium and cultured until OD₆₀₀ = 0.6, approx. 2-3 h. ASA was culture in 5 ml of Luria-Bertani (LB) medium, cultured shaking for 48 h at 24°C. The culture was diluted 1:100 in new LB medium and cultured until OD₆₀₀ = 0.6, for approx. 6-7 h. ASA was previously isolated from *Salmo salar* and characterized at Keldur (Gudmundsdottir et al. 1990). Bacterial growth curves were assessed by reading at OD₆₀₀ (Figure 7).

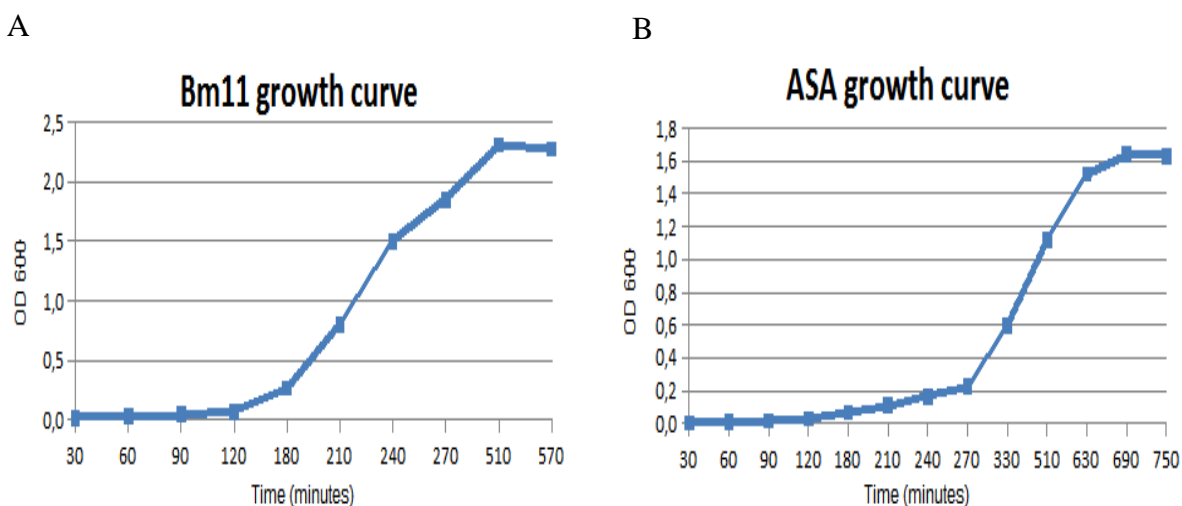


Figure 7: Growth curve of *Bacillus megaterium* (strain Bm11) and *Aeromonas salmonicida* ssp. *achromogenes* (ASA) in Luria-Bertani (LB) medium at 30°C and 24°C respectively.

3.8 Protein extraction from the cell medium

CHSE-214 were grown in 75 cm² flasks until 100% confluence and stimulated as described in paragraph 3.2, but in a final volume of 12 ml medium containing 10% FBS without antibiotics. Proteins from cell culture medium were enriched in OASIS HLB columns (Waters). The columns were activated with one volume of 100% acetonitrile (ACN) followed by three volumes of 0.1% trifluoroacetic acid (TFA) to equilibrate the columns. Samples were acidified with 0.1% TFA and loaded onto the columns. Afterwards salts were washed from the columns by adding first one volume of 0.1% TFA and then one volume of 20% ACN containing 0.1% TFA. Proteins were eluted by adding 1.5 ml of 80% ACN containing 0.1% TFA. Proteins extracted from one 75 cm² flask were aliquoted into two Eppendorf tubes, frozen and lyophilized overnight in a HETO Maxi dry lyo. Lyophilized samples were resuspended either in 9 µl of water for antimicrobial activity assay or in 30 µl of loading buffer (4x) for western blot assay. As negative control and in order to examine if proteins/peptides were responsible for the antimicrobial activity, one enriched extract from the Oasis columns was digested with proteinase (pronase E (Sigma-Aldrich)) following the instructions. Subsequently the digested samples were lyophilized as described before, and resuspended in 9 µl of water.

3.9 Protein extraction from CHSE-214 pellet

CHSE-214 cells from the 75 cm² flasks were washed with PBS. Three milliliters of Trypsin-EDTA (0.05%) were added to detach the cells. The cells were centrifuged at 1000 rpm, the supernatant was discarded and the cell pellet was resuspended in 3 ml of PBS. One milliliter of resuspended pellet was centrifuged again, discarding supernatant, and the pellet was resuspended in 0.5 ml of TRIreagent for qPCR analysis as described in paragraph 3.4. The remaining 2 ml were centrifuged and the pellet was resuspended in 300 µl RIPA buffer (PMSF 1 mM). This suspension was incubated for 10 min on ice and afterwards sonicated for 5 sec at an amplitude of 50%. Subsequently the sample was centrifuged at 14000 rpm for 20 min at 4°C and loading buffer (4x) was added to the supernatant. Protein concentration from the lysate solution was measured according to Bradford assay.

3.10 Protein extraction from Atlantic salmon sperm and gill

Gill samples were pooled from 10 randomly selected fish stimulated with HMB as explained in paragraph 3.5. A total of 100 mg of pooled samples were homogenized with a Kontes™ Pellet Pestle™ Cordless Motor in 500 µl RIPA buffer (PMSF 1 mM), incubated for 10 min on ice and sonicated 3 times for 10 sec at an amplitude of 50% using a cell sonicator (Sonics vibra from SONICS). Total lysate was spun at 14000 rpm for 20 min at 4°C. Total proteins were denatured in loading buffer by incubation for 10 min at 95°C. Protein concentration was measured according to Bradford assay.

Sperm samples were collected and pooled from 3 individuals (without stimulation). Extraction and protein concentration measurements were performed as described above with the exception of using 150 mg of tissue in 1.5 ml of RIPA buffer (PMSF 1 mM).

3.11 Inhibition zone assay

For the inhibition zone assay, ASA and Bm11 subcultures were diluted 1:10 and 50 µl of this dilution was added to 6 ml of 1% LB agar, previously melted and keep it at 47°C until the bacteria were ready. LB agar containing the bacteria was mixed and poured on petri dishes, letting the plates settle for 20 min at room temperature. Small wells (3 mm) were punched and 3 µl of lyophilized sample resuspended in water was loaded in the wells. As positive control 1.5 µl ampicillin (100 µg/ml) was used, as negative control 3 µl of 0.1% TFA.

Agar plates with Bm11 were cultured overnight at 30°C, while agar plates containing ASA were cultured 48 h at 24°C and subsequently bacteria-free zones were measured.

3.12 Gel overlay assay

Proteins were extracted with RIPA buffer from either Atlantic salmon sperm or gill described in section 3.10 or from homogenized cell lysates as described in section 3.9. Protein concentration was measured according to Bradford assay, denatured in loading buffer by incubation at 95°C for 10 min and 20-30 µg of soluble proteins were loaded onto SDS-PAGE gel, 10% acrylamide. Electrophoresis was carried out at 110 volts for 1 h. SDS-PAGE gel was performed in duplicate to perform overlay gel assay or coomassie staining. For the overlay assay proteins were fixed to the gel by immersion for 30 min in fixation solution containing 20% isopropanol and 10% acetic acid in distilled water. Gels were detoxed by washing in 200 ml dH₂O for 2 h at room temperature (4 times, 50 ml each). As control, loading buffer was loaded onto SDS-PAGE gel and the fixation and detox protocol was followed as described before. After detox, gels were placed in a petri dish and 10 ml of LB agar containing equal amount of bacteria (Bm11 or ASA) were poured over the gels. The agar was cooled down at room temperature and subsequently the petri dish were incubated either overnight at 30°C for Bm11 or 48 h at 24°C for ASA. After this time, bacterial-free zones were observed and recorded. In order to correlate the observed bacterial-free zones to protein size the overlay gels were superposed over the coomassie stained gels and the proteins were located by overlapping.

3.13 Synthetic peptides and antibody production

Two peptides, 14 and 13 amino-acid, for asCath2 (1: C-terminal and 2: N-terminal) were produced synthetically (GenScript) with over 85% and 93% purity, respectively (Figure 8). Affinity-purified antibody were produced against either the synthetic asCath2 C-terminal peptide (AB1) or the synthetic asCath2 N-terminal peptide (AB2) in rabbit by GenScript (Figure 8). Both antibodies were used at the final concentration of 5 µg/ml for the immunoassays.

A polyclonal mouse anti serum (asCath2AB) was generated previously (in Keldur) against a recombinant 6 His-tagged asCath2 fusion protein. A DNA fragment corresponding to full length of asCath2 was amplified and cloned in frame into a plasmid (pET100). Expression

was optimized in *E. coli* BL21 (DE3) as describe below in paragraph 3.17. His-tagged protein was purified by affinity in a nickel-NTA column. The asCath2AB dilution was 1:1000 for immunoassays.

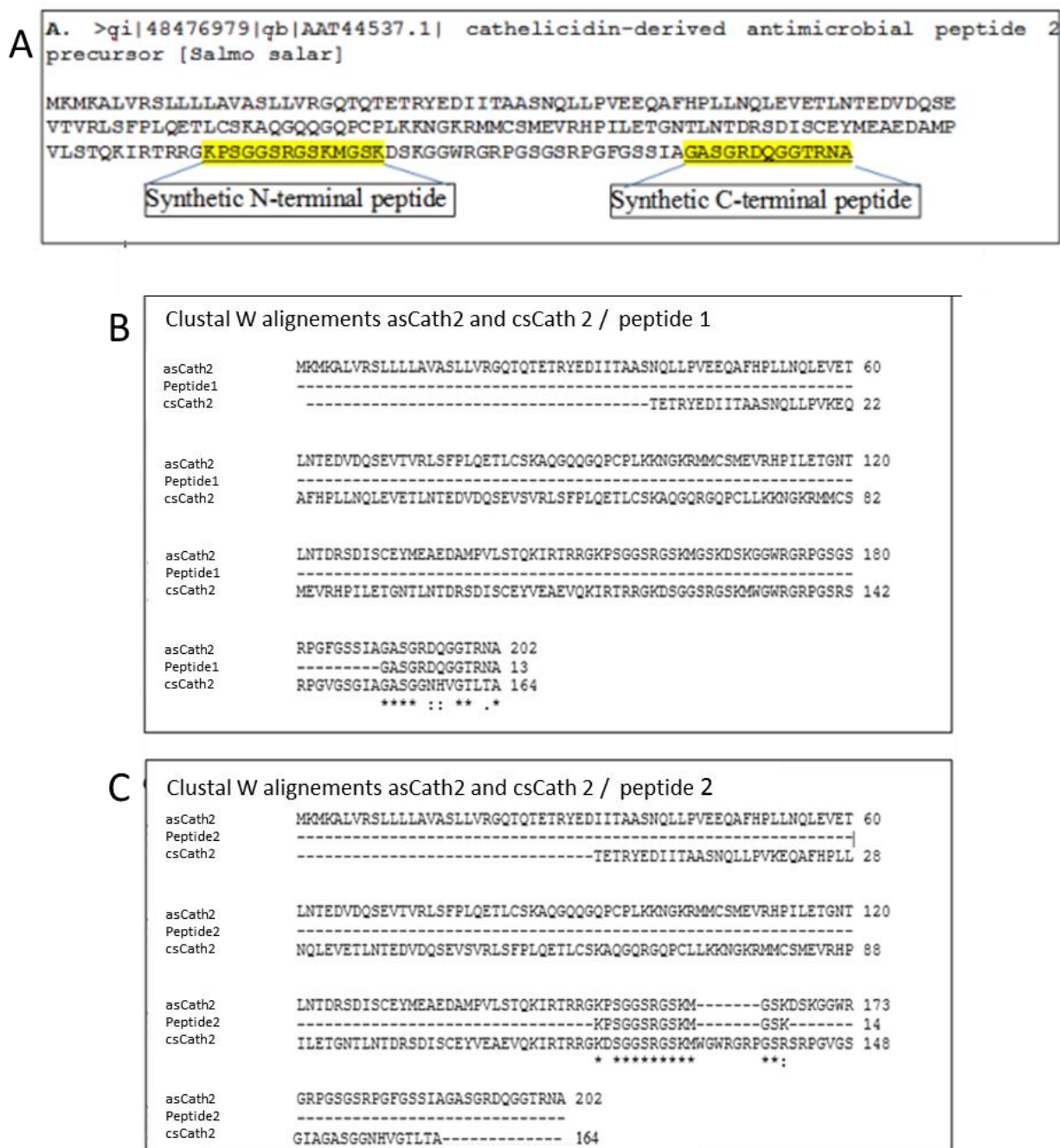


Figure 8: The *Salmo salar* cathelicidin antibodies used in this study were raised against the amino acid sequence highlighted in yellow (A). ClustalW alignments of synthetic asCath C-terminal peptide (B) and synthetic asCath N-terminal peptide (C) with the deduced amino acid sequences of asCath2 and csCath2.

3.14 Directional TOPO cloning

3.14.1 mRNA extraction from gill and cDNA preparation

Total RNA was extracted, using TRIzol reagent (Invitrogen), from approximately 30 mg of tissue from Atlantic salmon stimulated with HMB as explain in paragraph 3.5. Genomic DNA was removed by digestion using DNase I (New England Biolabs) following the manufacturer's instructions. Quantity and quality of the resulting RNA was measured using NanoDrop ND-1000 UV/Vis- Spectrophotometer (NanoDrop Technologies), agarose gel was also performed to assess the RNA quality. Total RNA was adjusted to a final concentration of 100 ng/μl. One μg of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences).

3.14.2 PCR, direct TOPO cloning and TOP10 Chemically Competent *E. coli* transformation

PCR was performed for asCath2 cDNA using *Taq* polymerase and specific primers, including the 4 base pair sequences (CACC) necessary for directional cloning on the 5' end of the forward primer (Table 2). PCR was performed by 95°C for 4 min followed by 39 cycles of 95°C for 1 min, 60°C for 1 minute and 72°C for 1 minute, with a final extension step of 4 min at 72°C. Agarose gel electrophoresis was used to check the integrity and yield of PCR products.

PCR products were directly cloned into the pET200 vector (Invitrogen) following the protocol. Fresh PCR product (3 μl), salt solution 1 μl, sterile water 1 μl and ® TOPO vector 1 μl were mixed gently and incubated for 5 min at room temperature. Afterwards the reaction was placed on ice and One Shot® TOP10 chemically competent *E. coli* were transformed following the protocol.

After transformation, more than 100 colony forming units (cfu) were obtained and 10 colonies were taken for "colony PCR" and at the same time put in LB with kanamycin (50 μg/ml) to grow overnight at 37°C. The subculture was spun at 6000 g for 5 min. The pellet was resuspended in LB-glycerol (30%) and frozen at -80°C.

Table 2: Primers to clone asCath2.

Oligo Name	Type	Sequence (5'-3')
asCath-2 Fwd	DNA	CAC CAT GAA GAT GAA GGC TCT GGT
asCath-2 Rev	DNA	CTA TGC ATT GCG AGT TCC ACC TTG G

3.14.3 Plasmid DNA extraction for sequencing

NucleoSpin Plasmid Miniprep kit (Macherey- Nagel) was used to extract plasmid DNA. TOP-10 transformed cells were taken from the glycerol stock and stroked out onto 10 ml of LB with kanamycin (50 μg/ml) to grow overnight at 37°C with shaking. Plasmid DNA extraction was performed according to manufactures recommendations. Plasmid DNA was recovered with 50 μl of AE buffer (5 mM Tris-HCl, pH=8.5). Quantity and quality of the

resulting DNA was measured using NanoDrop ND-1000 UV/Vis- Spectrophotometer (NanoDrop Technologies), agarose gel was also performed to assess the DNA quality. Plasmid DNA was submitted to sequence to Beckman Coulter Genomics.

3.14.4 Plasmid DNA extraction to transform BL21 (DE3) competent *E.coli*

Plasmid DNA was extracted from TOP10 *E. coli* using Quick Boiling Plasmid Miniprep protocol. 1.5 ml of overnight culture was centrifuged for 5 min at 14000 rpm. Supernatant was removed and 300 µl of STET buffer was added containing 200 µg of lysozyme. Reactions were incubated for 10 min on ice and then boiled for 2 min in closed tubes. Subsequently the reactions were centrifuged and 200 µl were transferred into a new tube and 200 µl of ice-cold isopropanol was added. DNA was precipitated for 30 min on ice and centrifuged for 10 min at 14000 rpm at 4°C. DNA was washed twice with Ethanol 70%. The dried pellet was resuspended in 50 µl of TE. Quantity and quality of the resulting DNA was measured using NanoDrop ND-1000 UV/Vis- Spectrophotometer (NanoDrop Technologies), agarose gel was also run to assess the DNA quality.

3.14.5 Transform BL21 (DE3) competent *E.coli* and protein expression

One hundred µl of BL21 (DE3) competent *E. coli* were defrosted on ice, 1 µl of plasmid DNA was added, mixed gently and incubated on ice for 5 to 30 min. Subsequently the cells were heat-shocked for 30 sec at 42°C. The tube was immediately transferred to ice and 250 µl of room temperature S.O.C. medium was added. After incubation at 37°C for 1 hour with shaking, 100-200 µl of bacterial culture was spreaded on a prewarmed selective plate with kanamycin and incubated overnight at 37°C. After transformation, more than 100 cfu were obtained and 10 colonies were taken for “colony PCR” and at the same time put in LB with kanamycin (50 µg/ml) to grow overnight at 37°C. The BL21 (DE3) cells were induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) at OD600 = 1.1 and grown for 6 h. Equal amounts of cells were selected for SDS-PAGE analysis and western blot before and after induction.

3.15 asCath2 isolation

BL21 (DE3) cells were harvested after induction, spun at maximum speed and supernatant was discarded. The pellet was resuspended in 10 ml of 10 mM Tris hydrochloride (Tris-HCl) (pH=7.4), sonicated 4 times for 30 sec at an amplitude of 40% and afterwards spun again. Supernatant was dialyzed in a Spectra/pore membrane (mwco: 12-14000) overnight against 1L 10 mM Tris-HCl. The dialyzed fraction was loaded on a DEAE-sephacel column. The column was washed with 2 volumes of 10 mM Tris-HCl and eluted by a gradient step (10 ml of each 75, 100, 125, 150, 200, 225, 250, 500 mM NaCl in 10 mM Tris-HCl (pH=7.4)) and all of them were fractioned into 1 ml fractions. The positive fractions for asCath2 were pooled and stored at -20°C.

3.16 Western blot and dot blot

Proteins extracted from cell medium and pellet (paragraph 3.8 and 3.9) from cells stimulated as described in paragraphs 3.2 and total proteins from transformed BL21 (DE3) culture described in paragraph 3.14.4 were denatured in loading buffer by incubation for 10 min at 95°C. Equal amounts of proteins, according to Bradford assay, were loaded onto the gels. Electrophoresis was performed at 110 V for 1 h and the proteins were blotted in a nitrocellulose membrane (0.1µm, Amersham) by wet transfer for 1 h at 30 V. Blotting was carried out with 5% fat free milk in PBS containing 0.05 % Tween20. The blot was incubated with asCath2 antibody (Keldur) and affinity purified antibodies AB1 and AB2 (GenScript) overnight at 4°C. After washing, the membrane was incubated with secondary antibody (Polyclonal goat anti-mouse IgG/HRP and Polyclonal goat anti-rabbit IgG/HRP, respectively). Bands were visualized with chemifluorescence and chemiluminescence (ECL) with Pierce ECL plus substrate using a Typhoon 9400 scanner.

3.17 Indirect immunofluorescence

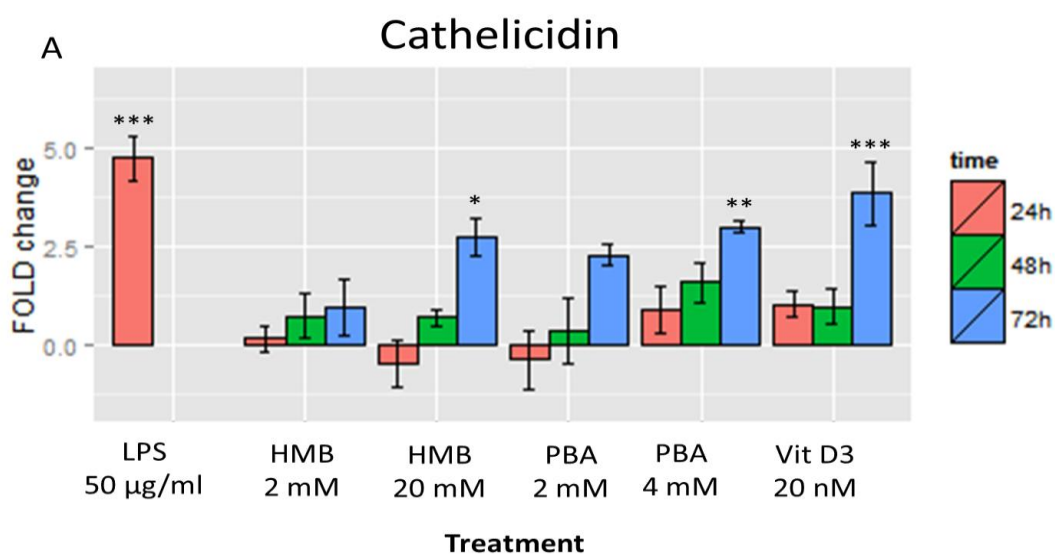
CHSE-214 cells from two 75 cm² flasks were split into 6 well plates and grown on sterile covers until 100% confluence. Cells were stimulated in 3 ml serum and antibiotic free media, with either 20 mM HMB, 4 mM PBA, 20 nM Vit D3 for 72 h or with O3FO and CLO for 3 h. Untreated cells were used as control. Experiments were performed by duplicate. After stimulation the cells were washed with PBS, fixed with 3.7% para-formaldehyde for 15-20 min at room temperature and subsequently washed again with PBS. Cells were permeabilized with 0.4% Triton X-100 in PBS for 10 min and then washed for 10 min. Free formaldehyde was blocked with 50 mM NH₄Cl for 10 min. After washing for 5 min cells were quenched with 10% FBS for 20 min at room temperature. CHSE-214 cells were incubated for 1 h at room temperature with asCath2 antibody (Keldur) and affinity purified rabbit antibodies (AB1 and AB2) (GenScript). Primary antibodies were removed by washing 4 times for 5 min and the cells were incubated for 1 hour at room temperature with Alexa Fluor® conjugated secondary antibody (diluted 1:1000 in PBS/ 1% BSA) and TOPRO (1:5000). After the cells were washed with PBS-Tween20, approximately 10 µl of fluoromount was added and covered with a coverslip. All washes were performed in PBS. Results were analyzed in a fluorescence microscope.

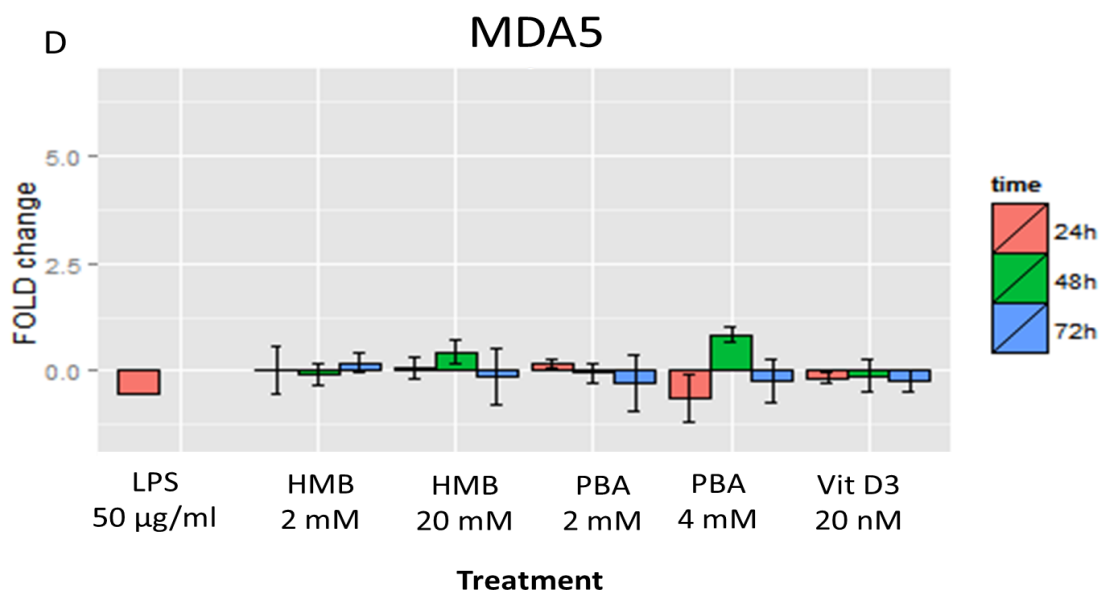
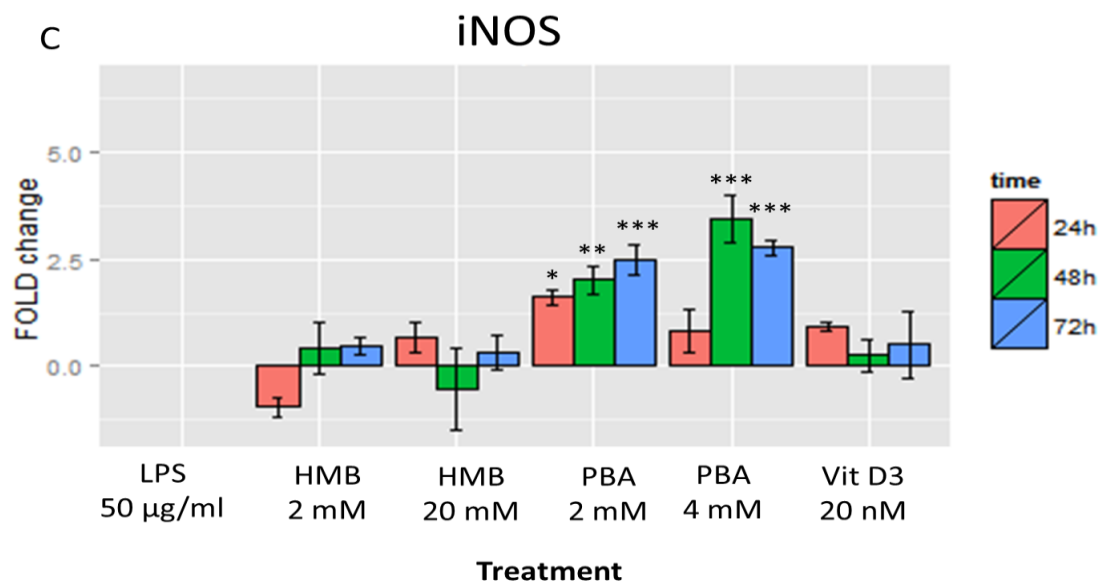
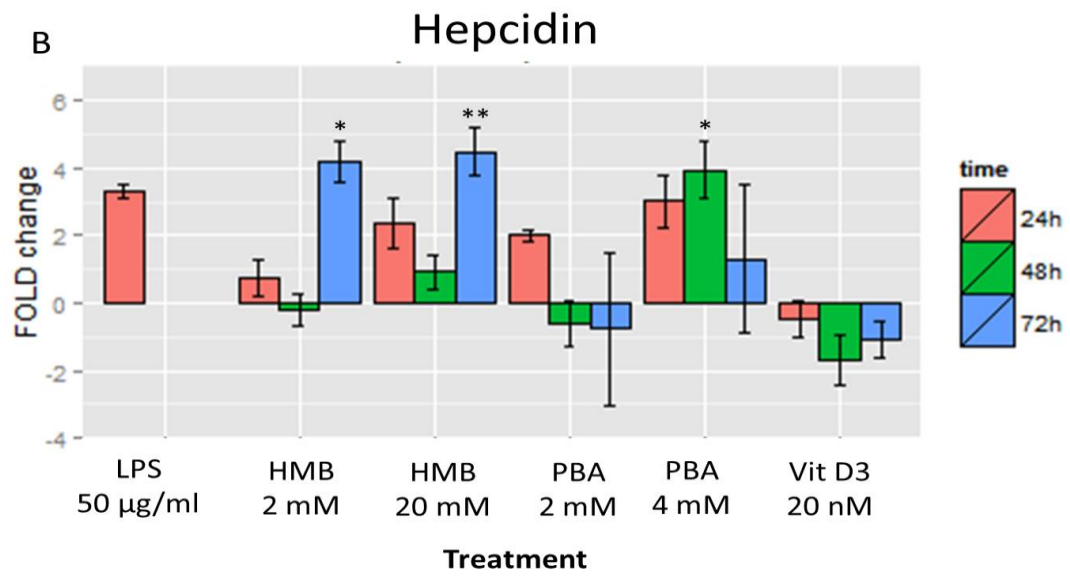
4 RESULTS

4.1 Gene induction

4.1.1 Induction of immune genes by butyrates and 1, 25-dihydroxyvitamin D3 (calcitriol)

In order to determine which inducer can stimulate gene expression in CHSE-214 cells, we treated the cells with different compounds and analyzed the expression of several genes involved in the fish immune system using qPCR. Cathelicidin 2 (csCath2) expression was dependent on the concentration of the inducers and length of the treatment (Figure 9A). csCath2 expression was significantly increased at 72 h with 20 mM HMB, 4 mM PBA and Vit D3. As a positive control LPS was used to increase csCath2 after 24 h as previously described (Maier et al. 2008). Hepcidin expression was up-regulated by LPS, HMB and PBA. Hepcidin was significantly increased after 72h treatment with HMB at both concentrations and with 4 mM PBA after 48 h. iNOS expression was up-regulated by PBA and this induction was found to be significant at all-time points except for 4 mM PBA after 24 h. Remarkable is the fact that after induction with LPS iNOS expression disappeared (Figure 9C). MDA5 expression was not affected by any of the treatments used here (Figure 9D). RIG1 expression was increased by HMB and PBA and this increase was dependent on concentration and time. A significant increase in RIG1 gene expression was found with 2 mM HMB at 72 h, 20 mM HMB at 48 and 72 h, 2 mM PBA at 72 h and 4 mM PBA at all three time-points. Vit D3 on the other hand had no effect on the expression of RIG1 whereas LPS induction resulted in a slightly and not significant down-regulation. (Figure 9E). ISG15 expression was found to be significantly increased with 20 mM HMB and 4 mM PBA after both 48 and 72 h, but LPS and Vit D3 had no effect on the expression of this gene (Figure 9F). These results show that most of the immune genes (antibacterial and receptors) analyzed in this study, except MDA5, were differentially regulated by the inductors over the course of 72 h post-induction.





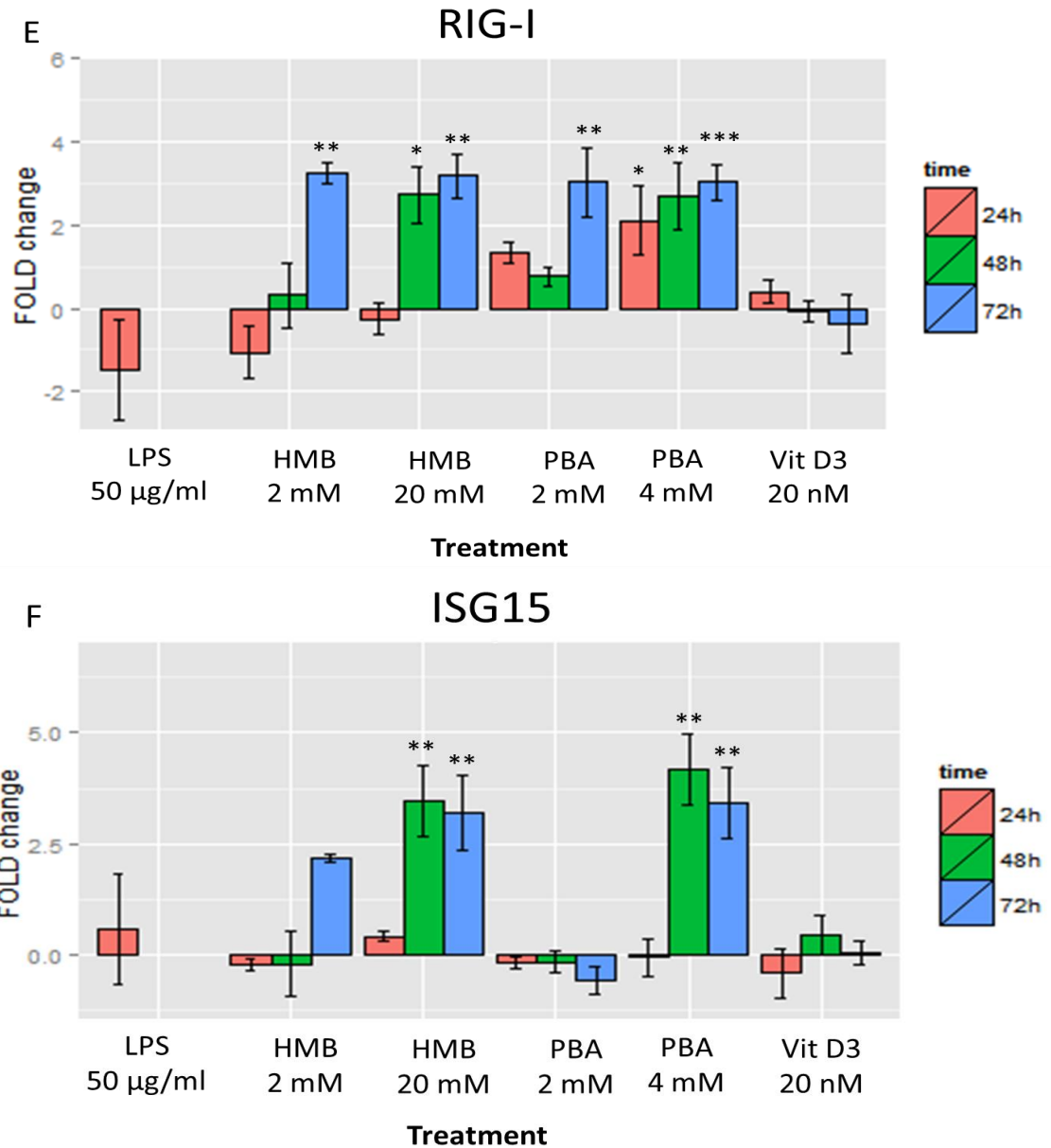
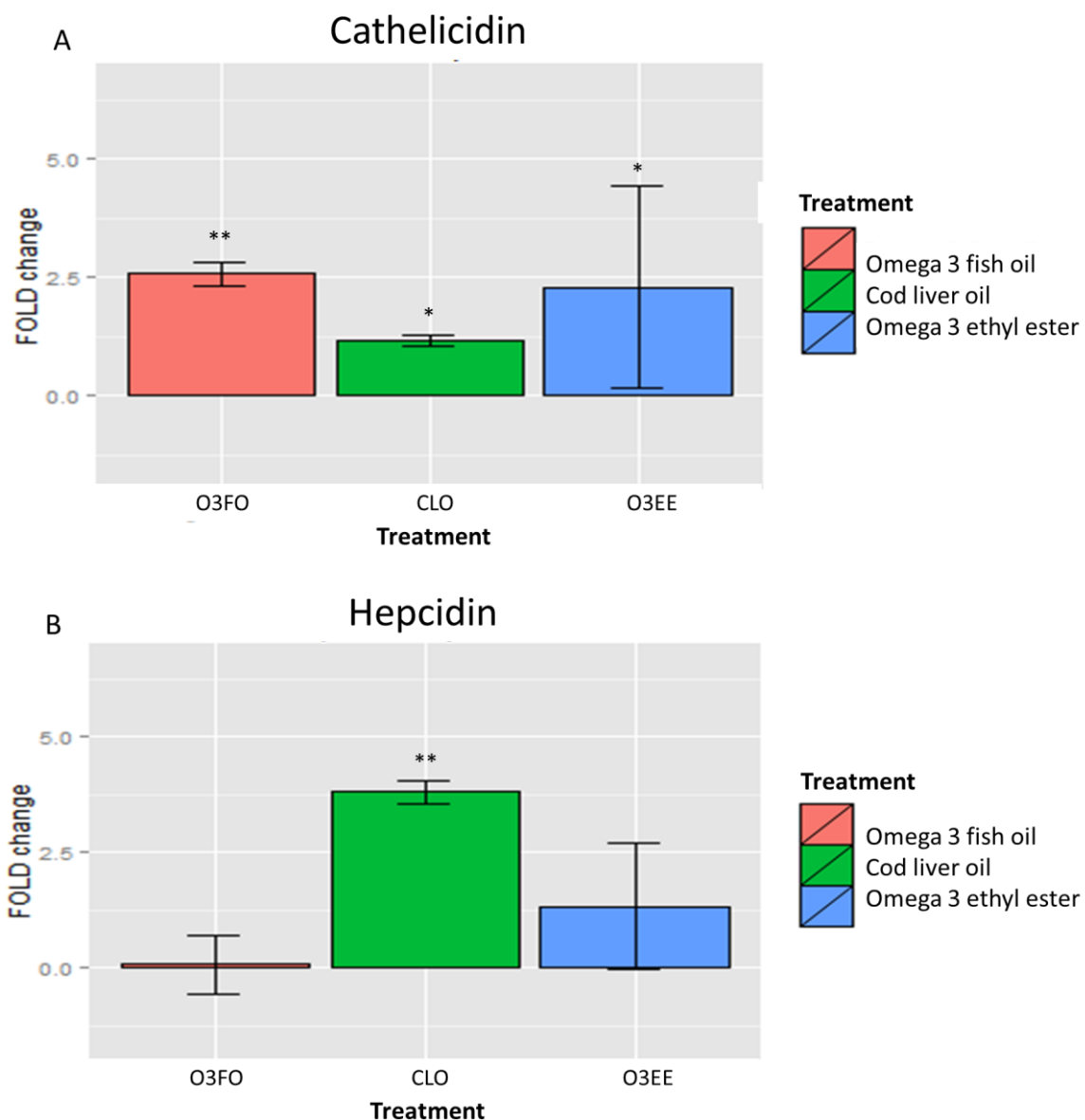
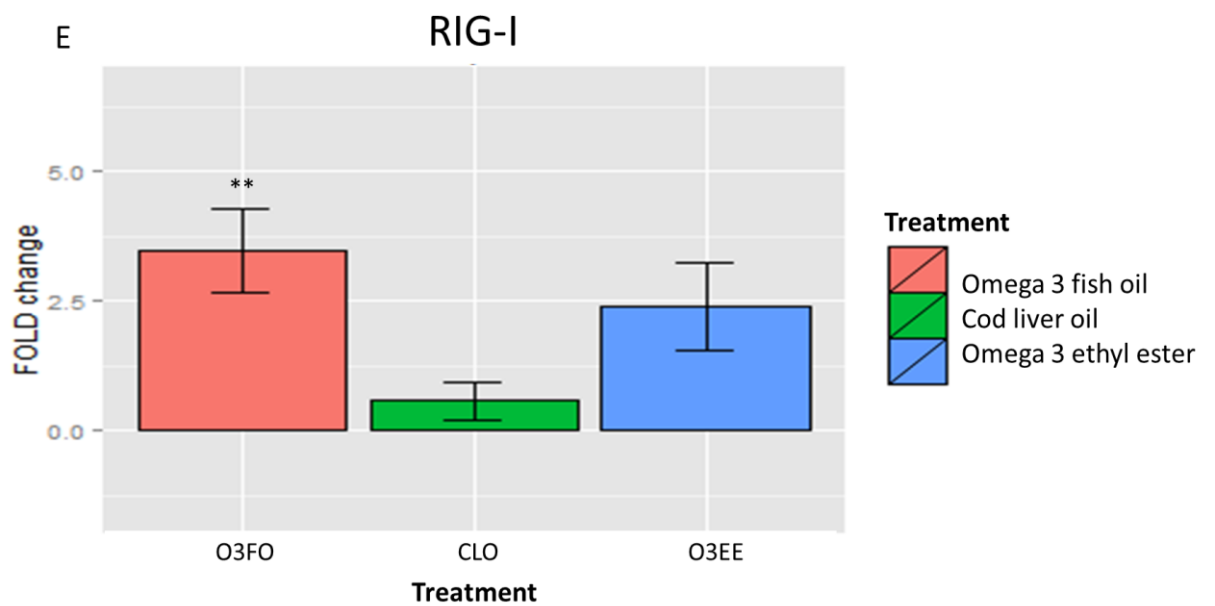
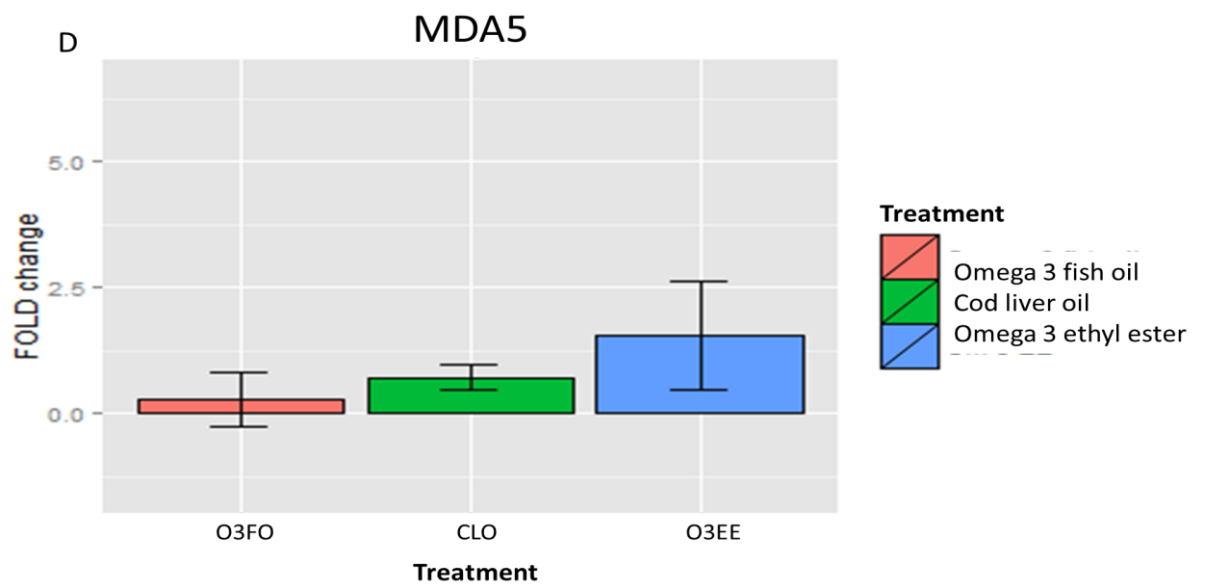
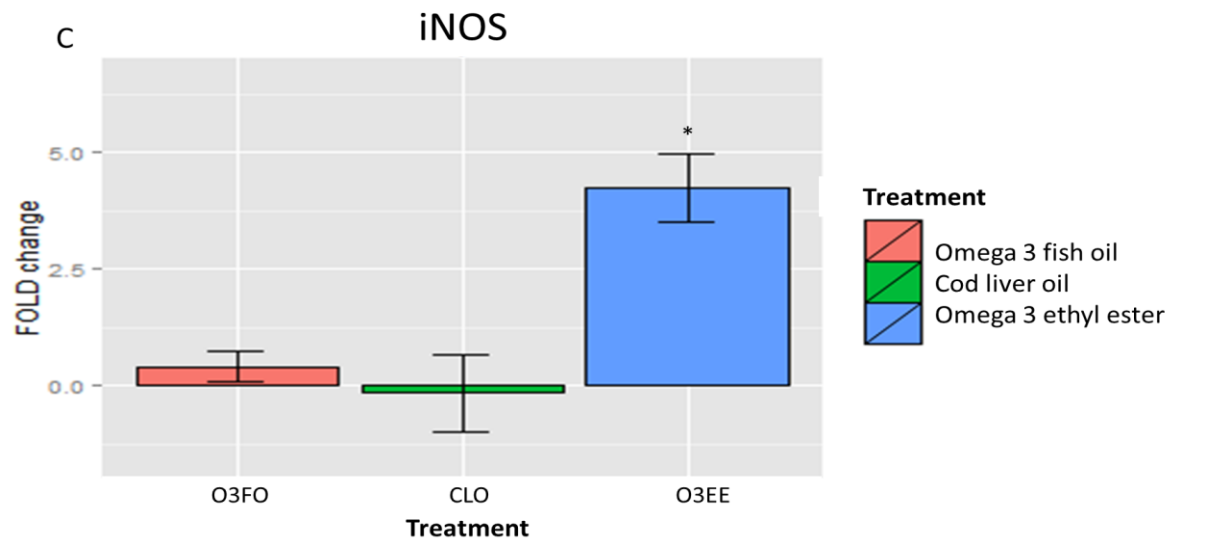


Figure 9: Effects of LPS, butyrates and vitamin D on gene expression in CHSE-214 cells. CHSE cells were treated for 24 h with LPS or for different times with HMB, PBA or Vit D3 at the indicated concentrations. Gene expression for csCath2 (A), Hepcidin 1 (B), iNOS(C), MDA5 (D), RIG1 (E) and ISG15 (F) was estimated using qPCR and compared to the controls at each time point. Statistical data analysis was calculated respect to the mean of all the three time point. Fold change means change compared to control treatments (medium). Barr plots show normalized data (Fold change is log RQ) as mean and standard errors of the mean from at least three independent experiments, significant differences in fold were tested by one-way ANOVA and Tukey test. P values showed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.2 The effect of lipids from fish oil on immune genes expression

To assess the effect of lipids from fish oil on CHSE-214 genes expression, cells were stimulated for 3 h with 5 ml of omega-3 (O3FO), cod liver oil (CLO) or omega-3 ethyl ester (O3EE) from fish oil. Mineral oil was used as control. CsCath2 expression was significantly up-regulated with the three lipids, while Hepcidin was up-regulated only by CLO (Figure 10 A and B). O3EE was found to stimulate iNOS mRNA expression and RIG1 was significantly up-regulated after stimulation with Omega-3 fish oil (Figure 10 C and E). Neither of those lipids had any effect on MDA5 or ISG15 (Figure 10 D and F). Fish oils therefore differentially regulated the immune genes which encode for antimicrobial peptides analyzed in this study. On the other hand, for the genes relevant for virus infection only RIG1 was up-regulated.





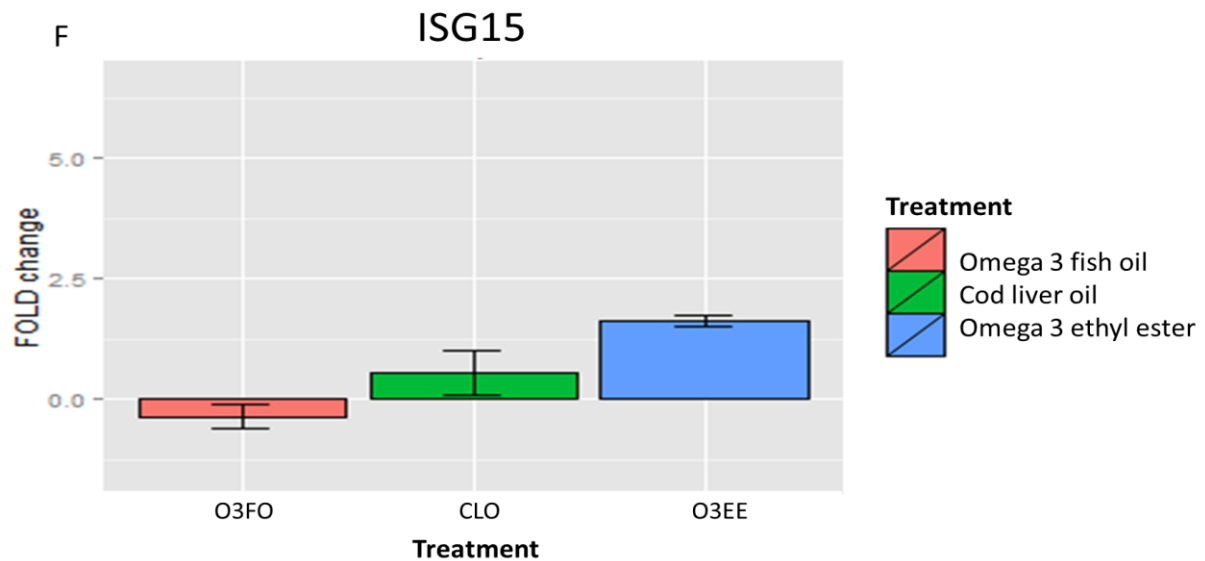


Figure 10: Effects of lipids from fish oil on gene expression in CHSE-214 cells. CHSE-214 cells were treated for 3 h with omega 3 (OM3FO), cod liver oil (CLO), and omega 3 (O3EE). Gene expression for csCath2 (A), Hepcidin 1 (B), iNOS(C), MDA5 (D), RIG1 (E) and ISG15 (F) was examined using qPCR and compared to the controls at each time point. Statistical data analysis was calculated respect to the mean of all the three time point controls. Fold change means change compared to control treatments (mineral oil). Barr plots show normalized data as mean and standard errors of the mean from at least three independent experiments, significant differences in fold were tested by one-way ANOVA and Tukey test. P values showed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2 Activity of antimicrobial peptides

4.2.1 Correlation between mRNA expression and antimicrobial activity

In order to correlate mRNA expression and antimicrobial activity, CHSE-214 were grown in 75 cm² flasks until 100% confluence. Cells were stimulated with butyrates or Vit D3 for 12, 24, 48 or 72 h and with OM3FO or CLO for 3 h (data not shown). The results for the gene expression were similar to the previous results (paragraphs 4.1.1 and 4.1.2) where csCath, hepcidin and iNOS were up-regulated by O3FO and CLO and by 20 mM HMB, 4 mM PBA or 20 nM Vit D3 at 48 and 72 h post induction. Proteins from pellet and medium from stimulated and unstimulated cells were extracted to test antimicrobial activity and correlate with mRNA expression.

4.2.2 Antimicrobial activity by inhibition zone assay against *Bacillus megaterium* of secreted components from CHSE-214 upon stimulation

Proteins extracted from CHSE-214 cell medium after stimulation were analyzed by inhibition zone assay against *Bacillus megaterium* (strain Bm11) in order to determine their antimicrobial activity and a possible correlation with csCath2, Hepcidin or iNOS mRNA expression. The bacterial-free zones were measured for medium from cells stimulated with 20 mM HMB showed the highest inhibition zone at 48 and 72 h, up to 4 fold respect to the control (Figure 11 A and B). Medium from samples stimulated with lipids also showed antimicrobial activity, bacterial-free zones were increased 2 fold with respect to the control. Cod liver oil had the highest measured antimicrobial activity among the lipids (Figure 11 A and B). As a positive control the antibiotic ampicillin (100 µg/ml) was included and resulted in bacteria free zones of ~ 21 mm. The negative control (0.1% TFA) did not show any antimicrobial activity. In order to know if proteins are responsible for the antimicrobial activity, samples were digested with pronase E. In conclusion, lyophilized proteins extracted from cell medium had antimicrobial activity against Bm11 and this activity was due to CHSE-214 secreted antibacterial components. This activity was eradicated after protein digestion indicating that the antimicrobial agents are proteins or peptides. There was correlation at 72 h between mRNA expression described in section 4.2.1 and the antimicrobial activity while at 48 h there was no apparent correlation suggesting the presence of different antimicrobial peptides/proteins not included in this study at this time point.

A Bm11

Time point	48 h		72 h	
SAMPLE	Mean(mm)±SD	Fold	Mean(mm)±SD	Fold
Control	2.5±3.5	1	2±1.4	1
HMB 20mM	11±7.1	4.4	8.25±10.3	4.1
PBA 4mM	8±2.8	3.2	4.25±0.4	2.1
Vit D3 20nM	3±4.2	1.2	1.25±2.5	0.625
C+: Amp	21.7±5.1		21±3.6	
C-: 0.1% TFA	0±0		0±0	
C-: Protease	0±0		0±0	

Time point	3 h	
SAMPLE	Mean(mm)±SD	Fold
Control	2±2.8	1
O3FO	4.3±1.8	2.1
CLO	4.8±1.8	2.4
C+: Amp	21.7±5.1	
C-: 0.1%TFA	0±0	
C-: Protease	0±0	

B

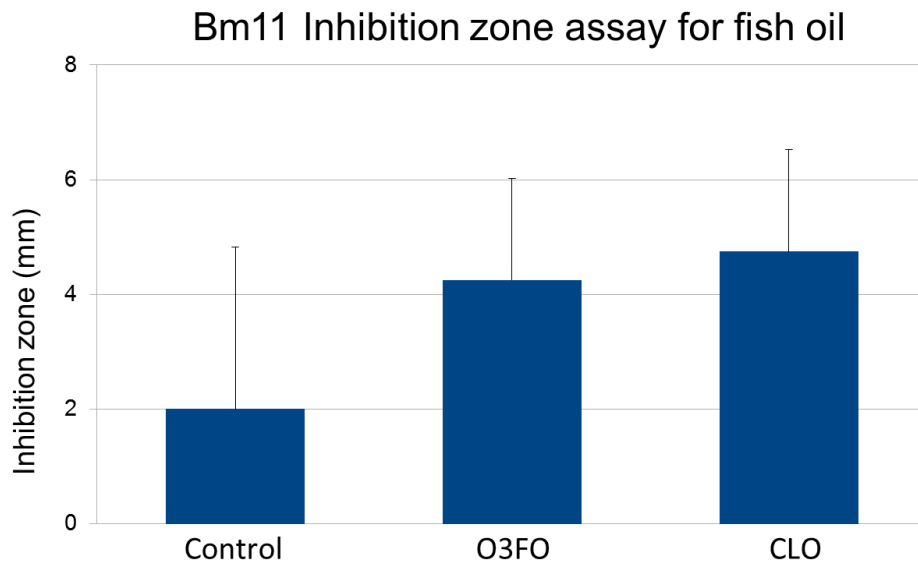
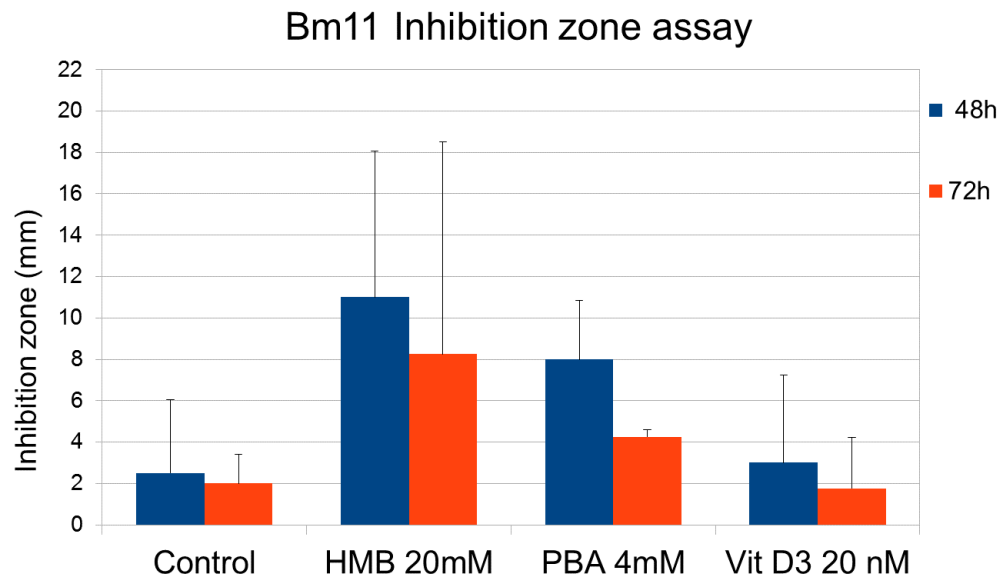


Figure 11: Antibacterial activity against *Bacillus megaterium* (strain Bm11). Lyophilized medium from non-stimulated (control) and stimulated CHSE-214 cell cultures was incubated with Bm11 on LB agar plates overnight at 30°C. Inhibition zones for bacterial growth were measured and expressed as mean and standard deviation (A) from three independent experiments. As positive control 100 µg/ml of ampicillin was used and as negative control 0.1% TFA and samples digested with protease. Fold was calculated with respect to the controls, where controls were given the arbitrary value of 1. (B) Bar plots show normalized data as mean and standard errors.

4.2.3 Antimicrobial activity by inhibition zone assay against *Aeromonas salmonicida* ssp. *Achromogenes* of components secreted from CHSE-214 upon stimulation

Inhibition zone assay against *Aeromonas salmonicida* ssp. *Achromogenes* (ASA) was performed to determine if there is correlation between csCath2, Hecpudin or iNOS mRNA expression in CHSE-214 cells and antimicrobial activity against a common bacteria associated with salmon. Lyophilized proteins extracted from cell medium were analyzed by measuring bacterial-free zones. Extracts from CHSE-214 cells induced with 20 mM HMB displayed the largest free zones, analogous to the results of inhibition zone assay against *Bacillus megaterium*. Inhibition zones at 72 h were found to show the largest free zones, with folds up to 4 for HMB and up to 3 for PBA and Vit D3 (Figure 12 A and B). Samples stimulated with CLO showed the largest free zone for the lipids, similar to the activity against *Bacillus megaterium*. Samples digested with protease had no effect on the bacterial growth suggesting that peptides/proteins were the antimicrobial agents responsible for the antimicrobial activity. These results show that CHSE-214 cells upon stimulation can produce peptides/proteins with antimicrobial activity against ASA. Compared to the expression results (Figure 9), the antimicrobial activity seems to begin earlier (48 h), this might suggest other proteins also playing a role in the antimicrobial effect.

A ASA

Time point	48 h		72 h	
SAMPLE	Mean(mm)±SD	Fold	Mean(mm)±SD	Fold
Control	6±0	1	4.5±2.1	1
HMB 20mM	15.5±3.5	2.2	18.5±2.1	4.1
PBA 4mM	14±4.2	2.3	14±7.1	3.1
Vit D3 20nM	7.75±4.6	1.2	14±7.1	3.1
C+: Amp	19.7±14.8		22±16.1	
C-: 0.1% TFA	0±0		0±0	
C-: Protease	0±0		0±0	

Time point	3 h	
SAMPLE	Mean(mm)±SD	Fold
Control	6±4.24	1
O3FO	7±5.7	1.1
CLO	10.25±9.5	1.7
C+: Amp	19.7±16.2	
C-: 0.1%TFA	0±0	
C-: Protease	0±0	

B

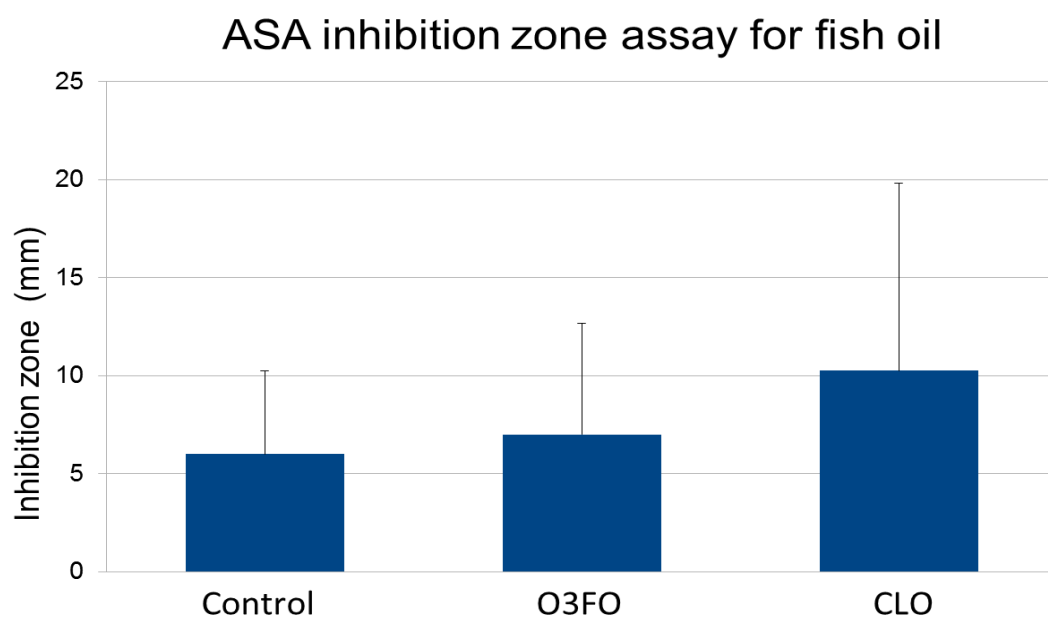
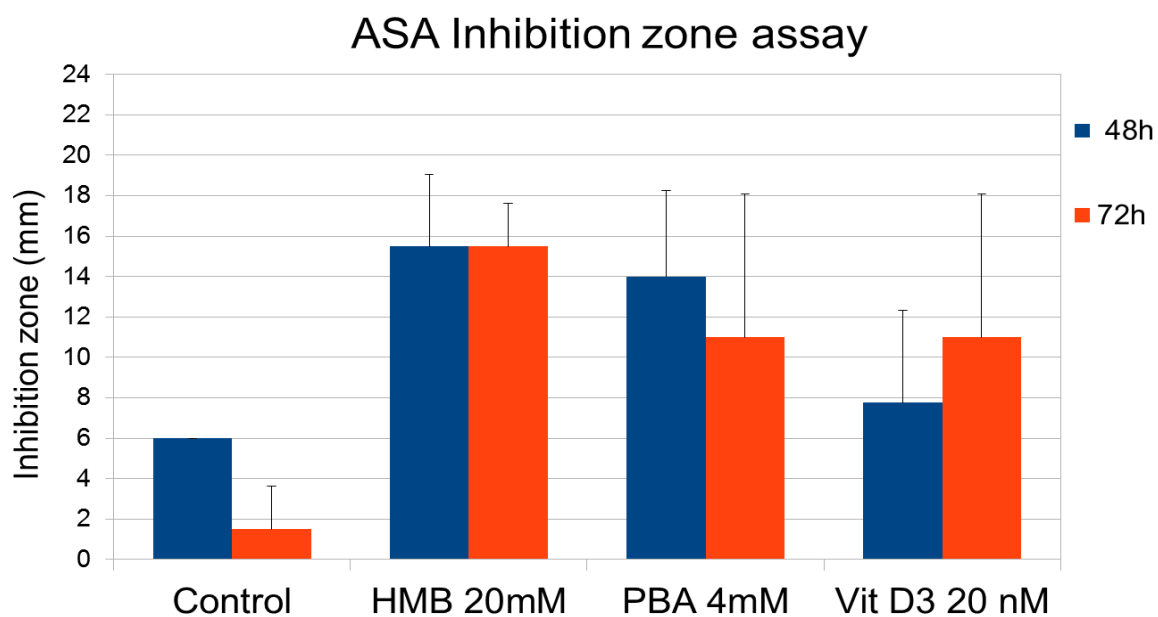


Figure 12: Antibacterial activity against *Aeromonas salmonicida* ssp. *Achromogenes* (ASA). Lyophilized medium from non-stimulated (control) and stimulated CHSE-214 cell cultures was incubated with ASA on LB agar plates overnight at 24°C. Inhibition zones for bacterial growth were measured and expressed as mean and standard deviation (A) from three independent experiments. As positive control 100 µg/ml of Ampicillin was used and as negative control 0.1% TFA and samples treated with protease. Fold was calculated respect to the controls, where controls were given the arbitrary value of 1. (B) Bar plots show normalized data as mean and standard error.

4.2.4 Antimicrobial activity of antimicrobial agents extracted from CHSE-214 cells upon stimulation by overlay gel assay

To assess if CHSE-214 fish cell line can produce antimicrobial peptides, overlay gel assays were performed against *Aeromonas salmonicida* ssp. *Achromogenes* (ASA) and *Bacillus megaterium* (strain Bm11). For the SDS-PAGE gel 20-30 µg of proteins extracted from treated or untreated cells as explain in section 3.10 were electrophoresed on a gel and incubated with ASA and Bm11. Overlay assay against Bm11 did not show inhibition zones while the assay against ASA showed clear and noticeable inhibition zones for samples treated with lipids from fish oil and with 20 mM HMB and 4 mM PBA at 48 and 72 h (pictures not show). According to the location on the SDS-PAGE gel, high molecular weight proteins are responsible of this antimicrobial activity. However, this result was only obtained once.

4.2.5 Antimicrobial activity of proteins extracted from Atlantic salmon gill and sperm tested by overlay gel assay

In order to determine antimicrobial activity of peptides or proteins from Atlantic salmon sperm and gill as described in section 3.10. Total proteins were loaded into a SDS-PAGE gel at different concentrations, the initial concentration was 30 µg, followed by a serial dilutions. Antimicrobial activity against Bm11 was observed for high molecular weight proteins in sperm, while in gill the observed activity originated from high and low molecular weight proteins. According to the location of the inhibition zone in the agar overlay and by overlapping with the SDS-PAGE gel (Figure not show) we found that there are agents in gill with pronounced antimicrobial activity. These antimicrobial agents have a molecular weight between 7-17 kDa and 58-80 kDa (Figure 13). However a digestion of the samples should be done to confirm if proteins are the antimicrobial agents.

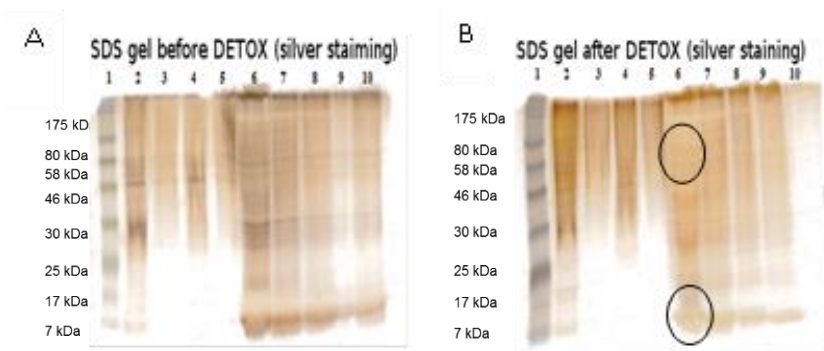


Figure 13: SDS PAGE gel analysis for proteins extracted from *Salmo salar* stimulated with HMB, proteins from sperm (lanes 1-5) and from gill (lanes 6-10) were loaded at different concentrations. Lane 1 corresponde to the protein lader. Labeled portions on the SDS PAGE showed antimicrobial activity against *Bacillus megaterium* (strain Bm11) in agar overlay gel incubated after detox overnight at 30°C. Silver stained SDS PAGE gel showing proteins before detox (A) and after (B).

4.3 Immunoassays

Several techniques were performed in order to test the effectiveness of the antibodies to detect the proteins in their native or denatured state. Dot blot and indirect immunofluorescence were performed to detect the protein in its native form, whereas western blots were performed to detect it in the denatured form.

4.3.1 asCath2 cloning and isolation

In order to obtain a positive control for the affinity purified antibodies and for further experiments and immunization, asCath2 was cloned into a TOPO cloning vector (pET200). Sequencing of the asCath2 plasmid was performed by the dideoxynucleotide procedure (Beckman Coulter Genomics) to confirm the correct insert. AsCath2 expression was optimized in BL21 (DE3). After induction with IPTG, 3 clones were selected to analyze expression of asCath2, whereas one sample was collected from the culture just before induction as negative control. Equals amounts of bacterial cells were selected for SDS-PAGE analysis and western blot. The induced bacteria express a protein (Figure 14) and the size corresponds to potential protein of 25 kDa. AsCath2 was isolated by DEAE ion-exchange column (Figure 15). In conclusion, asCath2 was successfully cloned and is suitable to use as positive control for immune assays and for antibody production.

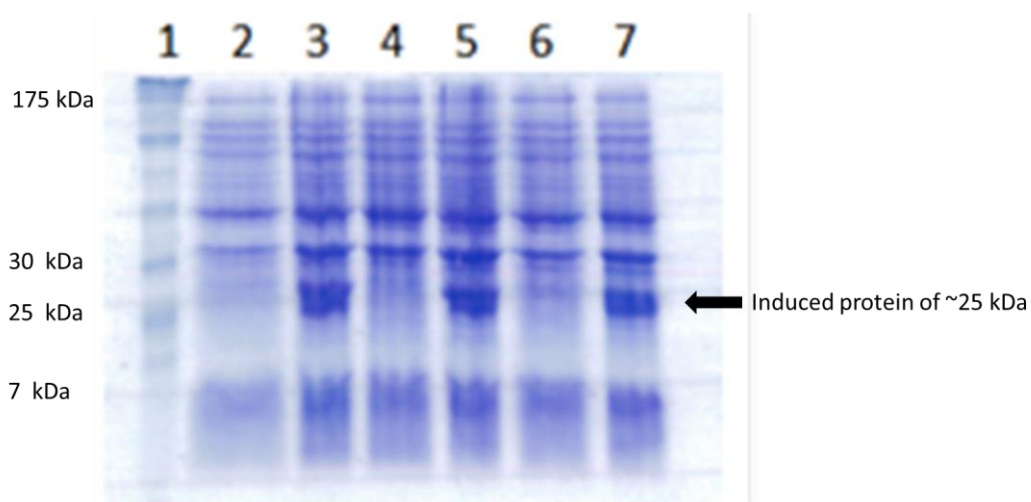


Figure 14: BL-21 (DE3) were cultured in LB with kanamycin (50 $\mu\text{g/ml}$) to grow at 37°C. The BL-21 (DE3) cells were induced with IPTG at OD600 = 0.6 and let growth for 6 h. After induction equals amounts of three different transformed cells were selected for SDS-PAGE analysis; lane 1: protein ladder, lanes 2, 4 and 6: transformed cells before induction; lanes 3, 5 and 7: transformed cells after induction.

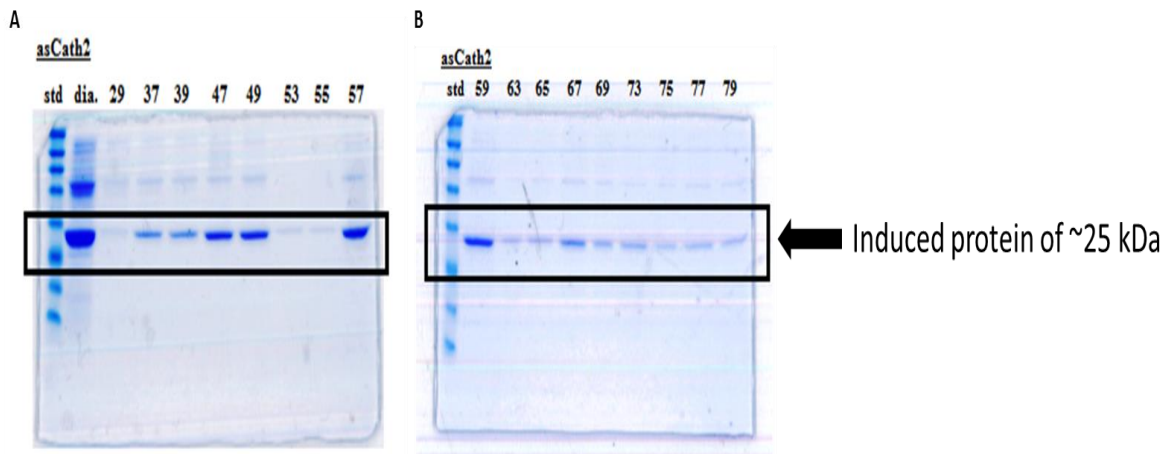


Figure 15: Induced BL-21 (DE3) cells were harvested and the dialyzed cell lysate was loaded on a DEAE sephadex membrane. SDS gel was performed to test the fractions which contain asCath2. (A) Lane 1: protein ladder (std); lane 2: dialyzed SN from induced BL-21 (DE3); lanes 3: fraction eluted with 125 mM Tris-HCl; lanes 4-5: eluted with 150 mM Tris-HCl; lanes 6-7: eluted with 175 mM Tris-HCl; lanes 8-10: eluted with 200 mM Tris-HCl. (B) Lane 1: protein ladder (std); lane 2: fractions eluted with 200 mM Tris-HCl; lanes 3-6: eluted with 250 mM Tris-HCl; lanes 7-10: eluted with 500 mM Tris-HCl.

4.3.2 Western blot and dot blot

To monitor the immunoreactivity of the commercially made affinity purified antibodies, serial dilutions of synthetic peptides were used for dot blot and western blot assays. No signal was detected for either antibody by western blot (Figure 16A) using serial dilutions of the synthetic peptides.

Dot blot was also performed to assess the immunoreactivity of the antibodies. A weak signal was observed in the dot blot after incubation of C-terminal peptide with AB1, raised against this synthetic peptide, nonetheless no signal was detected for the reaction between AB2 and N-terminal synthetic peptide. Cross reaction between synthetic peptides and antibodies was also tested and neither of the two antibodies showed any signal against the opposite peptide (Figure 16B).

This results show a very weak signal and therefore this antibody was not considered to be appropriate for further use.

To screen the presence of cathelicidin in fractions from Atlantic salmon (sperm and gill) and from CHSE-214 cells medium and pellet previously stimulated with 20 mM HMB as explain in sections 3.5 and 3.2 western blot analyse was performed with all the antibodies. Proteins from uninduced and induced BL21 (DE3) were loaded onto the SDS PAGE gel as negative and positive control. Cathelicidin was not detected in any of the fractions (Figure 17 A, B and C) nor did the positive control show a band. In conclusion the antibodies tested are not suitable for dot blot and western blot, although the dot nblots performed by the company (Genscript) were positive for the immunoreactivity of both antibodies against the synthetic peptides (Appendix A).

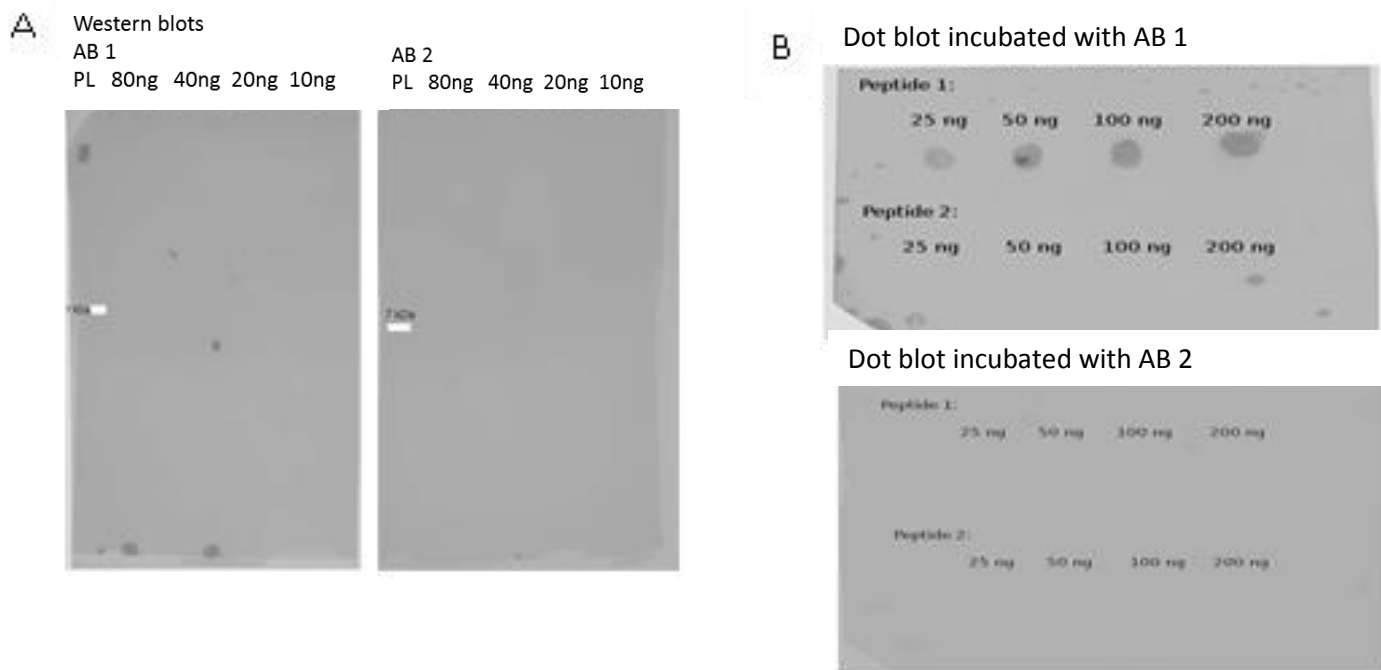


Figure 16: Western blot analysis showing different concentrations of synthetic peptides (peptide 1 was loaded in membrane A and peptide B was loaded in membrane B), membranes were incubated with antibody 1 (5 μ g/ml) and 2 (5 μ g/ml) respectively. SDS-PAGE gels (10%) were transferred to a nitrocellulose membrane by wet-transfer (A). Dot blot showing no cross reaction between antibodies and peptides (B).

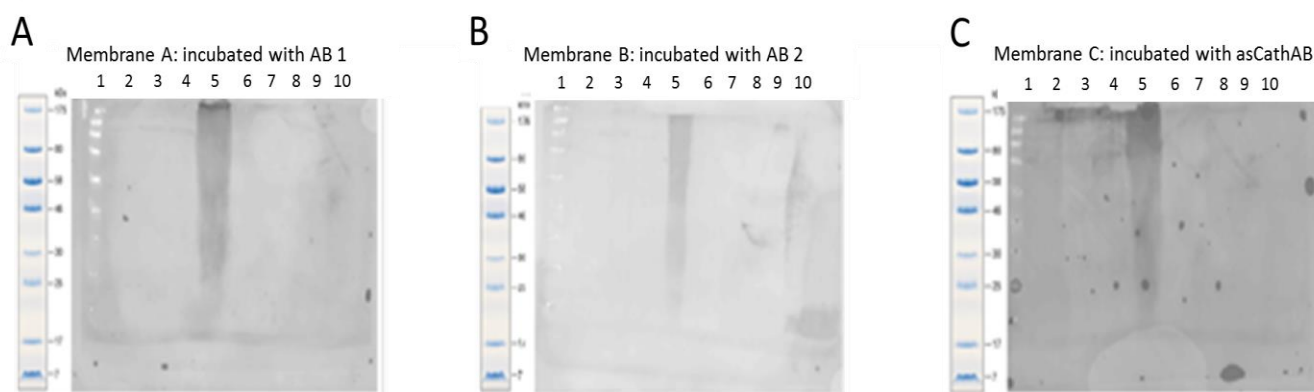
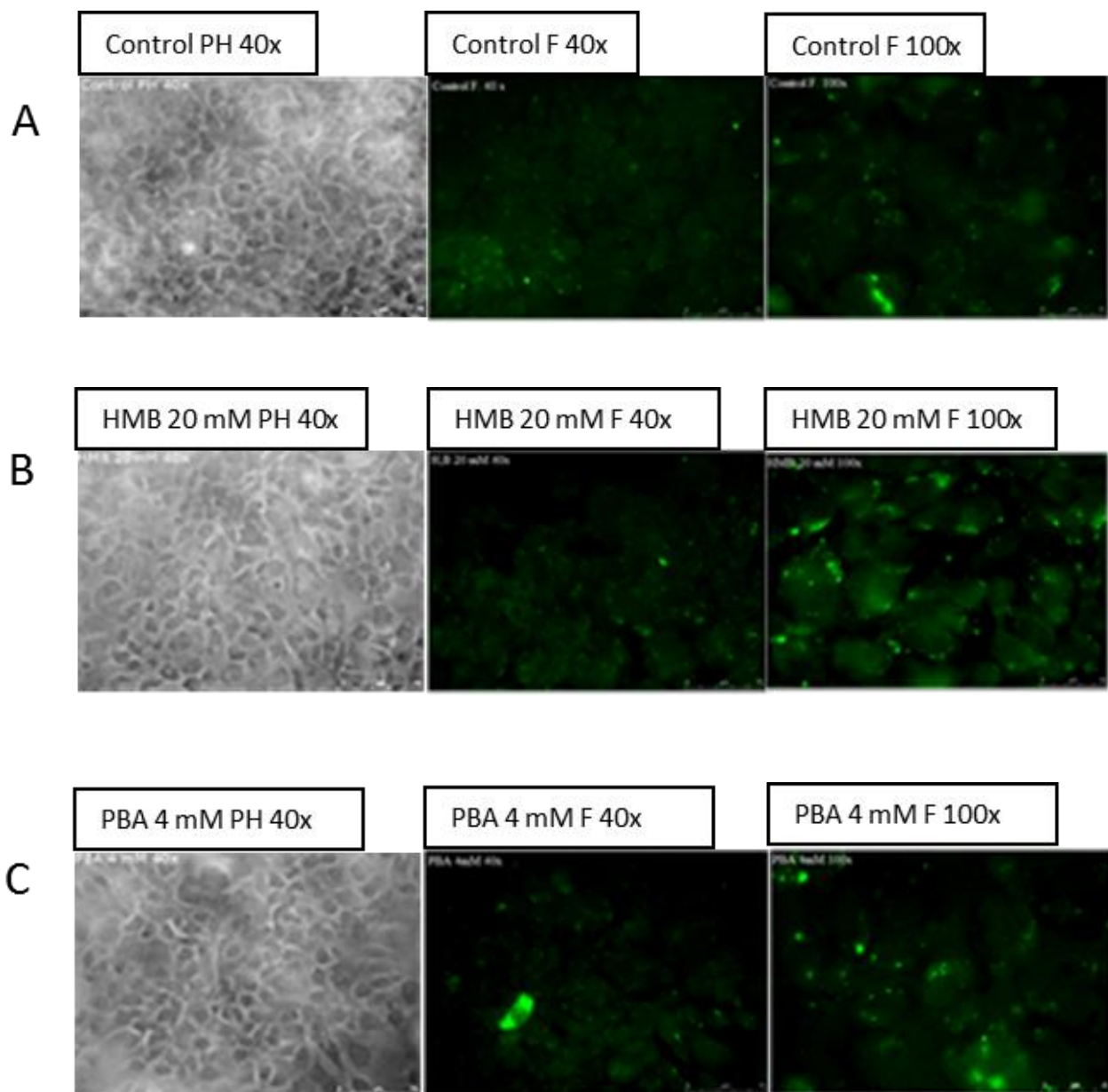


Figure 17: Affinity purified antibody 1 and 2 against synthetic peptides (A) and (B), and asCath2 antibody (made in Keldur) (C) was tested by western blot analysis to detect *Salmo salar* cathelicidin. Lane 1: protein ladder; lane 2: (C-) clone 2 BL21 (DE3) before induction; lane 3: (C+) Clone 2 BL21 (DE3) after induction; lane 4: Clone 4 BL21 (DE3) after induction; lane 5: proteins from Gill (*Salmo salar*); lane 6: proteins from Sperm (*Salmo salar*); lane 7: proteins from medium from CHSE-214 cell culture stimulated with 20 mM HMB; lane 8: proteins from pellet from CHSE-214 cell culture stimulated with 20 mM HMB; lane 9: synthetic Peptide (20 μ g/well).

4.3.3 Indirect immunofluorescence

In order to examine csCath2 in the native state for immunofluorescence, CHSE-214 cells were grown on coverslips and stimulated with butyrates and Vit D3 for 72 h at the indicated concentrations and with OM3FO or CLO for 3 h. The assay was performed with the three antibodies, asCath2AB against the recombinant protein, AB1 or AB2 against synthetic peptides. Lower fluorescence signal was detected for untreated cells while a weak signal was found for cells treated with butyrates (Figure 18B and C) and Vit D3 (Figure 18D). The fluorescence signal for csCath2 was higher for cells treated with lipids from fish oil (Figure 18E and F). However, this results are not conclusive to determine the efficacy of the antibodies tested for this assay.



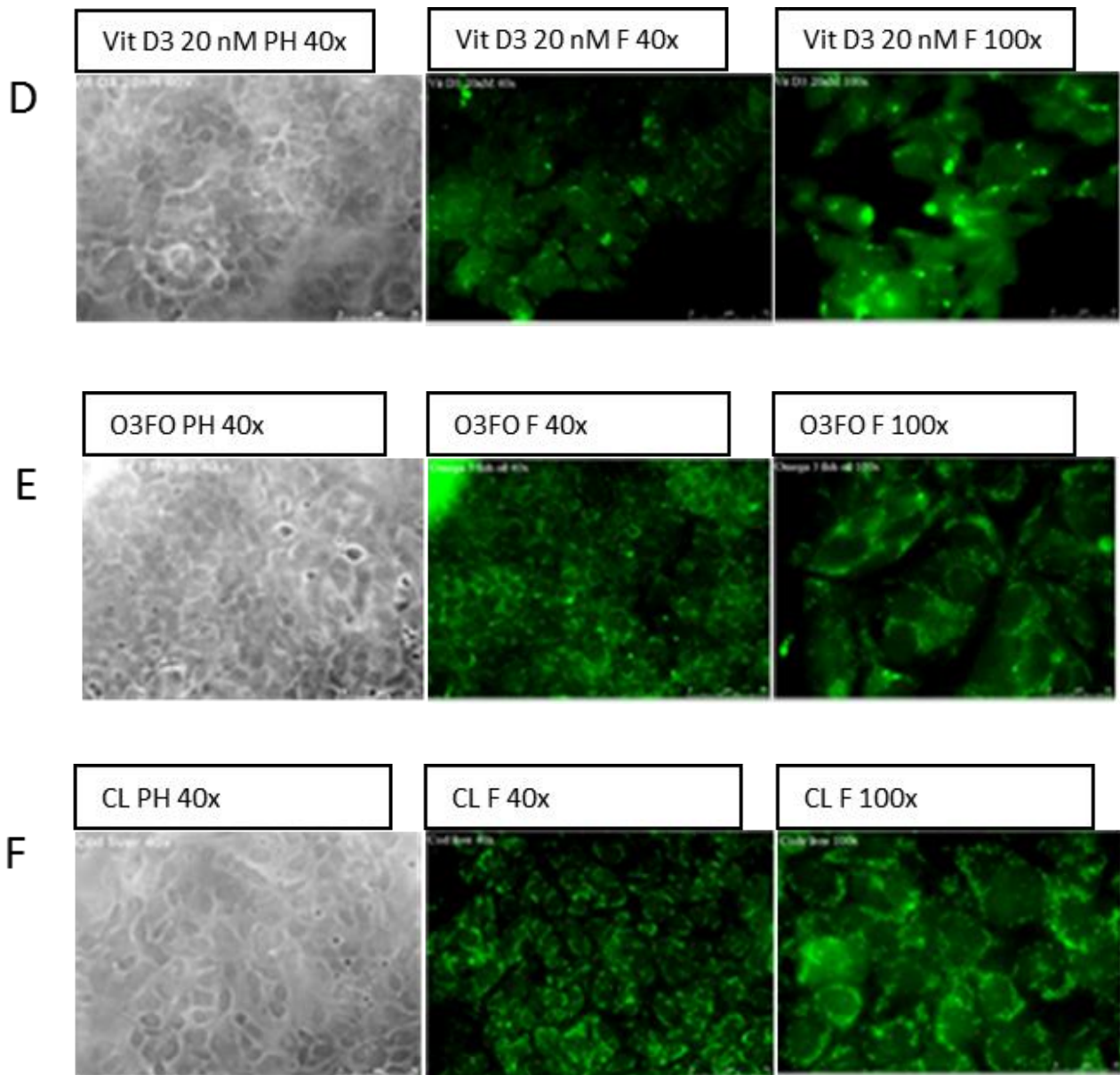


Figure 18: Indirect immunofluorescence of csCath2 in CHSE-214 cell cultures stimulated with 20 mM HMB (B), 4 mM PBA (C) or 20 nM Vit D3 (D) for 72 h and with omega 3 (OM3FO)(E) and cod liver oil (CLO) (F) for 3 h. Untreated cells were used as control (A). Commercially made affinity purified antibodies AB1 or AB2 (GenScript) and asCath2 antibody (made in Keldur) were used to test immunoreactivity. The pictures correspond to the assay made with asCath2 antibody and is representative for all three antibodies. From the left to the right, phase contrast 40x (grey), fluorescence 40x (green) and fluorescence 100x (green).

5 DISCUSSION

During the last few years many studies have focused on finding alternatives to vaccines and antibiotics in aquaculture. These alternatives might include compounds to boost the innate immune response of fish. The innate immune system is not only the first line of defence against infectious diseases, but also acts as immune modulator of physiological processes affected by different factors, such as stress. Stress is an important component in the fish farm industry caused by the human manipulation and can suppress the innate immune response (Noga et al., 2011). In order to study potential inducers of the innate immune system, in this study CHSE-214 cells were stimulated with different compounds and the mRNA expression of several immune genes was estimated. The genes examined are related to the production of AMPs, like cathelicidin and hepcidin, or antiviral proteins, like MDA5, RIG-I and ISG15, as well as the production of innate immune effectors like iNOS.

To define new inducers for the innate immune system of fish was the first aim of this thesis and for this purpose we chose different immune stimulants based on their effects on the immune system of animals, including humans. As candidates we have selected two butyrate-derived compounds (PBA and HMB) due to their ability to regulate the gene expression via inhibition of HDCA (Wächtershäuser & Stein 2000). Previous studies have demonstrated that butyrates like PBA, induce the expression of host defence peptides and modulate the immune response, while other studies have focused on the effect of HMB on the innate and adaptive immune system in animals (Siwicki et al. 2003; Siwicki et al. 2000). The present study is the first study focusing on the effect of HMB on gene expression. It has been clear for some time that HMB has the ability to prevent the stress-related suppression of immune function and this effect is directly measured by a reduction of mortality in animals (Nissen et al., 1993; Siwicki et al. 2003). Several studies in animals showed that HMB in the diet reduced the mortality induced by Motile *Aeromonas septicaemia* (MAS) in common carp by enhancing the nonspecific cellular and humoral immunity (Siwicki et al., 2011). In rainbow trout HMB enhanced microbial killing capability of leukocytes (ROS-response) and increased concentrations of plasma lysozyme and complement activity (Kunttu et al., 2009). HMB also reduced the mortality caused by *A. salmonicida* up to 62 % (Siwicki et al., 2003), showing an important role in the protection against furunculosis and also against *Y. ruckeri* in pikeperch, again by enhancing the nonspecific defence mechanism (Siwicki et al., 2006). PBA, an inhibitor of histone deacetylase, is an important inducer of cathelicidin in mammals (Schauber et al., 2004; Steinmann et al., 2009) and a recent study suggests that it can also induce cathelicidin in fish (Broekman et al. 2013). However the mechanism of action of PBA on gene expression is still unclear. Earlier results suggest that PBA modulates *CAMP* expression indirectly, promoting the expression of other genes encoding important factors for *CAMP* gene expression. Butyrate has been demonstrated to mediate its effects through the mitogen activated protein kinase (MAPK) signaling pathway in the *CAMP* gene expression (Steinmann et al. 2009), but little is known whether this also holds true for butyrate-derived compounds.

Nowadays, the effect of vitamin D₃ intake on the human health is clear, but little is known about the role of vitamin D₃ in fish. Calcitriol is the most potent vitamin D metabolite and exerts its transcriptional actions via the vitamin D receptor (VDR), a member of the nuclear receptor superfamily, nonetheless the function of calcitriol in fish is still unclear. A

functional vitamin D endocrine system was demonstrated in Atlantic salmon (Lock et al., 2007) and in other fish species like carp, cod and zebrafish (Swarup et al., 1984; Sundell et al., 1993; Lin et al., 2012). The reasons for choosing calcitriol as immune stimulant in this study is based on the presence of VDRs in several fish species, including salmonids, as well as a functional endocrine system. This data and the fact that vitamin D₃ can regulate the expression of antimicrobial peptides such as cathelicidin in humans (Liu et al. 2006), gave us reason to speculate that vitamin D₃ might also be an effective inducer of the innate immune system in fish.

Omega 3 fatty acids from fish oil was chosen based on the literature, but little is known about the effect of omega 3 from fish oil on the regulation of gene expression. However many studies point toward it being a powerful booster of the immune system (Miles & Calder 1998; Calder 2007). In the past decades, the interest in the effect of fish oils on human health has increased, but the mechanism of action of fish oil and its effect on the innate immune response is still unclear. Several studies have reported that EPA and DHA have a clear anti-inflammatory role in humans and can decrease production of reactive oxygen species by neutrophils, but the dose-response came up with conflicting results (Calder, 2001).

In this study we have shown that cathelicidin, an antimicrobial peptide with a broad spectrum antimicrobial activity, was up-regulated by HMB, PBA or Vit D₃, as well as by all the fish oils tested (OM3FO, CLO and O3EE). Cathelicidin is an important multi-functional peptide of the innate immune system that among other functions, plays an important role as immunomodulator of the immune system. In Atlantic salmon it was demonstrated that after the challenge with *Y. ruckeri* cathelicidin promoted the expression of the chemokine interleukine-8 (IL-8), a recruiter of immune cells (Bridle et al. 2011). In addition, cathelicidin is known by its ability to neutralize LPS, kill pathogens, like bacteria (Gram-negative and Gram-positive), protozoa, fungi or virally infected cells and tumoural cells (Zanetti et al. 1995). Cathelicidin was demonstrated to be effective against important pathogens such as *Y. ruckeri* or *A. salmonicida* (Martin et al. 2006; Maier et al. 2008a; Lu et al. 2011), both causatives of important economic losses in salmonid aquaculture.

However, cathelicidin is only one of several antimicrobial peptides of the innate immune system, hence we decided to analyze the expression of hepcidin, another antimicrobial peptide with a dual function. In the present study we show that hepcidin was up-regulated with HMB, PBA or CLO, thereby enhancing the innate immune response mediating by this antimicrobial peptide. Hepcidin is known to act as an iron modulator under stress conditions or during inflammation (Douglas et al. 2003), but also to act as an antimicrobial peptide by killing bacteria, viruses or certain fungi. Hepcidin has been demonstrated to be effective against Gram-negative and Gram-positive bacteria, for example, against *A. hydrophila* or *S. aureus* in medaka (Cai et al. 2012) or against *P. salmonis* or *A. salmonicida* in Atlantic salmon or rainbow trout (Douglas et al. 2003; Álvarez et al. 2014). Hepcidin as well as cathelicidin mRNA expression has been detected at early stages during embryonic development, suggesting an important role of these peptides in the innate immune system (Bo et al. 2011, Broekmann 2011). In spite of the fact that cathelicidin and hepcidin are both effective tools in the fight against pathogens, they are not solely responsible for the host protection. The innate immune system is a complex system, where the different immune compounds are working together to protect the organism. In order to

clarify the complexity of the innate immune response, we examined the expression of iNOS, an important ubiquitous gaseous signaling molecule. iNOS expression was up-regulated by OM3EE and PBA, the latter was found especially interesting, due to the effect being time and concentration independent. Interestingly HMB did not have any effect on the iNOS expression. Amino acids are known to have important functions on the regulation of the immune response and amino acids like arginine, asparagine, aspartate or phenylalanine modulate iNOS expression, but leucine does not (Li et al. 2007). HMB is a metabolite of leucine and this may be the reason why iNOS expression was not affected by the stimulation with HMB, but future experiments should be done to determine the validity of this hypothesis.

Increasing iNOS expression has special importance due to the role of iNOS in the defence against bacteria and parasitic infection. For example iNOS expression was demonstrated to be long-lasting up-regulated in rainbow trout after challenge with the Gram-negative bacteria *R. salmoninarum* (Campos-Perez et al. 2000) or by ectoparasites like *Neoparamoeba perurans*, the responsible agent for the amoebic gill disease (AGD) in cultured salmonids (Alvarez-Pellitero 2008). iNOS expression was also demonstrated to be up-regulated in salmon skin after infection with *Lepeophtheirus salmonis*, an important ectoparasite associated with salmonids and responsible for inflammatory reactions in the host (Braden et al. 2012). Therefore, there is no doubt that stimulating iNOS expression can lead to an increase of the host innate defense against a broad spectrum of pathogens, many of them responsible of important economic losses in aquaculture.

In addition to bacteria and parasitic infections, viruses are responsible for important diseases in cultured fish, therefore we selected three genes which encode for antiviral proteins. In this study we showed that HMB and PBA had effects on RIG-I and ISG15 expression, but not on MDA5, while OM3FO had effects only on RIG-I. Any of the three proteins can recognize ssRNA and dsRNA viruses and ISG15 can also recognize DNA viruses (Biacchesi et al. 2009). Infectious pancreatic necrosis virus (IPNV), a double stranded RNA (dsRNA) virus and infectious salmon anaemia virus (ISAV), a single-stranded RNA (ssRNA) virus, are two important virus causatives of severe economic losses in the salmon farming industry. Several studies have shown that the RNA expression of MDA5, RIG-I and ISG15 was up-regulated after the challenge with these viruses, demonstrating their importance in the recognition of the viruses and afterwards the triggering of the innate immune response (Workenhe et al. 2009; Skjesol et al. 2011). Interestingly despite RIG-I and MDA5 belonging to the same family, they show a clear divergence in respect to their expression upon stimulation in the present study. MDA5 and RIG-I share not only similarities in their protein structure, but also in their signaling pathway converging at the adaptor level, activating both the I IFN signaling pathway, however they are not very similar at the gene regulation level. HBM may be exerting its effect either at the upstream signaling cascade or at the transcriptional level.

In this study LPS, a constituent of Gram-negative bacterial cell wall, was used as positive control for cathelicidin expression, however it was interesting for us to test the effect of LPS on the expression of the other genes. Stimulation with LPS increased significantly the expression of cathelicidin at 24 h post stimulation and this result is in line with previous studies, where crude LPS (from *E. coli*), but not LPS digested with DNase, up-regulated the expression of cathelicidin in CHSE-214 (Maier et al., 2008a; Broekman et al. 2013) or

in rainbow trout head kidney cells (Chang et al., 2005). The discussion about how LPS affects the response of the immune system in fish is still open. Both bacterial DNA and peptidoglycan contamination in the LPS have been suggested to be the real trigger for cathelicidin transcription in fish cells (Maier et al., 2008b). Furthermore it is still unclear how TLR4 can recognize LPS and activate the immune response in fish, since fish TLR4-mediated signaling shows some particular alterations compared to vertebrates (Rebl et al., 2010). Previous studies show that either TLR4 does not recognize LPS in zebrafish (Sepulcre et al., 2009) or that TLR4 is not present in some fish species such as pufferfish (*Takifugu rubripes*) and possibly salmonids (Rebl et al., 2010). We found that LPS also has an effect on the other examined genes and remarkably iNOS expression was undetectable after the stimulation with LPS. The Regulation of iNOS expression could be modulated at the transcriptional and posttranscriptional level and depending on the cell type and on the stimulant it is regulated by different signaling pathways. For example, in mammals iNOS activity modulation is mediated by cellular receptors like TLRs (Janeway et al., 2002) and LPS stimulates iNOS via TLR4 interaction (Du et al., 2001). Another example is the regulation by IFN- γ via the Jak-Stat signaling pathway (Rao 2000). At the transcriptional level the iNOS expression is regulated by transcription factors and some stimulants like LPS can down-regulate or up-regulate the DNA binding activity of the transcription factors (Ye & Liu 2001). At the posttranscriptional level the iNOS expression is regulated by modifying its synthesis, stability and degradation through cyclic guanosine monophosphate (cGMP) or nitric oxide (NO) that regulate the iNOS stability by positive or negative feedback mechanisms (Pérez-Sala et al. 2001). In this study, LPS inhibited the iNOS expression and further experiments may be done to understand the mechanism of action of LPS on iNOS expression in CHSE-214 cell line and if it's exerting its action through some of these signaling pathways. Genes mostly implicated in the viral immune response were not stimulated by LPS in the present study. LPS stimulation was found to increase MDA5 expression in leukocytes from kidney and peripheral blood in Japanese flounder (Ohtani et al., 2011) and in channel catfish liver all three RLRs were induced by Gram-negative bacteria (Rajendran et al., 2012). ISG-15 on the other hand was not found to be up-regulated in black rockfish (*Sebastes schlegeli*) (Baeckel et al., 2008) or in Atlantic cod (*Gadus morhua*) (Holen et al., 2012) by LPS, in accordance with our results, suggesting differences in the ISG-15 activation pathway between different fish species. The fish low sensitivity to the toxic effects of LPS suggest a strategy to survive in the aquatic environment, since LPS can lead to septic shock and this is particularly important in the case of fish that are constantly exposed to pathogens (Forlenza et al., 2011).

For a more complete picture we analyzed the antimicrobial activity of un-induced and induced cells and also proteins from Atlantic salmon gill and sperm. Some studies have demonstrated a strong antimicrobial activity of salmonids antimicrobial peptides like cathelicidin or hepcidin against different bacteria (Chang et al. 2005; Álvarez et al. 2014). The present study is in line with previous studies showing a pronounced antimicrobial activity for the fractions from induced-cells. We could establish a relation between mRNA expression of the immune genes tested in this study at 72 h and antimicrobial activity. We found that the higher expression was at 72 h post induction for all of the genes and the strongest antimicrobial activity was found at 72 h for *Aeromonas salmonicida* and at 48 h for *Bacillus megaterium*. The strong antimicrobial activity at 48 h might be explained by the fact that there are more immune factors expressed at the same time than cathelicidin, hepcidin or iNOS, which can cause the bacterial killing. To confirm this hypothesis a mass

spectrophotometry test should be done for a more concrete analysis. It should be noted that the peptide/protein exerted its antimicrobial activity against both *Bacillus megaterium* and *Aeromonas salmonicida*, which is known to be an important fish pathogen. Proteins extracted from Atlantic salmon gill and sperm were screened for antimicrobial activity and our results show two bacterial free zones in gill corresponding to ~7 kDa and ~70 kDa, suggesting the presence not only of small peptides, but also proteins with antibacterial activity, however further experiments might be done to confirm if the antimicrobial agents are peptides/proteins. Regarding the antimicrobial activity from sperm peptides our results show, in contrast to earlier studies (Johansen et al. 1995; López-Solanilla et al. 2003), that peptides/proteins from Atlantic salmon sperm did not display antimicrobial activity.

The second aim of this thesis was to acquire an anti-as-cath antibody to allow a fast and efficient localization of the peptide. Based on previous studies where successful anti-cathelicidin antibodies were obtained, for example anti-trout cathelicidin was produced using the recombinant protein (Zhang et al. 2015) or the full-length synthetic peptide (Schmitt et al. 2015) and anti-cod cathelicidin was produced using a 14 aa synthetic peptide (Broekman et al. 2011), we decided to use either synthetic peptides (N-terminal and C-terminal synthetic peptides) or the full-length recombinant asCath to raise antibodies. Unfortunately, neither the commercially made affinity purified antibodies nor the antibody against recombinant anti-cathelicidin was able to detect the peptide. Different possibilities were screened in this project to find the most suitable antibody, such as to design the synthetic peptides for the N-terminal or the C-terminal part of the protein or to use the recombinant protein to immunize. Likewise, the antibodies were tested to examine cathelicidin in the denatured or native state by western blot or immunofluorescence respectively. The results for immunofluorescence show a stronger signal for cathelicidin for the samples stimulated with O3FO or CLO, but unfortunately it was not possible to determine if these results are related to the cathelicidin over-expression upon oils induction or they are an optical effect caused by the oils. In the light of the results, asCath was again cloned, expressed and successfully isolated. The size of the asCath peptide observed by western blot was slightly higher than its nominal mass and this could be due to the fact that cationic molecules may run slowly in SDS-PAGE, this effect was reported previously for cationic peptides (Broekman et al., 2011; Corrales et al., 2009). Therefore, we can conclude that asCath was successfully cloned and isolated and it is suitable for further immunization experiments.

6 Conclusions

In conclusion, we have found inducers of the salmon innate immune system. The immune genes stimulated have been demonstrated to fight against bacteria, viruses and parasites causative of important infectious diseases in aquaculture. Among the pathogens susceptible to be eliminated are the bacteria *Piscirickettsia salmonis* or *Aeromonas salmonicida*, the viruses ISAV or IPNV and the ectoparasite *Lepeophtheirus salmonis*. In addition, our results not only confirm the evidence that HMB enhances the immune response, but also they go more in depth and we can conclude that HMB exerts its effect by the stimulation of key elements of the innate immune system like cathelicidin, hepcidin, iNOS, RIG-I or ISG15. At the same time PBA reveals itself as a powerful immune inductor affecting the expression of all the genes tested except MDA5. In the case of fish oils, we can conclude that all of them are good inducers of the innate immune genes giving particular emphasis on cathelicidin, which resulted to be stimulated by the three of them. The strong and clear effect of vitamin D₃ on cathelicidin expression seen here, is the first time to our knowledge that the effect of vitamin D₃ on the innate immune system of fish has been examined. The effect that the inducers have on the innate immune gene expression, is further reflected at the protein level, with an increase of the antimicrobial activity.

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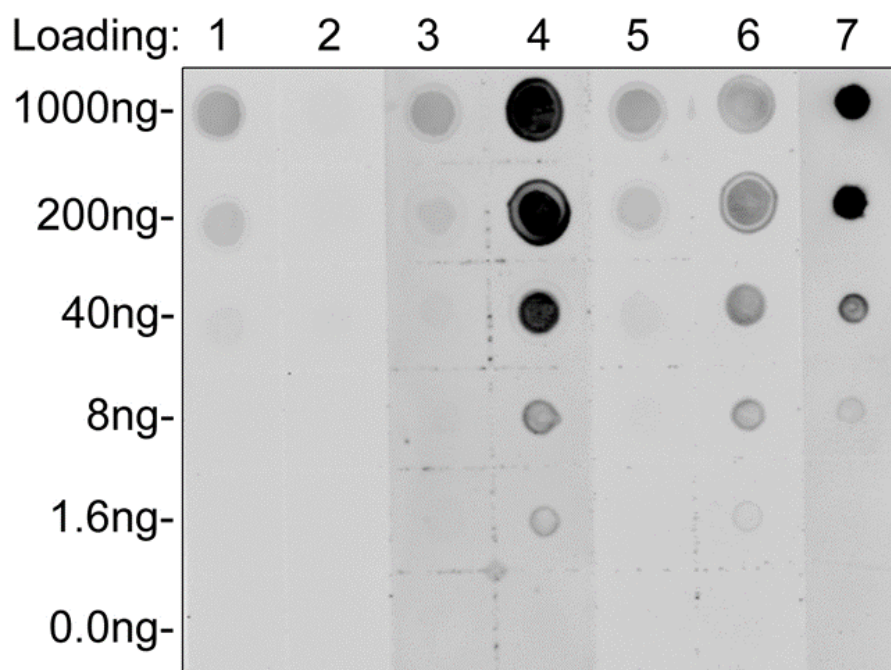
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Appendix A



Loading:

Lane 1,3,5: Peptide of 432424-2

Lane 2,4,6: Peptide of 432424-5

Lane 7: His tagged fusion protein(eGFP)

Primary Antibody:

Lane 1,2: Blank

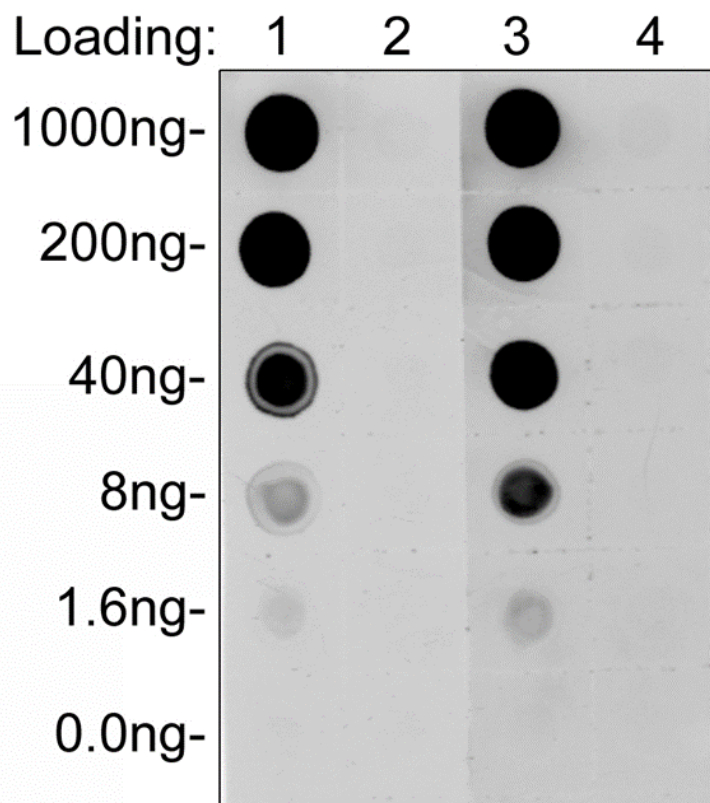
Lane 3,4: Purified antibody of 432424-5 5μg/ml

Lane 5,6: Purified antibody of 432424-5 1μg/ml

Lane 7: His-tag Antibody, pAb, Rabbit

(Genscript,A00174,Lot.13G000658, 1μg/ml)

by YXF 08/22/2014



Loading:

Lane 1,3: Peptide of 432424-2

Lane 2,4: Peptide of 432424-5

Primary Antibody:

Lane 1,2: Purified antibody of 432424-2 1 μ g/ml

Lane 3,4: Purified antibody of 432424-2 5 μ g/ml

by YXF 08/22/2014