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BIOLOGIA MOLECULAR E CELULAR

Novel antimicrobial peptides from sea bass (*Dicentrarchus labrax*) and their potential applications for the improvement of aquaculture production

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NOVEL ANTIMICROBIAL PEPTIDES FROM SEA BASS (*DICENTRARCHUS LABRAX)* AND THEIR POTENTIAL APPLICATIONS FOR THE IMPROVEMENT OF AQUACULTURE PRODUCTION

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Ao meu Pai,
À minha Avó,
Com saudade.

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List of Publications

The author declares that, in the elaboration of this thesis, published articles were included as listed below. The author also declares that participated actively in the conception and execution of the experiments that produced such data, as well as in their interpretation, discussion and manuscript writing and edition.

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Table of Contents

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Pink Floyd, "Hey You"

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Summary

Aquaculture is the food producing sector that grew the most during the last decades, with nearly half of fish currently consumed worldwide being raised on fish farms. However, the continuously growth of this sector has led to the appearance of disease outbreaks, mostly of bacterial and viral origin, in turn leading to significant mortalities and production losses. These outbreaks are often treated or even prevented with the use of antibiotics, but the misuse of these drugs in animal production is tightly associated with the appearance of microbial resistance, asides their inability in treating viral infections. Although there are some vaccines available for the prevention of some diseases, these methodologies are not always totally effective. Thus, it is necessary to develop novel sustainable prophylactic or therapeutic compounds to be used in aquaculture. As potential alternatives to antibiotics are included the antimicrobial peptides (AMPs), due to their wide antimicrobial action against different microorganisms, as well as immunomodulatory roles.

Although there are already many studies focusing on AMPs in different fish species, in the European sea bass (*Dicentrarchus labrax*), a commercially important species in the Mediterranean area, only hepcidin has been studied in detail. As such, in this thesis, we were able to isolate novel AMPs belonging to two different families: beta-defensins (Chapter II) and piscidins (Chapter IV). Furthermore, we also demonstrated that some of the piscidins isolated present a high antimicrobial activity against different microorganisms, including bacteria that cause disease outbreaks in aquaculture, as well as two parasites known to infect mammals (Chapter IV). We were also able to demonstrate the potential of sea bass synthetic peptides, namely hamp2 and piscidins 1 and 5, in the prevention or treatment of infection with *Photobacterium damselae* spp. *piscicida* (Chapters III and V). Fish treated with these peptides showed survival rates that range from 83 to 98%. On the contrary, hamp1, with a major role in the regulation of iron metabolism in fish, did not protect fish from infection. However, in iron overloaded animals, hamp1 attenuated some of the effects of excess iron (Chapter III). Considering the high diversity of AMPs found in sea bass, particularly hepcidins and piscidins, we raised the question if other peptides are also involved in iron metabolism, asides the type 1 hepcidin. The results presented in this thesis clearly points towards a role of piscidins 2 and 7 in the regulation of iron, while other peptides perform mostly antimicrobial roles (Chapter V).

This thesis opens the door for the development of novel prophylactic or therapeutic compounds derived from AMPs naturally produced by this fish species, as viable alternatives to the use of antibiotics in aquaculture. These peptides present many advantages, such as their efficacy in treating viral diseases and the reduced probability in

developing microbial resistance. However, the wide use of these peptides faces critical challenges, including the high production costs. As such, these peptides must be studied in detail, in order to establish the most effective doses and routes of administration, but with reduced costs and reduced impact for animals.

Resumo

A aquacultura é a indústria alimentar que tem verificado o maior crescimento, fornecendo cerca de metade do peixe atualmente consumido a nível mundial. No entanto, o contínuo aumento da produção de peixe tem levado ao aparecimento de doenças, nomeadamente de etiologia bacteriana ou viral, causando elevadas mortalidades e perdas económicas substanciais. Estes surtos são grande parte das vezes tratados, ou até mesmo prevenidos, com o uso de antibióticos, mas o uso descontrolado destes compostos na produção animal está intimamente associado ao aparecimento de microrganismos resistentes, para além de não serem eficazes no tratamento de doenças virais. Apesar de existirem vacinas para prevenção de algumas doenças, nem sempre estas se revelam totalmente eficazes. Por isso, é necessário desenvolver outros compostos terapêuticos ou profiláticos para a aquacultura. Como potenciais substitutos dos antibióticos encontram-se os péptidos antimicrobianos (AMPs), devido à grande atividade antimicrobiana em diferentes microrganismos e funções imunomodulatórias.

Apesar de já existirem vários estudos focados em AMPs de diferentes espécies, no robalo Europeu (*Dicentrarchus labrax*), uma espécie com elevado valor comercial na zona Mediterrânica, apenas a hepcidina tem sido estudada ao pormenor. Desta forma, nesta tese, foi possível isolar novos AMPs nesta espécie, pertencentes a duas famílias diferentes de péptidos: as beta-defensinas (Capítulo II) e piscidinas (Capítulo IV). Para além disso, foi também possível demonstrar a grande atividade antimicrobiana de algumas das piscidinas sintéticas contra vários microrganismos, incluindo bactérias causadoras de surtos em aquacultura, mas também contra duas espécies de parasitas conhecidas por infetar mamíferos (Capítulo IV). Foi possível também demonstrar o potencial de alguns péptidos sintéticos, nomeadamente a hepcidina de tipo 2 e piscidinas 1 e 5, na prevenção ou tratamento da infeção causada por *Photobacterium damselae* spp. *piscicida* (Capítulos III e V). Os peixes tratados com estes péptidos mostraram taxas de sobrevivência entre os 83 e os 98%. Por outro lado, a hepcidina de tipo 1, com principal função de regulação do metabolismo do ferro em peixes, não trouxe qualquer efeito benéfico aos animais infetados. No entanto, em animais com sobrecarga de ferro, a hepcidina de tipo 1 foi capaz de atenuar alguns dos efeitos causados pelo excesso de ferro (Capítulo III). Tendo em consideração a elevada diversidade de AMPs no robalo, principalmente de hepcidinas e piscidinas, colocamos a hipótese de existirem outros péptidos envolvidos no metabolismo do ferro, para além da hepcidina de tipo 1. Os resultados apresentados na presente tese apontam para uma função de regulação do ferro das piscidinas 2 e 7, enquanto outras estarão mais focadas na sua ação antimicrobiana (Capítulo V).

Esta tese abre portas para o desenvolvimento de novos compostos profiláticos ou terapêuticos baseados em AMPs naturalmente produzidos por esta espécie, como alternativas viáveis ao uso de antibióticos em aquacultura. Estes péptidos possuem várias vantagens, entre elas a eficácia em combater doenças virais e a reduzida probabilidade de criar resistência microbiana. No entanto, o seu uso generalizado enfrenta grandes limitações, como o elevado custo de produção. Desta forma, estes péptidos deverão ser estudados ao pormenor, de forma a estabelecer as doses e vias de administração mais eficazes, mas com custo reduzido e baixo impacto para os animais.

List of Abbreviations

AA - Amino acid
ACP - Alternative complement pathway
ACTB - Actin, beta
Ala - Alanine
AMPs - Antimicrobial peptides
Arg - Arginine
Asn - Asparagine
BMP6 - Bone morphogenetic protein 6
C3 - Complement component 3
CCP - Classical complement pathway
CCR2 - C-C Chemokine Receptor
cDNA - Complementary DNA
CFU - Colony-forming unit
CM - Cytoplasmic membrane
CW - Cell wall
CY - Cytoplasm
Cys - Cysteine
DC - Dendritic cell
DMT1 - Divalent metal transporter 1
DNA - Deoxyribonucleic acid
EDTA - Ethylenediamine tetraacetic acid
EPO - Erythropoietin
EPOR - Erythropoietin receptor
ERK - Extracellular-signal-regulated kinase
ESTs - Expressed sequence tags
EU - European Union
FPN1 - Ferroportin
FT - Ferritin
FTH - Ferritin H
GDF15 - Growth differentiation factor 15
gDNA - Genomic DNA
Gln - Glutamine
Glu - Glutamic acid
Gly - Glycine
GMQE - Global model quality estimate
HAMP- Hepcidin
Hamp1 - Hepcidin type 1
Hamp2 - Hepcidin type 2
HFE - Homeostatic iron regulator
HIF-1 - Hypoxia-inducible factor 1
HJV - Hemojuvelin
IFN - Interferon
IFN-γ - Interferon gamma
Ig - Immunoglobulin
IL-1β - Interleukin 1 beta
IL-6 - Interleukin 6
IL-6R - Interleukin-6 Receptor
IL-8 - Interleukin 8
IPNV - Infectious pancreatic necrosis virus
IREG1 - Iron regulated-transporter-1
JAK - Janus kinase
LCP - Lectin complement pathway
LEAP-1 - Liver-expressed antimicrobial peptide 1
LPS - Lipopolysaccharide
LRR - Leucine-rich repeats
Lys - Lysine
M.W. - Molecular Weight
MAC - Membrane attack complex
MAPK - Mitogen activated protein kinase
MBCs - Minimal bactericidal concentrations
MDR - Multidrug resistant
MHC - Major histocompatibility complex
MIC - Minimal inhibitory concentration
MRLs - Maximum residue limits

mRNA - Messenger RNA
MRSA - Methicillin-resistant Staphylococcus aureus
MTP1 - Metal transporter protein-1
MyD88 - Myeloid differentiation primary response 88
MΦ – Macrophage
NA - Nucleic acid
NF-κB - Nuclear factor kappa B
NK cell - Natural killer cell
NMR - Nuclear magnetic resonance
NNV - Nervous necrosis virus
NRAMP2 - Natural resistance-associated macrophage protein 2
OM - Outer membrane
ORF - Open reading frame
PAMPs - Pathogen-associated molecular patterns
PBS - Phosphate-buffered saline
PCR - Polymerase chain reaction
Pisc - Piscidin
PMN - Polymorphonuclear leukocytes
Poly (I:C) - Polyinosinic:polycytidylic acid
PRRs - Pattern-recognition receptors
QMEAN - Qualitative model energy analysis
RBCs - Red blood cells
RNA - Ribonucleic acid
ROS - Reactive oxygen species
Ser - Serine
Slc11a2α - Solute carrier family member 2 alpha
SLC40A1 - Solute carrier family 40 member 1
SMAD - Similar to mothers against decapentaplegic family member
STAT - Signal transducer and activator of transcription proteins
SVCV - Spring viraemia of carp virus
TCR - T-cell receptors
TF - Transferrin
TFR - Transferrin receptor
Th cell - T helper cell
TH - Tilapia hepcidin
Thr - Threonine
TLR - Toll-like receptor
TMPRSS6 - Transmembrane serine protease 6
TNFα - Tumor necrosis factor alpha
TSA - Tryptic soy agar
TSB - Tryptic soy broth
TWSG1 - Twisted Gastrulation BMP Signaling Modulator 1
UTR - Untranslated region
VER - Viral encephalopathy and retinopathy
VLRs - Variable lymphocyte-like receptors
VRE - Vancomycin-resistant Enterococcus
WGD - Whole-genome duplication
WGSS - Whole-genome shotgun sequence

List of Figures

List of Tables

Chapter I

General Introduction

Antimicrobial peptides: small molecules with great properties

An evolutionary perspective

All organisms live in an environment full of harmful pathogens and toxic substances. To protect themselves against these agents, several host mechanisms evolved, capable of recognizing and neutralizing them, called immune defenses. The immune system responses are found from invertebrates to higher vertebrates, with different degrees of complexity (Figure 1), being conceptually divided into innate (non-specific) and adaptive (specific) responses [1,2]. However, it is known that both responses work together: the innate precedes the adaptive, activating and determining the nature of the specific defenses and these in turn enhance the innate responses, making them more effective (Figure 2). The typical adaptive immunity, characterized by the capacity of antigen-specific memory, has been assumed to be absent in invertebrates, but their innate responses can be highly complex [3–5]. On the contrary, vertebrates fine-tuned the immune components, particularly the adaptive ones. These species were provided with a complex network of cells and receptors that orchestrate a system of antigen-specific responses, being capable of recognition and response after re-exposure to the same antigen, generating a long-lived immunological memory [2,4].

If the invading agent successfully overcomes the physical barriers, a series of innate immune responses arises. These are triggered by a number of germline-encoded pattern-recognition receptors (PRRs), that recognize pathogen-associated molecular patterns (PAMPs), and mediated by different cells, such as phagocytes, and by a series of soluble mediators, like cytokines or complement compounds [1]. While these mechanisms are older and are found in more primitive species, the adaptive immune system may have arisen approximately 450 million years ago in the first jawed vertebrates (Gnathostomata) [2]. Features of the specific immunity include specialized immune cells, such as T and B-lymphocytes, the major histocompatibility complex (MHC) and the generation of millions of different antigen-specific receptors trough the somatic rearrangement of variable (V), diversity (D) and joining (J) gene segments [1].

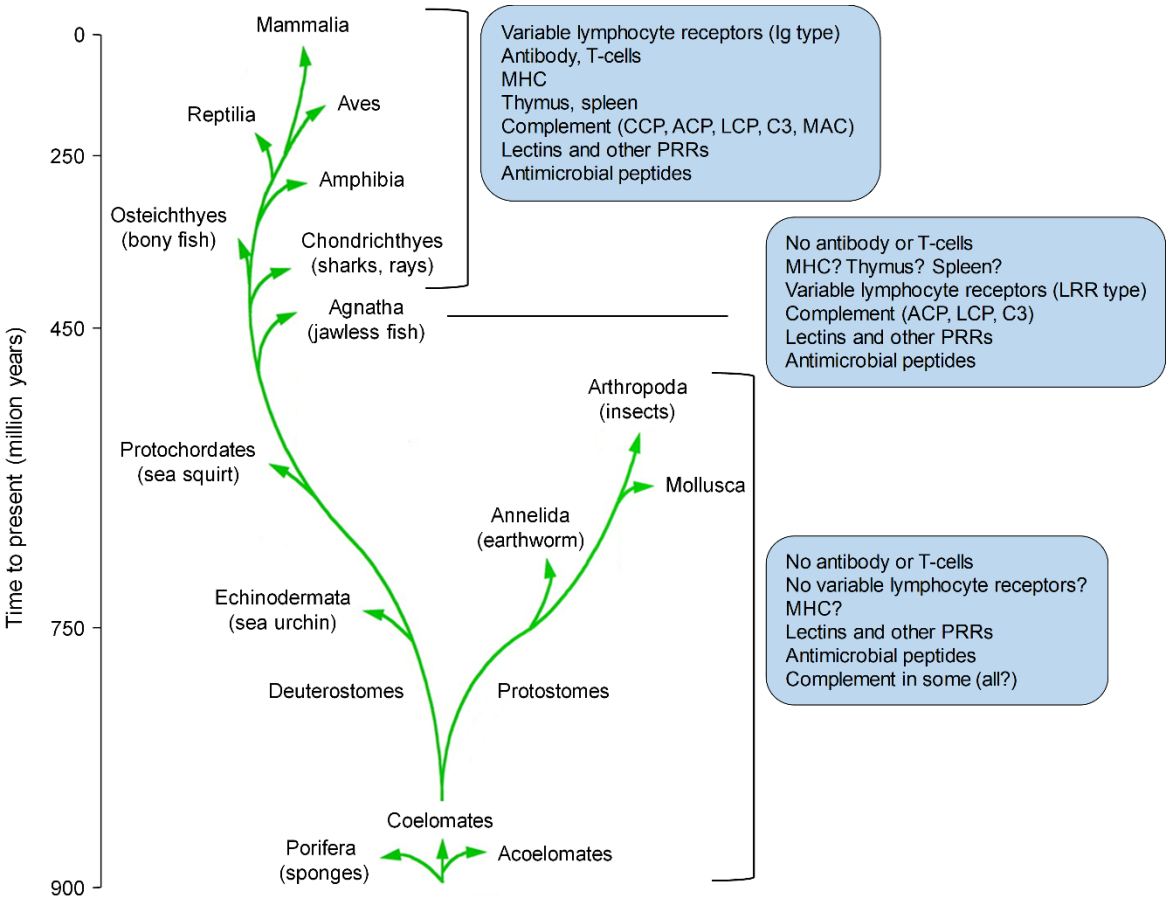

Figure 1. Immune system features of different groups of organisms (adapted from Sunyer et al [6]). ACP – Alternative complement pathway; CCP – Classical complement pathway; C3 – Complement component 3; Ig – Immunoglobulin; LCP – Lectin complement pathway; LRR - Leucine-rich repeats**;** MAC - Membrane attack complex; MHC – Major histocompatibility complex; PRRs – Pattern-recognition receptors.

The most primitive fish, the jawless vertebrates, are equipped with a series of innate molecules, but they lack true immunoglobulins (Igs), T-cell receptors (TCRs) and MHC molecules. Instead, they rely on variable lymphocyte-like receptors (VLRs), composed by leucine-rich repeats (LRRs) [2,7,8]. Fish are the most primitive organisms presenting both innate and adaptive responses. They present many of the components of the innate and acquired immunity found in mammals, but particular characteristics are observed in fish. These animals are free-living organisms since the early embryonic stages of life and are heavily dependent on their non-specific immunity to survive [9]. Their poikilothermic nature, restricted repertoire of antibodies, and slow proliferation, maturation and memory of fish lymphocytes, translate in a somewhat limited specific response [10,11].

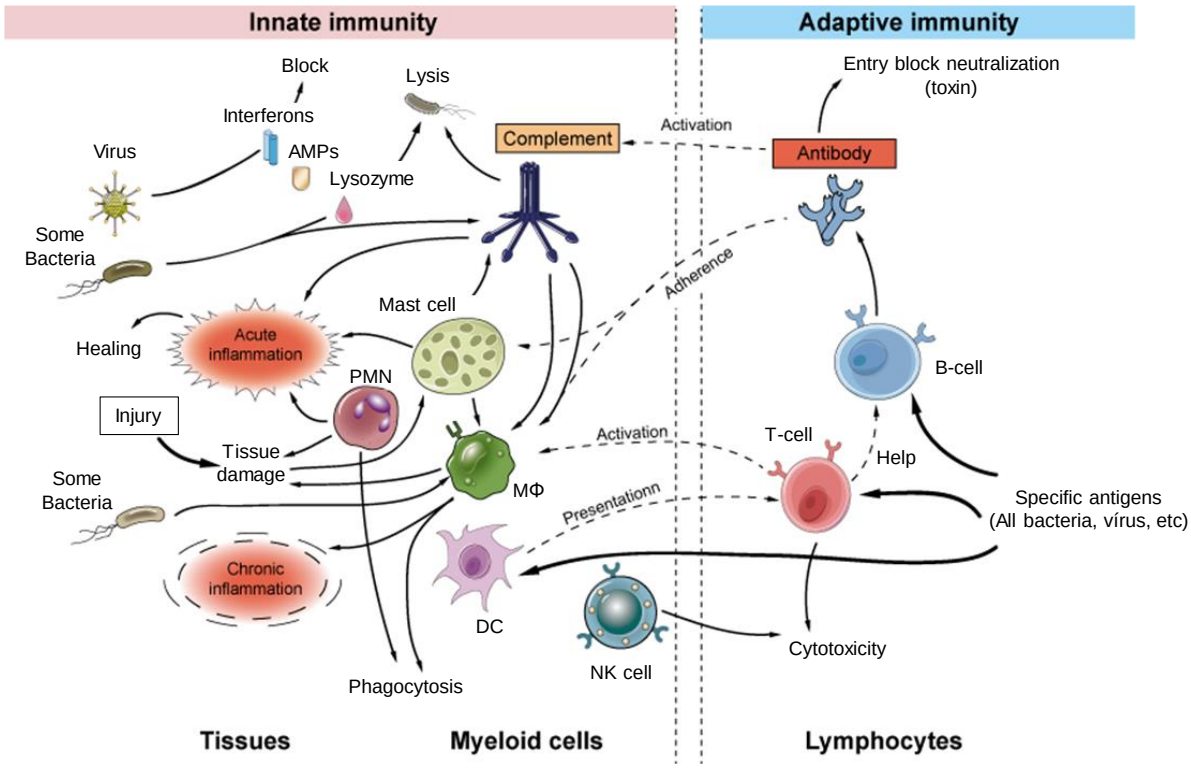

Figure 2. General components of both innate and adaptive responses (adapted from https://www.creative-diagnostics.com/innate-and-adaptive-immunity.htm). AMPs – Antimicrobial peptides; DC – Dendritic cells; MΦ – Macrophage; NK cell – Natural killer cell; PMN - Polymorphonuclear leucocytes.

A component of the innate immune response present in virtually all organisms are the antimicrobial peptides (AMPs), that are usually small, cationic and amphipathic. They show direct antimicrobial activity against a wide range of pathogens (several bacteria, viruses, fungi and parasites), but their functions go far beyond pathogen killing. Today, it is known that these peptides are integral part of immune responses, by modulating different immune mechanisms [12,13]. The presence of these small molecules along the evolutionary scale reflects the importance of these peptides in the fight against pathogens. We are currently dealing with an alarming situation regarding antibiotic resistance. As such, an in-depth study on AMPs, their complexity of sequences, structures and functions, advantages and disadvantages, would be a valuable information for the development of novel compounds.

A little bit of history

The discovery of antimicrobial substances goes back much further in history. In 1922, Alexander Fleming reported for the first time the existence of the antimicrobial protein lysozyme, after treating bacterial cultures with nasal secretions of a patient suffering from common cold [14]. Later, Fleming also discovered penicillin, a substance produced by the green mold *Penicillium notatum,* capable of inhibiting the growth of different bacteria [15]. This is perhaps one of the greatest scientific discoveries, hallmark of the modern medicine, which culminated with Alexander Fleming, along with Howard Florey and Ernst Chain, being awarded with the Nobel Prize for Medicine in 1945, for the discovery and the therapeutic properties of penicillin [16]. During that period, other antimicrobial molecules were discovered, including gramicidins and streptomycin [17–19]. In the so called “Golden Age” of antibiotics, many different classes of antibiotics were developed, including tetracyclines, penicillins, quinolones or sulfones [20]. However, with the discovery and use of antibiotics, the interest on the study of natural host peptides was lost, until the 60s, when the development of antibiotic-resistant microorganisms emerged [21]. With the growing interest on the study of AMPs, multiple authors isolated and characterized several molecules, in different species, ranging from bacteria to higher vertebrates.

During the 1950s, studies on AMPs started with the isolation of a cationic peptide from human polymorphonuclear leucocytes, with antibacterial activity, particularly on Gram-negative bacteria [22]. Years later, many other peptides were isolated, from insects to primates. In 1981, two AMPs were isolated in the hemolymph of the cecropia silk moth (*Hyalophora cecropia*) and named cecropins A and B [23]. In the same decade, six defensins were isolated in rabbit [24,25] and one defensin in human neutrophils [26]. Then, other defensin peptides were characterized, including the beta-defensin from bovine neutrophils [27] and human skin [28], primate theta-defensins [29] and, years later, the fish beta-defensins [30]. The plethora of novel AMPs isolated was not limited to defensins and soon other host defense peptides were also discovered: cathelicidin LL-37 [31], hepcidin [32,33] and the fish specific piscidins [34]. Today, over 3000 natural AMPs have been isolated, from bacteria, protists, fungi, plants and animals, with diverse activities [35].

Mechanisms of action of antimicrobial peptides: more than just pore forming

Antimicrobial peptides present unique characteristics - amino acid composition and secondary structure, charge and amphipathic character - that allow them to bind and

insert into pathogen membrane bilayers through different mechanisms, best understood for bacterial cells [36,37]. These membranes are organized in a way that the outer leaflet of the bilayer, exposed to the extracellular environment, is populated by negatively charged lipids, including lipopolysaccharides or anionic phospholipids in the Gram-negative bacteria, or the teichoic acids on the surface of Gram-positive bacteria [37–39]**.** On the contrary, most of eukaryotic membranes are formed by a variety of lipids, including neutral phospholipids and cholesterol, with most of the lipids with negatively charged headgroups positioned into the inner leaflet, facing the cytoplasm (Figure 3) [36,40,41]. The neutral charge, condensation of phospholipid bilayers by cholesterol and the asymmetric distribution of phospholipids in the membrane of eukaryotic cells lead to a reduced interaction of AMPs with these membranes. Thus, through hydrophobic and electroestatic interactions, peptides preferentially target the bacterial cells [41].

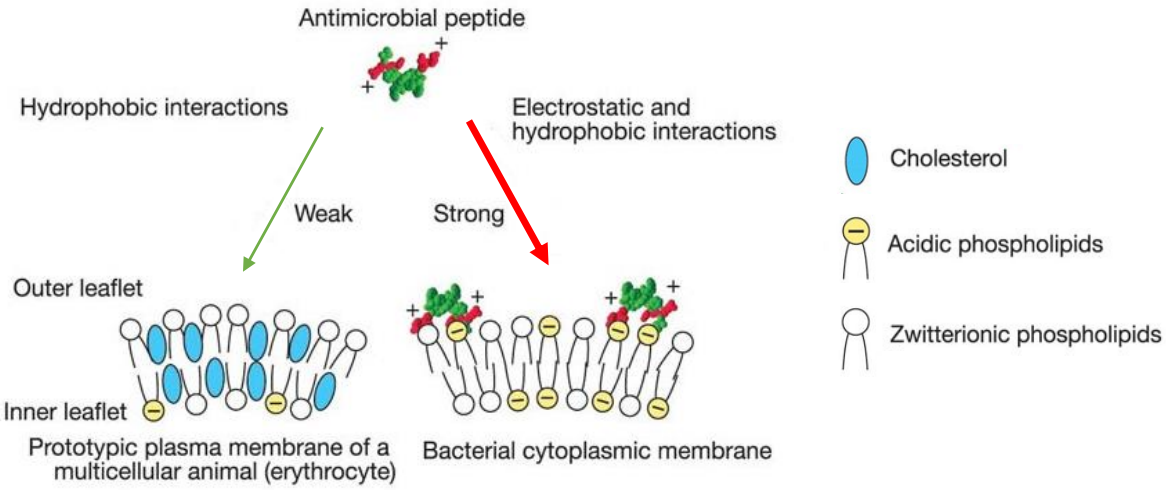

Figure 3. Interactions between eukaryotic and bacterial membranes and AMPs (adapted from Zasloff [36]).

Different models of membrane disruption were established to explain these interactions, including the carpet, toroidal-pore or barrel-stave models. Other mechanisms of membrane damage include electroporation, non-lytic membrane depolarization or membrane thinning/thickening [42] (Figure 4). The carpet mechanism consists in the accumulation of peptides on the bilayer surface, covering membrane surface in a carpet-like manner. When a threshold concentration is reached, the surface-oriented peptides are thought to disrupt the membrane, leading to the formation of micelles [37]. In the

toroidal-pore mechanism, peptides bind and displace the phospholipid head-groups, forcing them to bend to the interior of the lipid bilayer. In the barrel-stave model, helical peptides cross the membrane and stay parallel to membrane lipids, building a pore in an organized way, like a group of staves forming a barrel. AMPs can also interfere with membrane associated physiological events, leading to an impaired cell wall synthesis, or to a depolarization of transmembrane potential, with a consequent dysfunction, rupture and cell death [42–44]. These mechanisms are not necessarily exclusive of each peptide. Furthermore, action on pathogens can be simultaneously orchestrated by different peptides, having a complementary or synergistic effect, probably increasing the efficiency of AMPs [42,45].

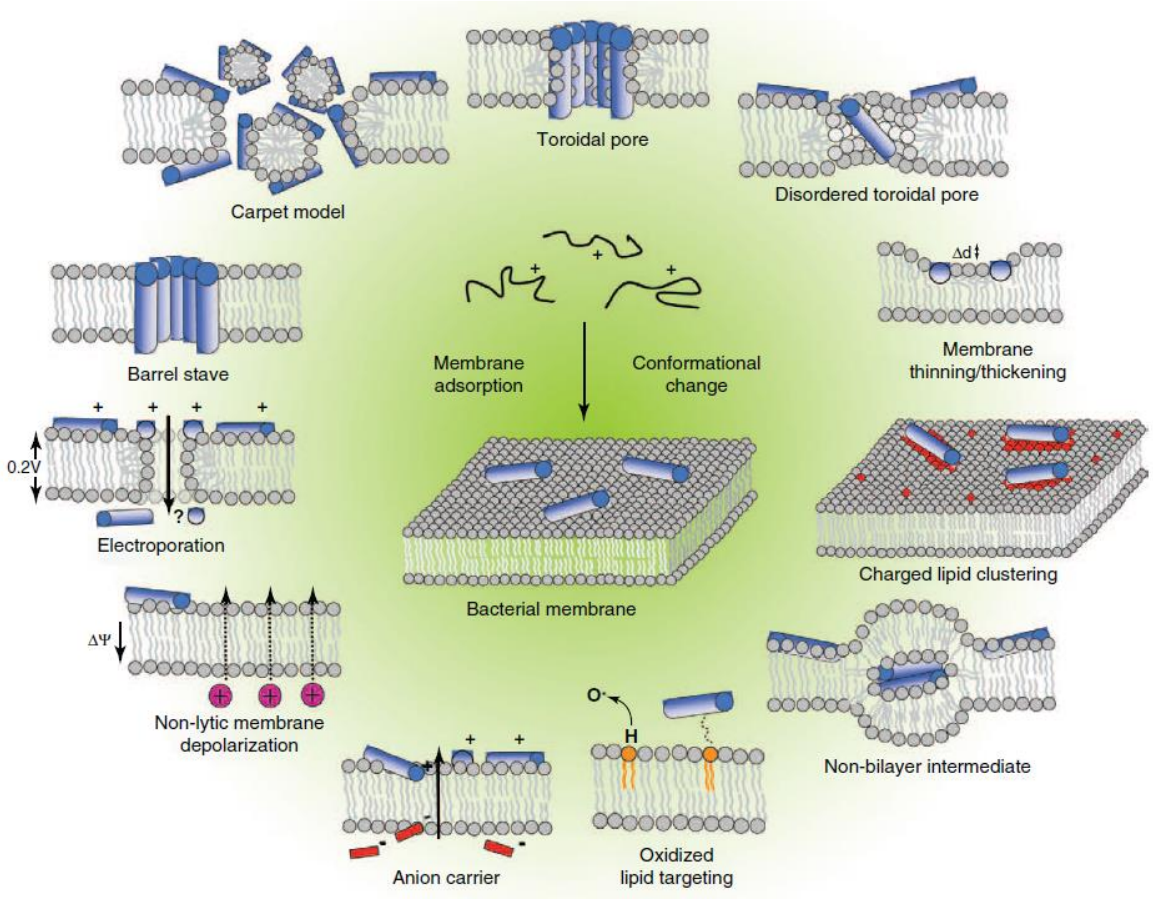

Figure 4. General mechanisms by which AMPs exert their antimicrobial action on membranes (adapted from Nguyen et al [42]).

Pore formation or membrane disruption can ultimately lead to cell death [43]. However, with the increasing knowledge on the functions and mode of action of these molecules, it

became clear that membrane targeting is not the only way that peptides have to attack pathogens. The mechanisms abovementioned essentially lead to membrane permeabilization or the formation of pores, which eventually lead to cell death, but can also allow the translocation of AMPs to the interior of the cell [44]. Once in the cytoplasm, these AMPs interact with their targets, interfering with vital cell processes, such as RNA, DNA and protein synthesis, and enzymatic activity [13,46]**.** For instance, killing of *Escherichia coli* by human defensin involves a series of events, starting with permeabilization of the outer membrane, followed by permeabilization of the inner membrane, and inhibition of RNA, DNA and protein synthesis [47].

Antimicrobial peptides not only have a direct pathogen killing activity, but also contribute indirectly, by modulating different mechanisms, targeting diverse cell types and pathways, leading to a recruitment and activation of different immune cells, regulation of inflammation or an increased phagocytosis [21,41,48,49]. For instance, human beta-defensins and the cathelicidin LL-37 present chemotactic activities towards different immune cell types [50–55]**.** The human beta-defensin 2 exerts its action towards immature dendritic and T-cells, through binding with the CCR6 [51]. More recently, it was demonstrated that human beta-defensins 2 and 3 and the mice orthologues beta-defensins 4 and 14 chemoattract monocytes, which do not express CCR6, in a CCR2 dependent manner [55]. As such, human beta-defensins exert their chemotactic activity by binding with different chemokine receptors. In another study, the authors described that murine beta-defensin 2 acts as an endogenous signaling ligand for Toll-like receptor 4 (TLR4), activating a signal for dendritic cell maturation through a pattern recognition receptor [56]. Hepcidin, a cysteine rich antimicrobial peptide, presents a major role as an iron regulator, inhibiting the function of the iron exporter ferroportin, regulating systemic body iron and promoting its availability or its limitation, depending on different physiological conditions [57–60].

Different AMPs are also potent anti-tumor molecules, killing cancer cells through membranolytic or non-membranolytic mechanisms [61]. Cancer cells present in general a negatively charged membrane, due to the increased presence of certain compounds, including phosphatidylserine or sialylated gangliosides, differing from the normal cell membrane, typically neutral in charge [61]. As such, AMPs kill these cancer cells through mechanisms similar to the ones involved in pathogen killing, that rely on membrane disruption and pore formation, destroying the cancer cells or leading to the leakage of cell content [62]. Still, the action of peptides on cancer cells is not limited to a simple pore formation or membrane disturbance, they can also trigger apoptotic pathways or promote cell necrosis [63–65].

Antibiotics *versus* antimicrobial peptides

The discovery of antibiotics changed the entire medical field, with many infections that were common causes of death and disease being easily treatable. Many antibiotics were commercialized during the "Golden Age", with activity against a wide range of bacteria [66]. However, the over-reliance on antibiotics in animal production and the over prescription in the medical practice are the two main factors that have led to the increasing development of resistant microorganisms [67]. Fleming himself warned that exposing microbes to non-lethal doses of penicillin could make them resistant to its action [68]**.** But Fleming's words were not taken seriously and currently, we are not only dealing with resistant bacteria, but also with the incapacity of scientific community in developing novel classes of antibiotics fast enough and in sufficient number to support our medical needs during the years to come [69]. Between the 1930s and 1960s, more than 20 novel classes of these drugs were produced. Since then, only three new antibiotics have been developed [67,69].

Antibiotics fall into different classes, depending on their target and mode of action. These drugs can exert their action at membrane level, or in certain bacterial mechanisms, necessary for their survival, such as protein, DNA and RNA synthesis [20]. Bacteria developed several mechanisms to overcome the action of antibiotics, that include active drug efflux, drug inactivation or target modification [66]. Antimicrobial peptides present a low tendency to induce pathogen resistance, but it is not nonexistent. There are advantages of AMPs over antibiotics: while antibiotics are mainly active against bacteria, AMPs present a wide range of action against different pathogens, including viruses; bacterial killing promoted by AMPs is rapid, usually less specific and may involve multiple targets, while antibiotics are more target-specific; AMPs have additional potential applications, including modulation of host's immune responses [70–72]. However, there are some factors hindering the introduction of AMPs: they are still expensive, when compared to antibiotics; they present a short half-life and susceptibility to protease degradation; some AMPs present a degree of toxicity, which can limit their clinical use [72].

Antimicrobial peptides can be used not only in animal production or medical field, but also as food preservatives. There is also a possibility to use them in combination with other molecules, to overcome some of the resistance mechanisms developed by pathogens [72,73]. However, microorganisms are capable of protecting themselves against any antimicrobial compound, by developing mechanisms of resistance against them. Thus, it is unrealistic to think that AMPs will not eventually promote some degree of microbial resistance [74]. Some bacteria can change specific outer membrane residues, in order to

reduce the negative charge and thus, lowering the electrostatic interactions between the membrane and cationic AMPs [75,76]. Bacteria also produce proteases capable of inactivate and degrade certain AMPs [77–79]. They can also produce outer membrane vesicles, creating a protective shield surrounding the outer membrane [80], transport AMPs to the extracellular environment through efflux pumps [81], or even suppress the expression of AMPs of the host [82], although some of these mechanisms are yet poorly understood (Figure 5). Still, bacteria acquire resistance towards host defense peptides at a much slower rate than antibiotics. This is possible because AMPs not only have multiple modes of action on pathogens, but also due to the role of AMPs as modulators of innate immunity, acting on the host and not directly on the bacteria, reducing the probability of resistance development [83]. Furthermore, as the connection between peptide-membrane relies on electrostatic and hydrophobic forces, significant alterations at membrane level, in order to prevent these interactions, seem unlikely and costly [36].

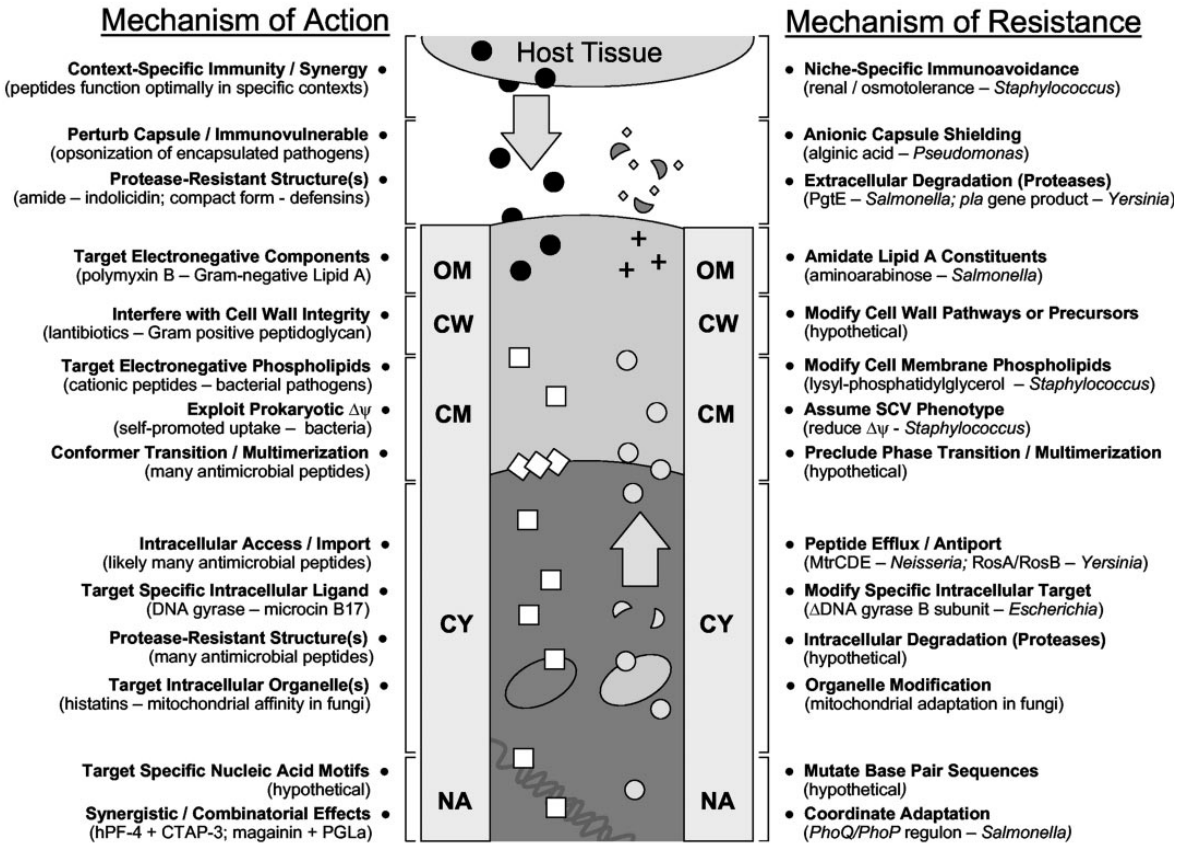

Figure 5. Mode of action of AMPS and mechanisms of microbial resistance (adapted from Yeaman and Yount [84]). CM: Cytoplasmic membrane; CW: Cell wall; CY: Cytoplasm; NA: Nucleic acid; OM: Outer membrane. Symbols: dark spheres: native antimicrobial peptides; Light squares: activated or conformation-transformed peptides; ovals:

organelles; double helix: nucleic acids; light spheres: inactive peptides; Crescents: degraded peptides.

Fish antimicrobial peptides: the future comes from the sea

Prophylactic and therapeutic strategies: for a sustainable aquaculture

With the rapid depletion of natural stocks, due to the increasing global needs for seafood products, aquaculture has become the fastest growing food producing sector in the world. According to the latest *The State of World Fisheries and Aquaculture*, worldwide fish production reached about 179 million tons in 2018, contributing with 46% for the total production (total fisheries and aquaculture) [85]. To cope with this striking growth, farmers have developed intensive aquaculture practices, where fish are exposed to stressful conditions, thereby contributing to an increased susceptibility to diseases [86]. Thus, as aquaculture is continuously increasing, so do the problems, particularly the emergence of disease outbreaks, impairing production and leading to substantial production losses [87]. To prevent and/or treat these disease outbreaks, few measures can be implemented, including water treatment systems, quarantine for newly arrived fishes, high hygiene standards at the facilities, use of immunostimulants, antibiotics and vaccination for a limited number of pathogens [88–90]. However, the misuse of antibiotics brought severe environmental and health-related implications. Among them are included residue accumulation in the surrounding environment, microbial selection for antibiotic resistance and the emergence of multi-resistant strains, with a consequent development of antibiotic resistance for important human bacterial pathogens [91]. Different classes of antibiotics have been used in aquaculture, such as erythromycin, fluoroquilonones or tetracyclins, with some fish bacteria presenting a degree of resistance against these drugs, including *Photobacterium damselae* spp. *piscicida*, *Vibrio anguillarum* or *Aeromonas salmonicida* [92–94]. Therefore, the use of novel prophylactic or therapeutic agents is urgent to make aquaculture a more sustainable industry.

In developed countries, the use of antibiotics is currently highly controlled. In Europe, European Union (EU) Council regulation prohibited the use of certain antibiotics and established maximum residue limits (MRLs) for other compounds, together with a severe monitoring of the sale and use of veterinary antibiotics [86,95]. Furthermore, the use of vaccines became a popular – and necessary – measure to prevent possible disease outbreaks, and the number of novel vaccines has increased during the last years. For instance, there has been a profound reduction in the use of antibiotics in the Norwegian

production of Atlantic salmon (*Salmo salar*), since the introduction of vaccines, that consist mainly in inactivated formulations for the main salmon infecting viruses and bacteria [96]. However, vaccines available are yet insufficient. Most of licensed vaccines are formulated using inactivated microorganisms with adjuvants, mainly administered via injection, but also via immersion or oral routes [89,97,98]. Most of the vaccines were produced to control bacterial infections, with some being specific for viral diseases. However, there is still a lack of efficient vaccines to control the variety of infections afflicting the multiple fish species that are currently produced in aquaculture [96]. Issues involving the use of these vaccines may include allergic reactions to formalin, development of a poor protection in some cases, or the administration of multiple applications to boost the immune response [97,99]. Furthermore, there are some constraints regarding the route of administration. The intraperitoneal route is by far the most effective one, but it presents some limitations: it is stressful and is not practical in small fish, and usually very time consuming [100]. Other routes, such as immersion or bath, represent an easier way to deliver vaccines, particularly in smaller fish. However, most are not yet compatible with the use of adjuvants and the lack of more knowledge of the mucosal responses is hindering the production of efficient mucosal vaccines [96,101]. A potential alternative relies on the use of attenuated vaccines. Smaller doses of the attenuated microorganism are usually required to induce a strong response, they can be easily delivered through natural routes, and the amount of adjuvant required is minimal [97,102]**.** However, and although they are generally considered safe, potential risks include the possibility to revert to virulent forms [96]. Due to the advances in the fields of immunology, genetics, and molecular biology, there has been an increasing interest in developing next-generation vaccines for aquaculture, that includes the recombinant, RNA, DNA and peptide vaccines, or nanovaccines [97,101]. To promote the best results, there are several factors that must be taken into consideration: pathogen, type of vaccine, route of administration, duration of protection, safety to animals and surrounding environment, stress to fish and labor costs [101].

The need for novel therapeutics in the medical field and animal production has compelled the scientific community to study the functions, mechanisms of action and potential beneficial effects of antimicrobial peptides of different organisms, fish included (reviewed in [99,103–105]). Their direct roles against pathogens and modulation of host's immunity promote a better response against infection and, consequently, increased survivals of infected fish. AMPs can be also used in the development of vaccines, acting as natural pathogen inactivators and replacing the conventional methods, such as formalin, overcoming the possible issues related with its use [106]. Use of AMPs as biomarkers of fish health is also a possibility. Alterations in the expression or production of AMPs are

closely related with different types of stress. Assessing these alterations could be a promising approach to monitor fish health, giving farmers the possibility to anticipate a possible disease outbreak and introduce the proper measures to control these outbreaks, before the establishment of the disease [107,108]. To date, many fish AMPs are characterized in different fish species, in varied number and showing different functions. They belong to different groups of AMPs and some of them – beta-defensins, hepcidins and piscidins – will be described in detail bellow.

Beta-defensins: peptides with several functions

Defensins are perhaps the most well-studied group of antimicrobial molecules. They not only present activity against different pathogens, but also show diverse immunomodulatory functions. Defensins constitute a diversified group of cysteine-rich peptides found in vertebrates. In mammals, and considering the intramolecular disulphide bonds, defensins can be classified in three different classes: alpha-, beta- and the cyclic theta-defensins [109]. However, in phylogenetically earlier vertebrates such as teleost fish, so far only beta-defensins were isolated, presenting six conserved cysteines that bind in a particular pattern of Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6, folding into a beta-sheet conformation [30,110–116]. Thus, while alpha- and theta-defensins appear to be specific to mammals, with theta-defensins being found only in some non-human primates [29,117], beta-defensins are widely distributed in different vertebrate species, from fish, to birds, mammals and reptiles [27,30,118,119]. The presence of beta-defensins in mammals was reported for the first time in 1993 [27]. However, only in 2007 fish beta-defensin-like peptides were characterized, with the isolation of three beta-defensins in zebra fish (*Danio rerio*), two in spotted green pufferfish (*Tetraodon nigroviridis*) and one in tiger pufferfish (*Takifugu rubripes*) [30]. Then, several other peptides were characterized in different fish species, in a diverse number: one defensin in Nile tilapia (*Oreochromis niloticus*) [113], Gilthead seabream (*Sparus aurata*) [120], Atlantic cod (*Gadus morhua*) [112] or meagre (*Argyrosomus regius*) [121]; three in common carp (*Cyprinus carpio*) [122,123]; four in rainbow trout (*Oncorhynchus mykiss*) [110,124]; and seven in Atlantic salmon (*Salmo salar*) [125]**.**

Fish beta-defensins are usually comprised by a signal peptide with 18 to 26 amino acids and a 39 to 45 mature peptide [30,110–112,120,125–127]. Contrary to other AMPs, such as piscidins and hepcidins (see below), beta-defensins do not present a proregion, with the exception of the peptides isolated in olive flounder (*Paralichthys olivaceus*). These are constituted by 67 to 77 amino acids, in which a 5 to 15 amino acid proregion is also

present, that is then cleaved to produce the active peptide [128]. Thus, olive flounder defensins present a different amino acid sequence and the resulting putative peptide shows anionic properties, while other fish defensins present in general a conserved amino acid sequence, that folds into a cationic protein. Still, six cysteines are retained in fish beta-defensins, at conserved positions, that fold into three anti-parallel beta-strands [30,112–116,127,129], although some peptides may also present an extra alpha-helix at the N-terminus of the three beta-strand structure [30,112,116,129]. Genes encoding for these AMPs in fish are comprised by three exons and two introns of varied sizes, differing from the mammalian counterparts, that show a gene structure with two exons and one intron [30,110,112,113,125,130,131].

These AMPs can be detected in several organs, but the more prominent expression of beta-defensins seem to be in the immune and mucosal tissues, mainly the head-kidney, skin, spleen, intestine, gill and liver [113,116,120,125,129,131,132], but they can also be found in the brain, eye, muscle, heart, stomach, pituitary, swim bladder or gonads [30,110,112,113,125,131–134]. Still, as observed for other AMPs, the expression of beta-defensins seems to be dependent on the fish species, tissue and gene analyzed. Beta-defensin transcripts can also be detected at the first stages of fish embryogenesis, suggesting that these AMPs may be part of fish defenses during development [112,121,128,135]. After infection by different pathogens or stimulation by PAMPs, an alteration in beta-defensin expression is observed, also dependent on the infectious agent, tissue or defensin analyzed [110,116,121,126,132,135–137].

The potential applications of beta-defensins in fish are poorly explored. It is known that these AMPs are active against bacteria and viruses (Appendix 1). Still, other major functions are attributed to beta-defensins, particularly chemotaxis [120,138–140], phagocytic activity stimulation [112,126,140] or regulation of inflammatory genes [126,132,138,141]. Beta-defensins may not exert their action through a direct antimicrobial activity, but rather trough stimulation of the abovementioned immune pathways. Fish beta-defensins present proved antiviral activity against betanodavirus and rhabdovirus [124,132,141]. However, *in vivo*, the function of defensins may rely on the modulation of immune responses rather than a direct antiviral activity, as demonstrated by García-Valtanen et al. [141], after treating zebrafish with the plasmid construct pMCV1.4-zfBD2 (zebrafish beta-defensin 2). This AMP elicited the activation of the type I interferon (IFN)-system, the transcription of immune-related genes, such as tumor necrosis factor alpha (*tnfα*) or interleukin 1 beta (*il1β*), as well as the recruitment of cytotoxic cells to the site of injection. Grouper beta-defensin also elicits antiviral activity *in vitro* by reducing the progression of viral replication and infection, as well as by inducting a type I IFN-response [132]. Atlantic cod beta-defensin did not show an *in vitro* antibacterial activity against *V.*

anguillarum. Still, the gene transcription was up regulated in the head kidney of challenged fish with this bacterium, and the administration *in vitro* of defensin stimulated the phagocytic activity of cod head kidney leukocytes. According to the authors, cod defensin was induced in the presence of *V. anguillarum* because it stimulates the phagocytic activity of head kidney leukocytes in response to the bacterial challenge [112]**.** Gilthead seabream head kidney leukocytes were attracted to the recombinant beta-defensins, but not to the recombinant human ones, suggesting a certain degree of specificity by fish receptors [120]. It is known that mammalian beta-defensins are chemotactic for different cells. Furthermore, the tertiary structure of beta-defensins presents similarities to chemokines, although the alpha-helix is located at the C-terminal of these proteins and the quaternary structures differ significantly between each sub-family [142]. Some of the players of the pathways by which human and mice beta-defensins promote their chemotactic activity are present in fish. Orthologues of CCR6 have been identified in in different fish species [143–145], but the presence of others, including CCR2, is not yet clear in fish [143,146]. Still, previous functional studies point towards the presence of CCR2-like orthologues in fish [147,148]. The relationship between fish chemokine receptors and beta-defensins is yet to be described, or if beta-defensins exert their chemotactic activity trough other pathways.

Hepcidin: the central player in the iron game

Hepcidin (HAMP) is a small cysteine rich peptide, predominantly secreted by the liver. It was first described in humans and named LEAP-1 (for liver-expressed antimicrobial peptide 1) by Krause and collaborators [32]. Later, it was isolated by other authors and named hepcidin, due to its hepatic expression and antimicrobial activity [33]. However, the antimicrobial function of this peptide was quickly relegated to the background and it is now considered to be the central player of systemic iron regulation.

Iron is an essential element for most organisms, involved in many processes, including oxygen transport, catalysis of many cellular metabolic reactions and enzymatic activities. However, excess of intracellular iron may result in the generation of reactive oxygen species (ROS), that can lead to protein or DNA damage. Since there is no active pathway of iron excretion (even in individuals suffering from iron-overload disorders), a tight control of body iron absorption, transport, storage and utilization for erythropoiesis and other physiological functions is maintained [149–151]. These pathways are coordinated by different cells and proteins, well conserved in vertebrate species, including fish. Many of the molecules involved in the uptake, export, transport and storage of iron were already

characterized in some fish species. Among these include the solute carrier family member 2 alpha, divalent metal transporter 1 or natural resistance-associated macrophage protein 2 (Slc11a2alpha/ DMT1/ Nramp2) [152–156]; ferroportin (Fpn1), also known as solute carrier family 40 member 1, iron regulated-transporter-1 or metal transporter protein-1 (Slc40a1/ IREG1/ MTP1) [157–159]; transferrin (Tf) [160–165] and transferrin receptor (TfR) [158,162,166]; or ferritin (Ft) [160,167–169].

Hepcidin regulates iron by inhibiting the function of the only known iron exporter, ferroportin, inducing it internalization in the hepatocytes, duodenal enterocytes and macrophages, decreasing the export of cellular iron [57–59,170]. Hepcidin expression increases during iron overload and infection or inflammation and decreases during anemia or hypoxia, through different pathways and involving several molecules, best understood in mammals (Figure 6) [171]. Still, many of the players involved in *hamp* regulation were also described in different fish species and thus, these mechanisms are mostly conserved between fish and mammals [172–176]. Most mammals present a single hepcidin, with the mouse being the only known exception [177,178]. Human hepcidin is predominantly produced in the liver as an 84-residue prepropeptide, which is later cleaved into the active 25-amino acid (aa) mature peptide [32]. However, fish present a diverse group of hepcidins, usually divided into two different groups, the type 1 hepcidin (Hamp1) and the type 2 hepcidin (Hamp2) [179].

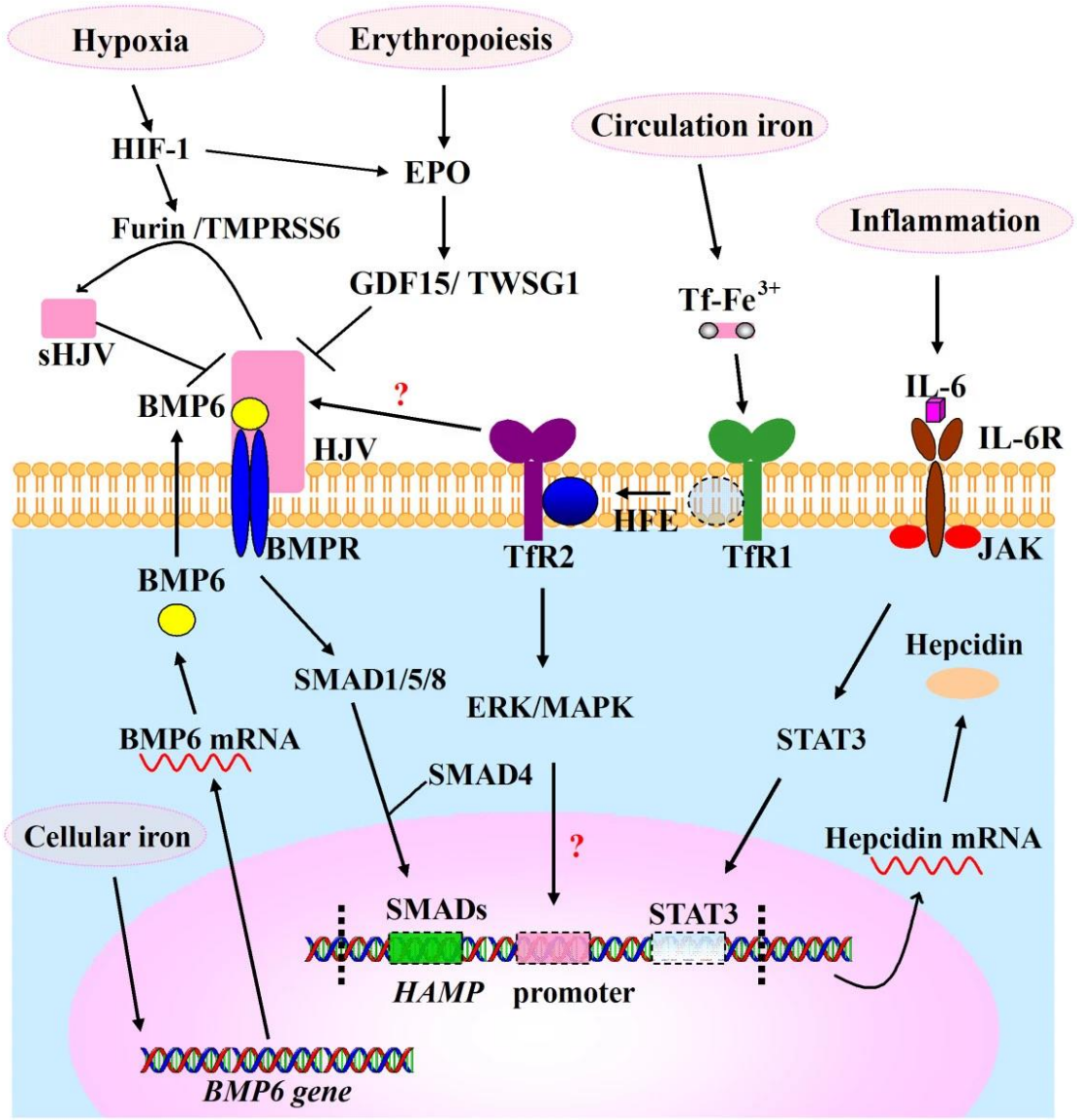

Figure 6. Pathways of *hamp* expression regulation in mammals (adapted from [180]). BMP6: Bone morphogenetic protein 6; EPO; Erythropoietin; ERK: Extracellular-signal-regulated kinase; GDF15: Growth differentiation factor 15; HFE: Homeostatic iron regulator; HIF-1: Hypoxia-inducible factor 1; HJV: Hemojuvelin; IL-6: Interleukin-6; IL-6R: Interleukin-6 receptor; JAK: Janus kinase; MAPK: Mitogen activated protein kinase; SMAD - Similar to mothers against decapentaplegic family member; STAT: Signal transducer and activator of transcription proteins; Tf: Transferrin; TfR: Transferrin receptor; TMPRSS6: Transmembrane serine protease 6; TWSG1: Twisted Gastrulation BMP Signaling Modulator 1.

The general characteristics of hepcidin are well conserved among vertebrates. Hepcidin genes are comprised by three exons/two introns, with sizes that vary between species [33,181–186]. The peptide is produced as an 81 to 98-aa prepropeptide, with a 22 to 24-aa signal peptide, a 38 to 50-aa prodomain and a 19 to 26-aa mature peptide [181–183,185–194]. The mature peptide normally includes 8 cysteines, at conserved positions, although hepcidins containing 4 and 6 cysteines were also isolated [182,195–197]. The resulting hepcidin mature peptide consists in a beta-hairpin-like peptide, after formation of the disulphide bridges, similar to the human counterpart [198–206]. Like mammals, the primary site of hepcidin expression in fish is the liver, but these AMPs can also be detected in other tissues, such as spleen, intestine, kidney, brain, gill or skin, depending on the species and hepcidin gene [183,186,187,189,195,204,205,207,208]. Hamp transcripts are also detected early during embryogenesis and in some species, its expression increases during development [121,183,193,209,210]. Fish hepcidin type 1 is characterized by the presence of a hypothetical iron regulatory sequence Q-S/I-H-L/I-S/A-L, is likely present in all species and is the homologous of the mammalian hepcidin [181–183,185,188,189,193,200,202–204,208,211–213]. However, hepcidin type 2 seems to be restricted to Acanthopterygian species and lacks the presence of this motif [121,190–192,194,195,207,211–216]. In some cases, multiple Hamp2-like genes are found, while hepcidin type 1 is usually present as a single gene [186,207,212,217]. This diversity of hepcidins is likely a result of genome duplications and positive selection, particularly in Perciform and Pleuronectiform species [179,218–220]. On the other hand, non-Acanthopterygian species, such as Salmoniforms, Cypriniforms or Gadiforms lack the presence of Hamp2-like orthologues.

Modulation of hepcidin expression is dependent on the physiological condition, either iron modulation, infection by different pathogens, or stimulation by PAMPs [121,159,184,186,187,200,207,213,221–223]. However, and considering that many fish species present a diverse and large number of hepcidins grouped into different types, these peptides can perform those functions independently. In fact, some studies support the hypothesis that *hamp1* is overexpressed during iron overload, while *hamp2* expression increases significantly during infection [159,186,187,223]. Still, *hamp1* can also be up-regulated in infected fish, to lead to iron withholding, limiting pathogen proliferation [186,187]. In anemic fish, *hamp1* expression presents significant down-regulations, allowing the ferroportin-mediated iron release for compensatory erythropoiesis [159,186,224,225]. Teleost fish presenting two different hepcidin types show a high degree of subfunctionalization: Hamp1 has iron regulatory roles, inhibiting the action of ferroportin, and showing little or any direct antimicrobial activity [158,159,186,221,226]; while different Hamp2 are significantly up-regulated during

infection and mostly perform antimicrobial functions, in a peptide and pathogen dependent manner [186,190,221,226–229], with no direct effect on ferroportin [159]. However, this may not be so straightforward, as in some fishes, asides the function on iron metabolism, hepcidin type 1 also presents significant antimicrobial roles [202,204,206,230,231]. Many studies already demonstrated the direct microbial killing of fish hepcidins, against fish and non-fish specific pathogens, or even cancer cell lines, and are described in Appendix 2. Hepcidin, usually cationic in charge, can bind and act on pathogens trough mechanisms similar to other AMPs. Previous studies showed that fish hepcidins act directly on bacterial membrane surface, causing cell disruption and cytoplasmic thinning [204,226,232], or thought bacterial DNA hydrolysis [189,202,206,221]. Furthermore, the importance of the Q-S/I-H-L/I-S/A-L motif in the regulation of iron was also demonstrated, as synthetic Hamp1 lacking the presence of this motif resulted in the loss of iron regulatory functions [189,206], similarly to what as previously observed for human hepcidin [233]. On the other hand, the antimicrobial capacity of these fish hepcidins lacking the iron regulatory motif seems to be retained or even improved.

Some studies have shown the protective effects of hepcidin administration during infection with different pathogens. Treatment of turbot with either hepcidin 1 or 2 before infection with *Edwardsiella tarda* or megalocytivirus RBIV-C1 led to an increased resistance, with fish presenting reduced bacterial and viral loads in different tissues, more pronounced in the group treated with Hamp2 [226]. In the mudskipper (*Boleophthalmus pectinirostris*), only Hamp2 reduced tissue bacterial load on fish infected with *E. tarda,* demonstrating the function of hepcidin type 2 as an antimicrobial molecule [221]. Tilapia hepcidin (TH) 1-5 also proved it value as a potential compound for the control of nervous necrosis virus (NNV, *Nodaviridae*) or pancreatic necrosis virus (IPNV, *Birnaviridae*), both *in vitro* and *in vivo* [227,234,235]. In the grouper and medaka, the co-treatment with this AMP and NNV promoted an increased fish survival after re-infection with this virus [227,235]**.** More recently, the use of the single hepcidin 1 of grass carp (*Ctenopharyngodon idella*) was tested, either as a prophylactic or therapeutic molecule. Administration of this hepcidin led to an increased survival of this fish species against *Aeromonas hydrophila,* by a direct action on bacteria and by reducing iron availability for the pathogen [206].

Piscidins: the fish specific antimicrobial peptides

Many fish species present a particular group of small, linear and amphipathic peptides, first described in the winter flounder (*Pleuronectes americanus)* [236]*,* that presents a distant relationship with the insect peptide cecropin [237]. Later, several other piscine

AMPs were also found in varied teleost families, such as Moronidae [238–241], Serranidae [242,243], Sciaenidae [244] and Gadidae [245]. Members of this group include the pleurocidins, moronecidins, gaduscidins, epinecidins and dicentracin and, although they show a low degree of similarity in terms of the amino acid sequences, the linear and alpha-helical nature of the mature peptides, with a high number of positive and hydrophobic residues, place all these small proteins on the same evolutionary related family, called “piscidin”.

Piscidins can be found in a diverse number, depending on the fish species. While Cyprinid and Salmonid species seem to lack the presence of piscidin orthologues, in other species, such as Nile tilapia (*Oreochromis niloticus*), white bass (*Morone chrysops*), striped bass (*Morone saxatilis*) and the hybrid striped bass (*M. saxatilis* × *M. chrysops*) five to six piscidins were already isolated [241,246]. Piscidin genes are mainly constituted by four exons/three-introns of varied sizes, with the first exon being usually constituted by the 5’ UTR, and the coding sequence starting at the second exon [238,246–250]. However, deviations from the common organization were also described, being the most significant the piscidin genes isolated in Nile tilapia and in the grouper (*Epinephelus coioides),* that show a three-exon/two-intron and five-exon/four-intron structure, respectively [246,251]. Piscidin peptides are usually produced as a 64 to 89 amino acid protein precursor comprised by a signal peptide, a mature peptide and a prodomain. This prepropeptide undergoes proteolytic cleavage to remove the signal peptide and the prodomain, resulting in an active 18–26 amino acid mature peptide [238,243,247,248,250,252,253]. However, in species belonging to the Moronidae family, piscidins with mature peptides comprised by 44 to 55 amino acids were characterized, with evident differences in the amino acid composition [240,241,254]. Piscidin mature peptides are highly diverse, suggesting that they are undergoing a positive Darwinian selection, through gene duplication and diversification. These AMPs adapted to rapidly changing pathogens that are coevolving with their host [218,255], supporting the reduced sequence identity amongst the mature peptides of piscidin family members.

The expression of piscidins varies depending on the gene, tissue and fish development. Studies performed in winter flounder, Atlantic cod (*Gadus morhua*), striped bass, meagre (*Argyrosomus regius*) and the European sea bass (*Dicentrarchus labrax)* have shown that some piscidins can be detected from the very first stages of fish development, while other piscidins are only expressed in juvenile/adult stages [121,210,249,256,257]. In juvenile to adult fish, piscidin constitutive expression is more significant in mucosal and immune tissues including the gills, skin and skin mucus, intestine, head kidney and spleen [188,241,242,244–246,248,250,253,258–262], but they can also be detected more broadly, in organs such as the liver, muscle, gall bladder, pyloric caeca, stomach, heart

and brain [246,249,250,253,256,258,261,263,264]. Piscidin producing cells include gill, intestine and skin mast cells, peripheral, head kidney or spleen phagocytes [242,263,265–272]. Piscidins can be found in tissues within the lethal concentrations for pathogens [107,273]. Furthermore, as evidenced by Mulero et al. [268], piscidins are delivered to phagosomes of granulocytes upon phagocytosis of bacteria, suggesting that piscidins are capable of acting against both extra- and intracellular bacteria. Thus, piscine peptides constitute an important compound of the fish immune responses against multiple infections.

Increases in the expression of piscidins are frequently observed after infection with different pathogens, or stimulation with different PAMPs, such as lipopolysaccharide (LPS) or poly (I:C) [242,244,246,248,253,260–262,269,274]. However, some reports also show decreases in the expression of piscidins or even no significant alterations. Different patterns of expression can be observed in the same fish species, during different infectious stimuli, with piscidins increasing in some tissues, at different time points, while in others the expression decreases or remain unchanged [121,243,246,258,259,275]. Thus, modulation of piscidin genes is not only dependent on the infectious agent, but also on the tissue and gene analyzed.

The functions and mode of action of different vertebrate AMPs have been studied since their discovery, with fish specific peptides being no exception. These fish AMPs are not only capable to kill or inhibit the growth of different fish and non-fish pathogens, but are also cytotoxic to different cancer cells. The main pathogens and cancer cell lines showing susceptibility to synthetic, recombinant or peptides derived from piscidins are shown in Appendix 3. Among these infectious agents are included important clinical pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [238,252,256,276–279], vancomycin-resistant Enterococcus (VRE) [238,278,279], *Klebsiella pneumoniae* Carbapenemase [278–280] or multidrug resistant (MDR) *Helicobacter pylori* [281,282]. Thus, it is widely recognized that piscidins have a direct pathogen killing activity. The amino acid sequence, secondary structure, amphipathicity and membrane composition are important factors for the action of piscidins. These peptides mostly adopt an amphipathic alpha-helical conformation, depending on the membrane composition [283–291]. Piscidins act on pathogens by disrupting their membranes [281,282,291–294] and may act through the toroidal or in-plane mechanisms, or via micellization [282,285,295]. Piscine peptides also act on cancer cells trough interaction with negatively-charged components of their membranes [296]. However, piscidin activity goes far beyond membrane disruption and pore formation. Pleurocidin is capable of translocate trough bacterial membranes and act intracellularly, inhibiting crucial mechanisms for pathogen survival, including protein synthesis [297]. Likewise, tilapia piscidin 4 induces the killing of

lung carcinoma cells by interfering with microtubule network of these cells [298]. Different piscidins can also induce the death of pathogens and different cancer cell lines by triggering apoptotic or necrotic mechanisms [65,299–303]. The mode of action of the 22-aa piscidins 1 and 3 of bass has also been addressed. Piscidin 3 presents in general higher minimal inhibitory concentration (MIC) values, when compared to piscidin 1 [34,241]. However, it was more recently described the relationship between the amino-acid sequences and structures of piscidins 1 and 3 with their antimicrobial functions, with both peptides acting through different mechanisms: while piscidin 1 is more membranolytic, piscidin 3 shows stronger interactions with DNA [304–306].

The antimicrobial and immunomodulatory functions of piscidins have been also widely studied, either in fish or mammalian models. Epinecidin treatment resulted in higher percentages of survival, lower bacterial loads and modulation of immune responses in mice infected with MRSA or *Helicobacter pylori* [276,281]. *In vitro,* pleurocidin NRC-04 induced mast cell migration, adhesion, degranulation and production of chemokines [307]. In fish, administration of piscidins resulted in lower mortalities, when compared to the infection alone [227,235,308,309]. Piscidins can be used as pathogen inactivators and can be useful in the development of inactivated vaccines. For instance, the use of a mixture with epinecidin and NNV or *Vibrio vulnificus* resulted in increased survival rates of fish after re-infection with these pathogens [227,235,308]. A similar experiment was made using tilapia piscidins 3 and 4, with fish showing a higher resistance to infection and higher survival percentages [309]. These results are accompanied with the regulation of immune-related genes, including *il1β*, *il6, il8*, *tnfα*, *ifnγ*, nuclear factor kappa B (*nfκb*), myeloid differentiation primary response 88 (*myd88*) and TLRs [308–311]. However, transcriptional changes of these genes are dependent not only on the pathogen, but also on the piscidin and route of administration.

European sea bass (*Dicentrarchus labrax*)

The European sea bass (*Dicentrarchus labrax,* family Moronidae) (Figure 7) is an euryhaline (3‰ to full strength sea water) and eurythermic (5-28 °C) fish, found in shallow waters (up to 100 m depth), from the north-eastern Atlantic Ocean to the Mediterranean and the Black Sea. They inhabit coastal inshore waters, estuaries and brackish water lagoons, being sometimes found in freshwater areas [312,313].

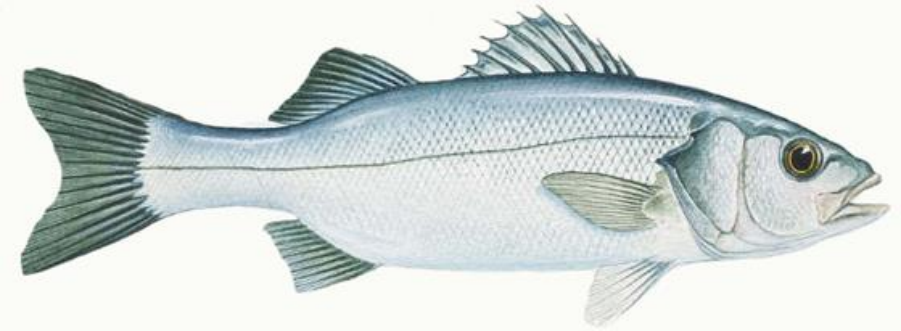

Figure 7. The European sea bass (*Dicentrarchus labrax*).
Source: https://thisfish.info/fishery/species/european-seabass/.

This fish species dominates the Mediterranean aquaculture, together with the gilthead sea bream (*Sparus aurata*), representing more than 90% of fish produced in this region [90]. Sea bass was historically cultured in coastal lagoons and tidal reservoirs before the introduction of intensive techniques and production expansion, during the 1980s. This fish species is currently intensively produced in ponds, tanks, cages and raceways, with the reproduction techniques being also well developed in some countries, providing a significant number of fry to fish farmers [312,314]. This was possible not only due to the high investment in sea bass production, but also to an increased knowledge of the species. During the last years, many researchers focused on the study of several aspects of sea bass, from nutrition [315] to the immune system [316,317], genome [318] and reproduction [319].

In 2019, production of sea bass in the Mediterranean area reached 208197 tons, representing an increase of 60000 tons since 2014 (approximately 148000 tons of sea bass produced). Turkey is the biggest producer, contributing with 105000 tons. In the European Union, Greece and Spain are the main producers, contributing with a total of 82535 tons. On the contrary, Portugal produced only 882 tons of sea bass in 2020, in a total of 6657 tons of fish produced, being one of the main importers of fish [320,321]. This intensification in the production of sea bass, as well as the gilthead sea bream, represented a huge development of aquaculture in the Mediterranean region. However, it brought some issues, including loss of production due to disease outbreaks [90,314]**.**

Fish produced under intensive aquaculture practices are kept at a high number in a relatively small production unit (about 100000–500000 fish per unit, being a tank, pond, or cage), for a long period of time (12–15 months) [90]. Fish maintained under these stressful conditions are particularly susceptible and outbreaks are frequent, the most common for sea bass being vibriosis (*Vibrio* spp.), photobacteriosis (*Photobacterium*

damselae spp.), tenacibaculosis (*Tenacibaculum maritimum*), and viral nervous necrosis (NNV) [90,322]. The use of antibiotics is inevitable to fight some of these diseases, and includes drugs such as tetracyclines or quinolones [323–325]. However, if for salmonid species vaccination to prevent the major disease outbreaks is already well implemented, for the species produced in the Mediterranean area this practice still needs to be improved [90,100]. Commercial vaccines available were designed essentially for the control of photobacteriosis and vibriosis, with some vaccines already developed against Nodavirus, using inactivated pathogens, administered through injection or the oral route, with varying efficacy: AlphaJect 2000™ (Aquavet SA), AquaVac™ Vibrio-Pasteurella (MSD Animal Health), Aquavac Vibrio Oral (MSD Animal Health), Alpha ject micro®1Noda (Aquavet SA) and Icthiovac®VNN (Hipra) [89,326]. Other vaccines have been studied, with varied degrees of protection, to be potentially used for the control of nervous necrosis virus/viral encephalopathy and retinopathy (VER) [327–330]; tenacibaculosis and mycobacteriosis [331–333]**.** Other approaches being tested include the use of probiotics, symbiotics, and other types of immunostimulants [334–339].

Antimicrobial peptides are also strong alternative candidates to the use of antibiotics in sea bass production. In fact, one report aimed to test the efficacy of an AMP as a potential prophylactic agent for sea bass [340]. In this study, the authors tested the use of a synthetic hepcidin in sea bass, before infection with *V. anguillarum*, with a clear reduction of mortalities in peptide-treated fish, highlighting the potential of host defense peptides as alternatives to antibiotics in sea bass aquaculture. However, and despite the plethora of fish peptides already identified, detailed studies regarding sea bass AMPs were mostly focused on hepcidin [159,175,186,225]. Other studies include the characterization of dicentracin, a sea bass piscidin-like peptide, and the transcriptional changes of this AMP during stress or infection [239,275,341] and evidences for the existence of other piscidins in sea bass, particularly orthologues of piscidin 3 [271] and piscidin 4 [270]. Still, a limitation to the potential use of AMPs in the production of sea bass, asides issues inherent to all peptides, is the clear lack of knowledge on the different families of peptides of this fish species.

Aims

As highlighted in the previous section, antimicrobial peptides (AMPs) are considered as promising alternatives to the use of antibiotics in animal production, including aquaculture. However, in the European sea bass (*Dicentrarchus labrax*), a commercially important species, an in-depth study on AMPs is limited to the hepcidin family, but the potential applications of hepcidins, as well as other sea bass AMPs, are not well explored.

In this thesis, we aimed to identify, characterize and assess the potential applications of different sea bass AMPs, namely piscidins, beta-defensins and hepcidins. To accomplish this, we proposed to achieve the following objectives:

1) Identify and characterize the different sea bass piscidin and beta-defensin coding genes at genomic DNA, cDNA and protein levels.

2) Evaluate the characterized AMPs constitutive transcription levels and tissue expression patterns for sea bass.

3) Investigate the modulation of the different AMPs during sea bass response to different experimental conditions.

4) Synthesize the relevant peptides, and evaluate their activity *in vitro*, against a range of fish and mammalian pathogens.

5) Evaluate the effects of synthetic peptides *in vivo,* in fish subjected to different physiological conditions, namely infection and iron modulation.

In the present thesis, we expanded the number of novel peptides from sea bass, belonging to different AMP families. This research on different novel AMPs in sea bass will contribute to the understanding of their roles and their interactions within the immune system of teleost fish. The new relevant antimicrobial peptides produced and tested in this study are expected to yield potential applications for aquaculture, as they can be employed as prophylactic or therapeutic agents, being promising alternatives to the current treatments used in aquaculture.

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Chapter II

Antimicrobial peptides: identification of two beta-defensins in a teleost fish, the European sea bass (*Dicentrarchus labrax*)

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Abstract

Beta-defensins consist in a group of cysteine-rich antimicrobial peptides (AMPs), widely found throughout vertebrate species, including teleost fish, with antimicrobial and immunomodulatory activities. However, although the European sea bass (*Dicentrarchus labrax*) is one of the most commercially important farmed fish species in the Mediterranean area, the characterization of its beta-defensins and its potential applications are still missing. In this study, we characterized two members of the beta-defensin family in this species. Phylogenetic and synteny analysis places sea bass peptides in the beta-defensin subfamilies 1 and 2, sharing similar features with the other members, including the six cysteines and the tertiary structure, that consists in three antiparallel beta-sheets, with beta-defensin 1 presenting an extra alpha-helix at the N-terminal. Further studies are necessary to uncover the functions of sea bass beta-defensins, particularly their antimicrobial and immunomodulatory properties, in order to develop novel prophylactic or therapeutic compounds to be used in aquaculture production.

Introduction

Defensins are a group of small cysteine-rich and cationic antimicrobial peptides (AMPs) found throughout nature. These peptides are divided into three different types: alpha-, beta- and the cyclic theta-defensins, a classification based on the cysteine pairing to form intramolecular disulphide bonds [1]. However, in phylogenetically earlier vertebrates such as teleost fish, only one defensin type has been isolated. Fish defensins present six conserved cysteines linked in a particular pattern of Cys1–Cys5, Cys2–Cys4 and Cys3–Cys6 [2–5], resembling the beta-defensin family members found in birds, reptiles and mammals [6–9]. Thus, while alpha- and theta-defensins appear to be more restricted to mammals, with theta-defensins being found only in some non-human primates [10,11], beta-defensins are widely distributed in different vertebrate species.

Beta-defensins were first isolated in zebrafish (*Danio rerio*), tiger pufferfish (*Takifugu rubripes*) and spotted-green pufferfish (*Tetraodon nigroviridis*) [2]. These AMPs were later found in several other fish species, with only one gene copy being characterized in Atlantic cod (*Gadus morhua*) [4] or Nile tilapia (*Oreochromis niloticus*) [5], to the seven defensins isolated in the Atlantic salmon (*Salmo salar*), distributed into five different subfamilies [12]. In zebrafish, three beta-defensins were isolated, with the beta-defensin 3 gene being found in a different chromosome, indicating that multiple defensin loci are

present in this fish species [2]. Fish defensin genes are formed by three-exons/two-introns, differing from the mammalian counterparts, that are encoded by two exons [2,5,12,13]. The resulting prepeptide is composed by a signal peptide with 18 to 26 amino acids and a 39 to 45 mature peptide [2–4,12,14,15], the defensins isolated in olive flounder (*Paralichthys olivaceus*) being an exception. This fish species presents a particular group of beta-defensins, constituted by peptides with 67 to 77 amino acids, in which a 5 to 15 amino acid proregion is also present that is cleaved to produce the active protein, which shows anionic properties [16]. Nevertheless, fish beta-defensin mature peptides share the six conserved cysteines and have an overall cationic net charge, folding into three anti-parallel beta-strands stabilized by the disulfide bonds, although some peptides may also possess an extra alpha-helix at the N-terminus of the three beta-strand structure [2,4,5,17,18].

Fish beta-defensins have been shown to be involved in several functions, including antibacterial, antiviral and immunomodulatory activities [19]. A high basal expression of beta-defensin genes can be observed in different tissues, including the spleen, gills, kidney or skin [5,20], and their expression is altered after stimuli with pathogen-associated molecular patterns (PAMPs), or by infection with different pathogens [21–23]. *In vitro*, recombinant or synthetic defensins are able to inhibit the growth of Gram-positive and Gram-negative bacteria, as well as some viruses [24–26]. The immunomodulatory functions of vertebrate beta-defensins include enhanced phagocytic activity [4,27], chemotaxis of fish leukocytes [14,28] and human immature dendritic cells and T cells [29], and also modulation of immune-related genes [28].

The European sea bass (*Dicentrarchus labrax*) is one of the most commercially important and intensively farmed fish species, particularly in the Mediterranean area. These fish are often subjected to disease outbreaks, mainly of bacterial and viral origin [30], and the current prophylactic and therapeutic approaches to deal with these outbreaks are limited. As such, novel compounds are necessary, with AMPs being promising candidates [31]. However, a comprehensive study of the different AMPs in sea bass is still lacking, and, thus, in this study, we characterize two beta-defensin family members in sea bass. These peptides fall into two different types, showing high similarities with other Perciform be-ta-defensins in terms of amino acid sequences and structure. Further studies are necessary to understand the functions of these peptides, in order to develop novel potential drugs to prevent or treat diseases in sea bass aquaculture.

Results

Molecular Characterization of Sea Bass Beta-Defensins

Two different beta-defensin genes were obtained by PCR amplifications using intestine, kidney and gill cDNA (Figure 1). Potential cleavage sites were determined using Sig-nalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/, accessed on April 2021).

Beta-defensin1 coding DNA (deposited in GenBank under accession number MZ198753) consists of an open reading frame (ORF) of 201 bp and encodes a 66-aa prepeptide. A potential cleavage site for the signal peptide was predicted between Ala24 and Ala25 (NEA/AS). Thus, the beta-defensin1 prepeptide consists of a 24-aa signal peptide and a 42-aa mature peptide. The mature peptide has a predicted M.W. of 4493.4 Da, an isoelectric point of 8.13 and a net charge at pH 7 of 1.8.

Beta-defensin2 coding DNA (accession number MZ198754) consists of an ORF of 192 bp, and encodes a 63-aa prepeptide. The potential cleavage site for the signal peptide was predicted between Gly20 and Asn23 (GEG/ND). Thus, beta-defensin2 is formed by a 20-aa signal peptide and a 43-aa mature peptide. The mature peptide has a predicted M.W. of 5205.9 Da, an isoelectric point of 8.77 and a net charge at pH 7 of 3.9.

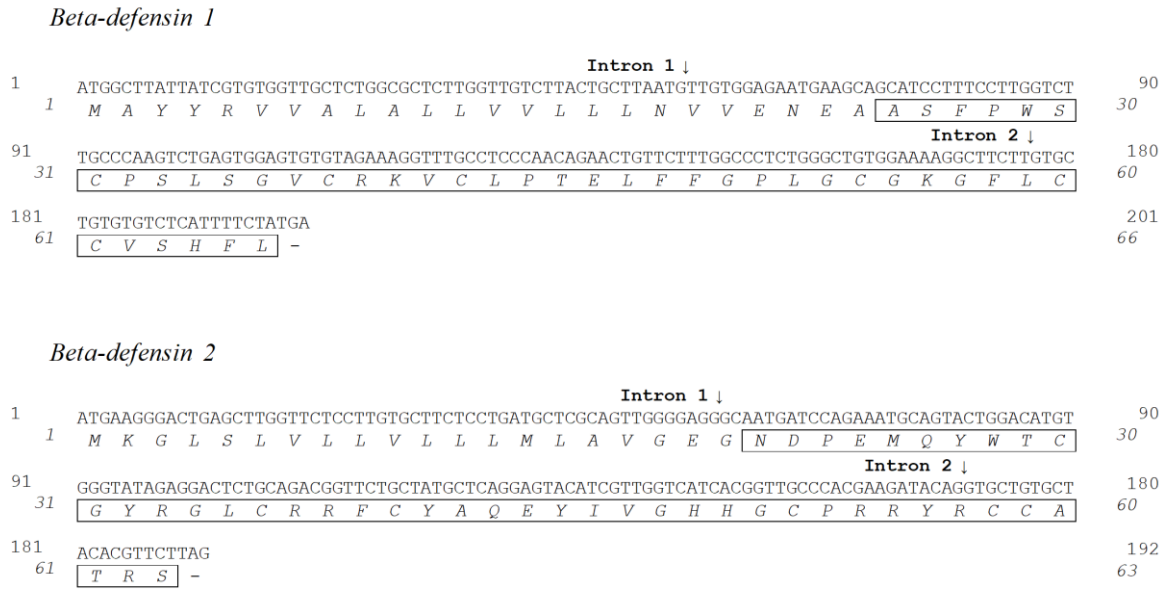

Figure 1. Sea bass beta-defensin coding DNA and amino acid sequences. Nucleotides are indicated in the upper row, and amino acids are indicated in italic in the lower row. Mature peptides are boxed, and intron positions are indicated by arrows.

Alignment between seabass beta-defensin peptides revealed several amino acid differences (identity score of 29.4%). However, both mature peptides retain the characteristic six cysteine residues at conserved positions. These six cysteines are predicted to form intramolecular disulphide bonds between Cys1–Cys5, Cys2–Cys4 and Cys3–Cys6 (Figure 2).

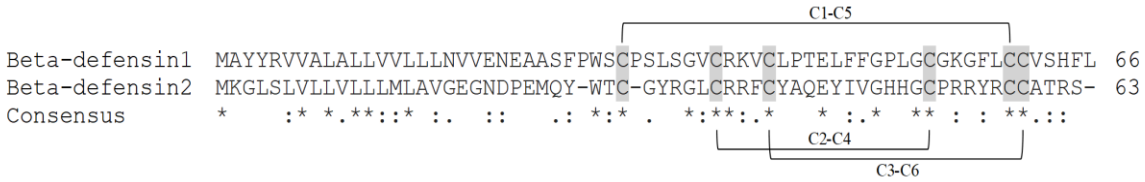

Figure 2. Alignment of sea bass beta-defensins. Cysteines are shaded gray, and the predicted disulphide bridges are also shown. Identical residues are denoted by (*), conserved substitutions by (:) and semi-conserved substitutions by (.).

Molecular Modeling of Sea Bass Beta-Defensin Peptides

The three-dimensional structures of both sea bass beta-defensins were predicted using the SWISS-MODEL server (Figure 3). The beta-defensin 1 model was predicted using as a template the NMR structure of human beta-defensin 6. According to the predicted tertiary structure, sea bass beta-defensin 1 folds into three antiparallel beta-sheets, with some disordered areas and an extra alpha-helix in the N-terminal. These secondary structures are stabilized by the disulphide bonds formed between Cys31–Cys60, Cys38–Cys54 and Cys42–Cys61. The beta-defensin 2 model was predicted using as templates the crystal structure of human beta-defensin 4 and a NMR structure of oyster big defensin. This peptide presents disordered areas, and the N-terminal is not predicted to fold into an alpha-helix. Still, beta-defensin 2 presents the three antiparallel beta-sheets, with the six cysteines forming the disulphide bonds between Cys30–Cys58, Cys36–Cys52 and Cys40–Cys59.

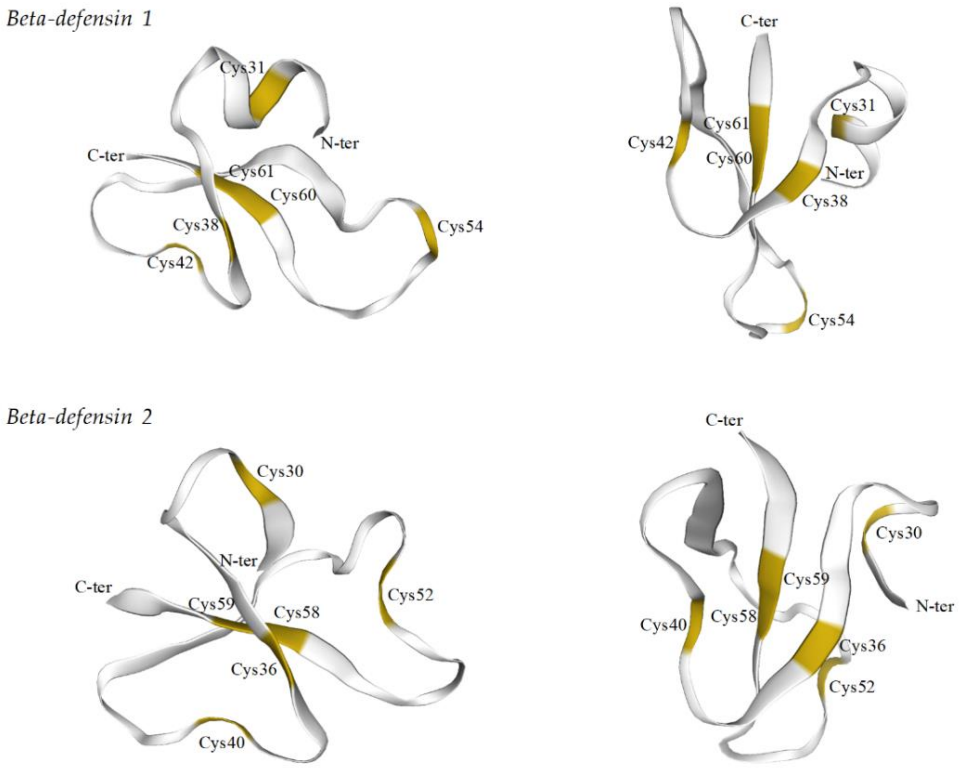

Figure 3. Three dimensional models of sea bass beta-defensins 1 and 2. Conserved cysteines are highlighted. Beta-defensin 1 shows an additional alpha-helix at the N-terminal. Models were predicted using as templates human be-ta-defensin 6 (2lwl.1.A) for beta-defensin 1, and human beta-defensin 4 (5ki9.1.A) and oyster big defensin (6qbk.1.A) for beta-defensin 2.

Genomic Organization

Sea bass beta-defensin genes are constituted by three exons and two introns. The beta-defensin 1 gene consists of exons of 55, 121 and 25 bp and introns of 97 and 92 bp. The beta-defensin 2 gene presents exons of 58, 112 and 22 bp and introns of 213 and 162 bp (Figure 4A). The signal peptides are encoded by exons 1 and 2, and the mature peptides are encoded by exons 2 and 3. Both genes present exons with similar sizes, exon 2 being the longest exon and exon 3 the smallest one. The most significant differences are observed between introns, with beta-defensin 2 presenting longer introns when compared to beta-defensin 1. Sea bass beta-defensin genes were compared with the ones of other vertebrates (Figure 4B), with fish species presenting a similar structure of three exons/two introns, as well as birds and reptiles. Mammalian beta-defensins present only two exons,

with similar sizes to fish exons 1 and 2, and one intron, that is much larger than fish introns.

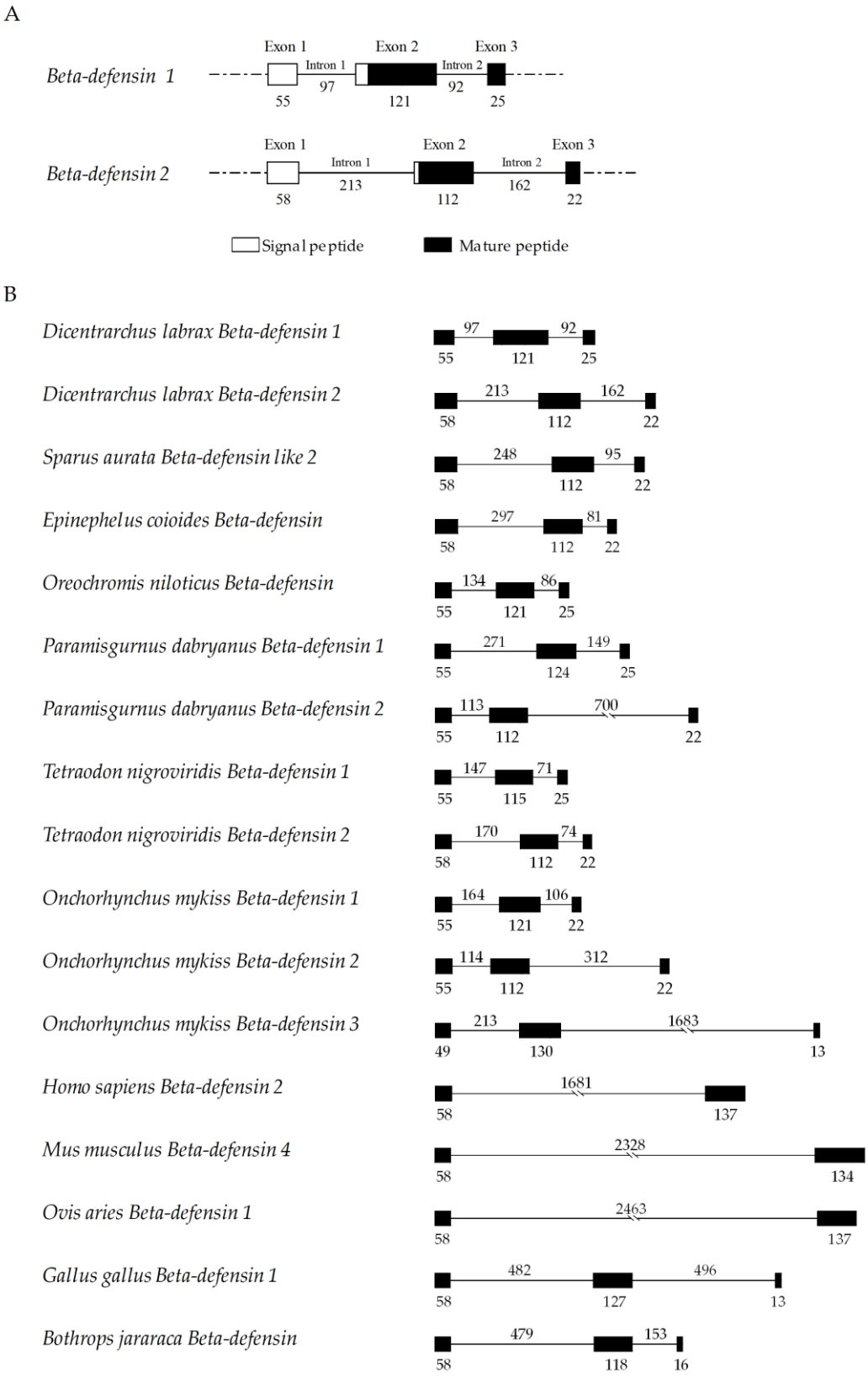

Figure 4. Genomic organization of sea bass beta-defensin genes. (A) Exon/intron diagram of sea bass beta-defensin genes. (B) Comparative view with other vertebrate beta-defensins. Exons are shown as boxes and introns as solid lines, with sizes of exons/introns in base pairs.

Sequence Comparison, Phylogenetic and Syntenic Analysis

Sequence comparison with other vertebrate beta-defensins showed a high degree of identity within each subfamily. Beta-defensin 1 shares an identity with other vertebrate peptides between 28% and 97% and beta-defensin 2 between 33% and 99% (Supplementary Figure 1). The characteristic six cysteines are retained in all peptides at conserved positions. Furthermore, beta-defensin 2 shares a similar motif between other type 2 beta-defensins, namely the CPRR(Y/L/F)K motif that is found between Cys52 and Cys58. (Figure 5).

Phylogenetic analysis separated fish beta-defensins from peptides belonging to other vertebrate species and *Crassostrea gigas* big defensin (Figure 6). Within fish defensins, two larger clades can be observed: the first is a heterogeneous group composed by two smaller clusters, one including all beta-defensins type 2 and also beta-defensin 5a from *S. salar*, and the other group includes all beta-defensins type 3, particularly from Cyprinid species, and beta-defensin 2 from Salmoniformes. The second clade includes all beta-defensins type 1 from the different species, as well as peptides belonging to *P. olivaceus* and also beta-defensins 3 and 4 from Salmonid species, that are included in two small groups. In both clades, a division between the different species is clear: Perciformes cluster with species belonging to Tetraodontiformes, Pleuronectiformes (with the exception of *P. olivaceus*), Centrarchiformes or Carangiformes, being separated from Salmoniformes, Cypriniformes or Gadiformes.

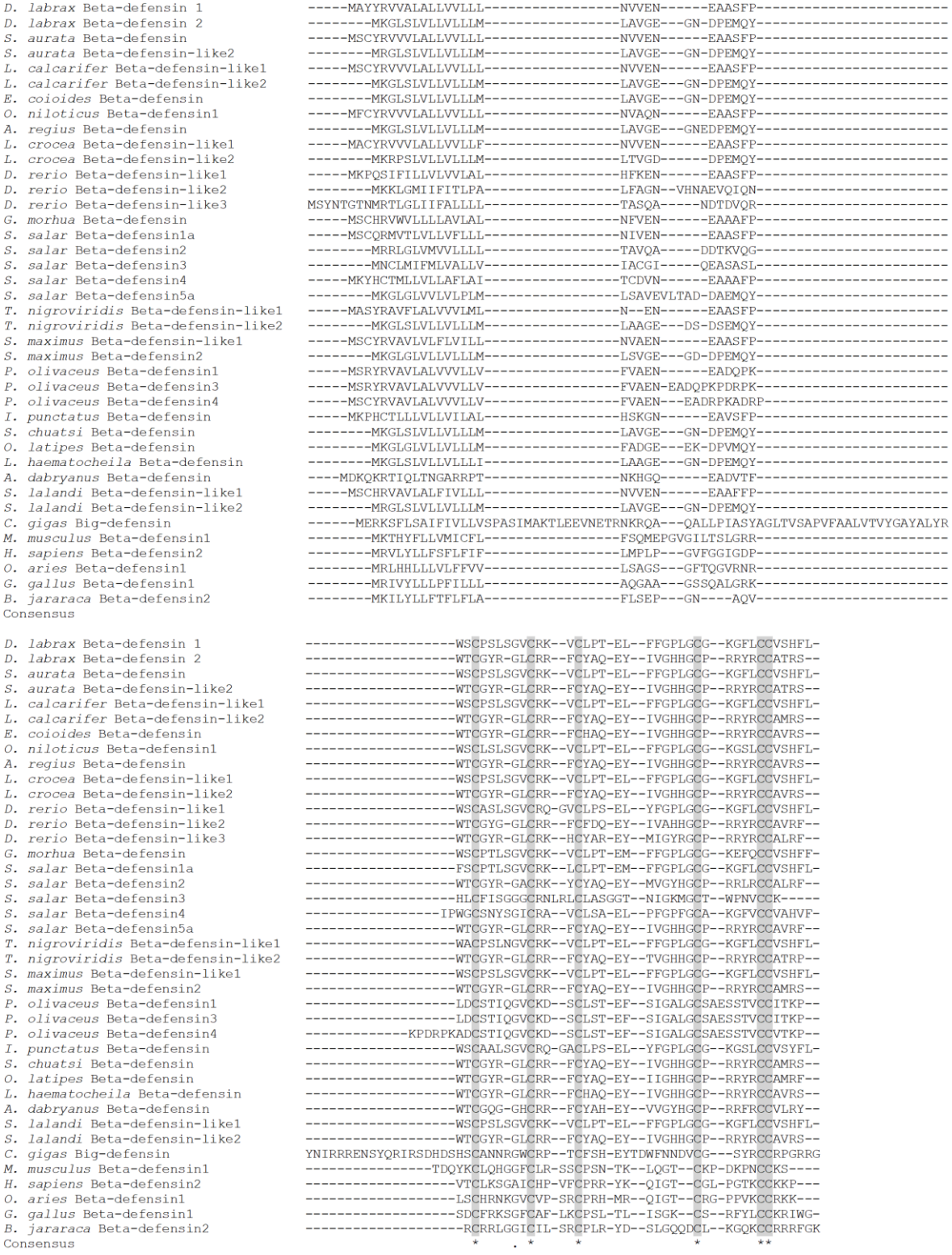

Figure 5. Alignment of sea bass beta-defensins with peptides from other vertebrate species. Cysteines are shaded gray and are denoted by (*) and semi-conserved substitutions by (.).

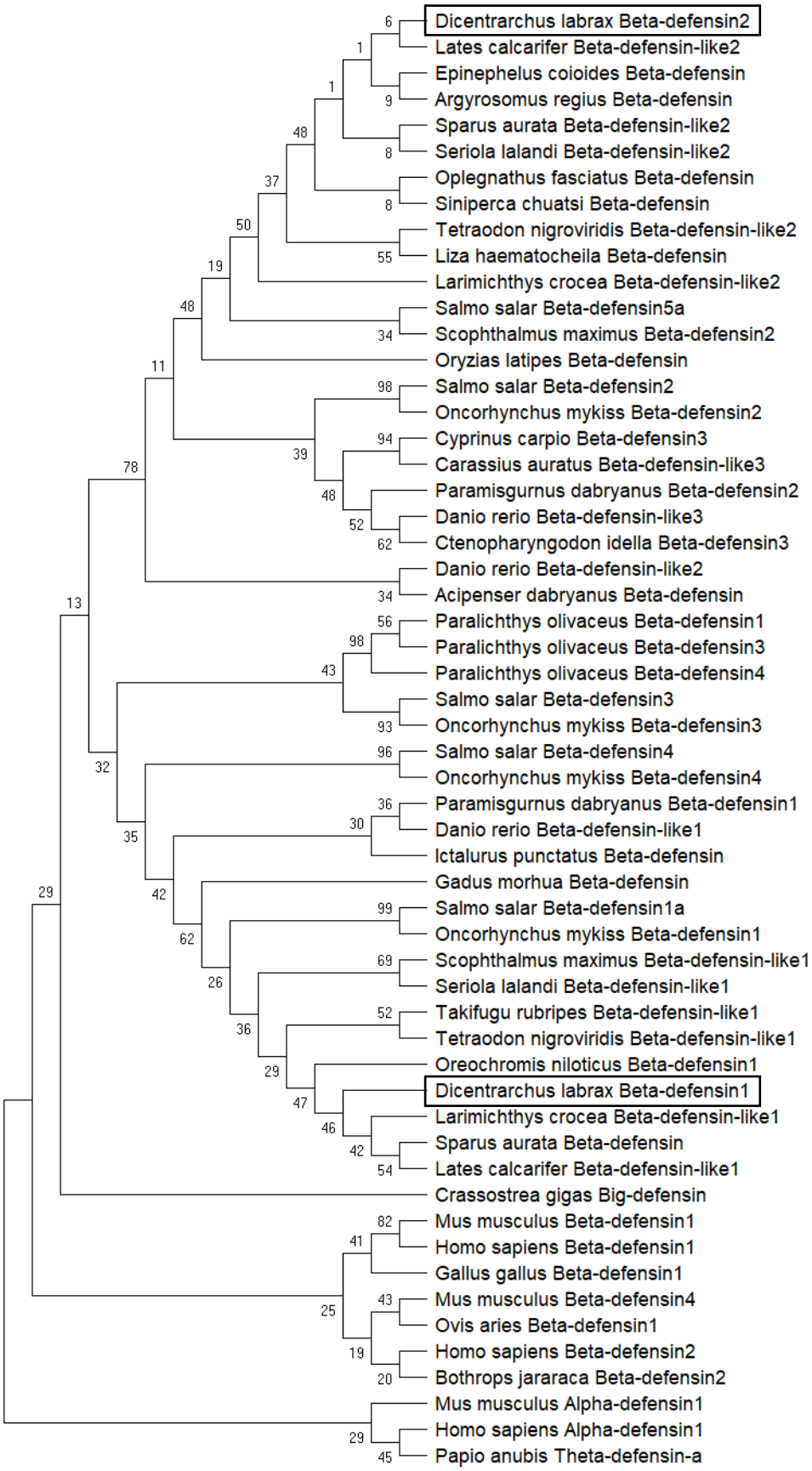
Dicentrarchus labrax Beta-defensin2
Lates calcarifer Beta-defensin-like2
Epinephelus coioides Beta-defensin
Argyrosomus regius Beta-defensin
Sparus aurata Beta-defensin-like2
Seriola lalandi Beta-defensin-like2
Oplegnathus fasciatus Beta-defensin
Siniperca chuatsi Beta-defensin
Tetraodon nigroviridis Beta-defensin-like2
Liza haematocheila Beta-defensin
Larimichthys crocea Beta-defensin-like2
Salmo salar Beta-defensin5a
Scophthalmus maximus Beta-defensin2
Oryzias latipes Beta-defensin
Salmo salar Beta-defensin2
Oncorhynchus mykiss Beta-defensin2
Cyprinus carpio Beta-defensin3
Carassius auratus Beta-defensin-like3
Paramisgurnus dabryanus Beta-defensin2
Danio rerio Beta-defensin-like3
Ctenopharyngodon idella Beta-defensin3
Danio rerio Beta-defensin-like2
Acipenser dabryanus Beta-defensin
Paralichthys olivaceus Beta-defensin1
Paralichthys olivaceus Beta-defensin3
Paralichthys olivaceus Beta-defensin4
Salmo salar Beta-defensin3
Oncorhynchus mykiss Beta-defensin3
Salmo salar Beta-defensin4
Oncorhynchus mykiss Beta-defensin4
Paramisgurnus dabryanus Beta-defensin1
Danio rerio Beta-defensin-like1
Ictalurus punctatus Beta-defensin
Gadus morhua Beta-defensin
Salmo salar Beta-defensin1a
Oncorhynchus mykiss Beta-defensin1
Scophthalmus maximus Beta-defensin-like1
Seriola lalandi Beta-defensin-like1
Takifugu rubripes Beta-defensin-like1
Tetraodon nigroviridis Beta-defensin-like1
Oreochromis niloticus Beta-defensin1
Dicentrarchus labrax Beta-defensin1
Larimichthys crocea Beta-defensin-like1
Sparus aurata Beta-defensin
Lates calcarifer Beta-defensin-like1
Crassostrea gigas Big-defensin
Mus musculus Beta-defensin1
Homo sapiens Beta-defensin1
Gallus gallus Beta-defensin1
Mus musculus Beta-defensin4
Ovis aries Beta-defensin1
Homo sapiens Beta-defensin2
Bothrops jararaca Beta-defensin2
Mus musculus Alpha-defensin1
Homo sapiens Alpha-defensin1
Papio anubis Theta-defensin-a

Figure 6. Phylogenetic analysis of beta-defensin peptides. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [32]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [33]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [33]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 56 amino acid sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 37 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [34].

The genomic location of sea bass beta-defensins was analyzed and compared with two perciform species (*Lates calcarifer* and *Sparus aurata*) and two other species, *Seriola lalandi* and *Scophthalmus maximus*, that belong to the Carangiformes and Pleuronectiformes orders, respectively (Figure 7). Based on available date from the Ensembl database, both sea bass defensins are expected to be located in the same chromosome, as well as *L. calcarifer*, *S. aurata* and *S. maximus*. On the contrary, defensins of *S. lalandi* were found in different locations. Still, synteny analysis reveals a high degree of conservation of these genetic loci, with all of the genes found in the vicinity of sea bass beta-defensins also being found in the other species analyzed.

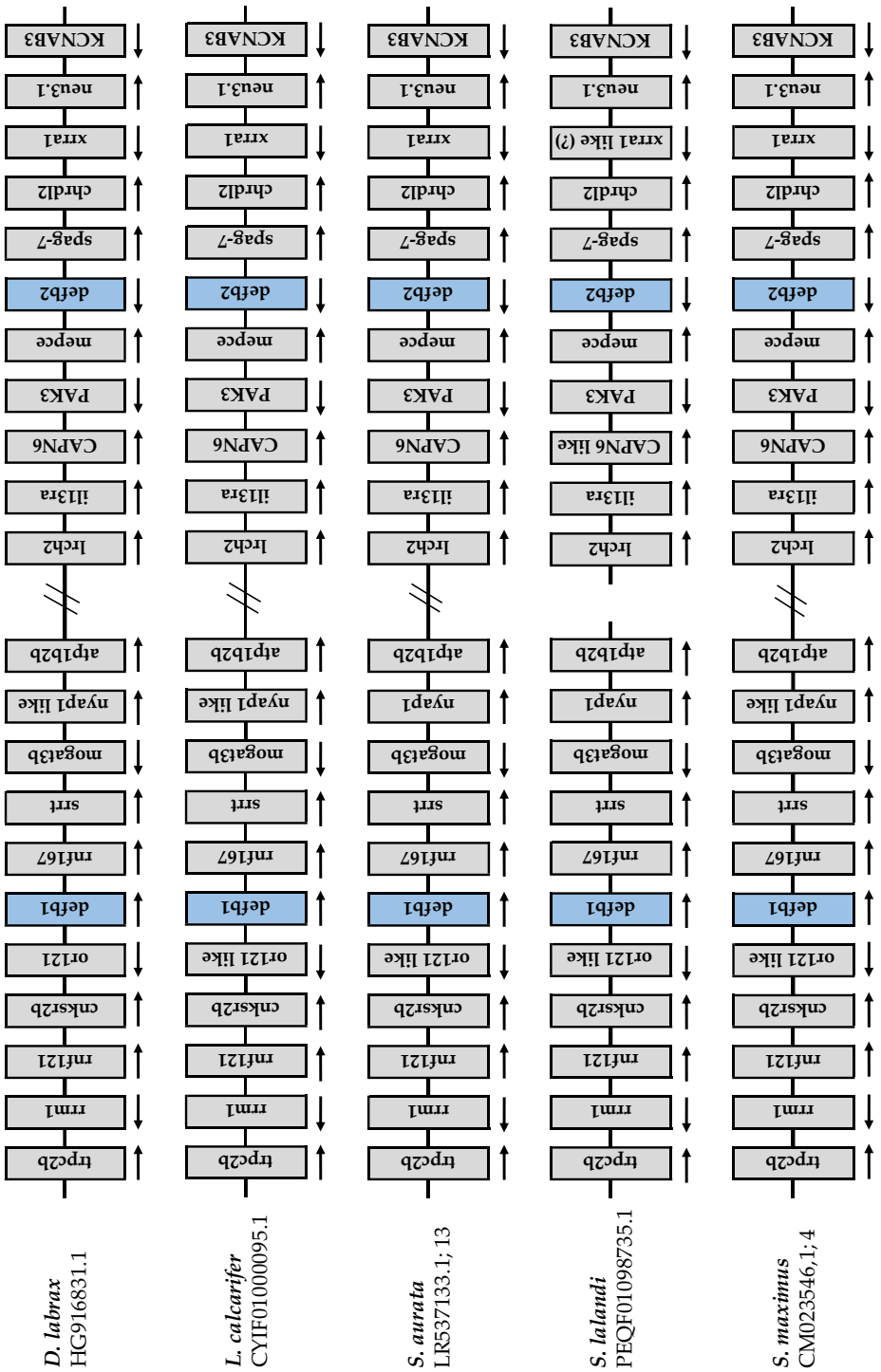

Figure 7. Analysis of beta-defensin loci in the European sea bass (*D. labrax*, location HG916831.1), Barramundi perch (*L. calcarifer*, location CYIF01000095.1), Gilthead seabream (*S. aurata*, location LR537133.1; 13), Yellowtail amberjack (*S. lalandi*, locations PEQF01098735.1 and PEQF01099151.1 for beta-defensins 1 and 2, respectively) and Turbot (*S. maximus*, location CM023546.1; 4). Analysis was performed using sequences available in the Ensembl genome browser 103, and the corresponding accession numbers of each gene are described in Table S2. *S. lalandi* xrra1: incomplete gene.

Basal Expression of Sea Bass Beta-Defensins

Different tissues of healthy sea bass were used to evaluate the constitutive expression of beta-defensins, namely the liver, spleen, head kidney, intestine, pyloric caeca, gill, heart and brain (Figure 8). Both defensins were detected in all tissues analyzed, with the spleen, head kidney and gills being the tissues with the highest relative basal expression of both beta-defensins. For beta-defensin 1, basal expression is also high in the pyloric caeca and heart, followed by the intestine, with the lowest expression being observed in the brain and liver. For beta-defensin 2, a high expression is also observed in the intestine, followed by the heart and pyloric caeca, with the liver and brain being the tissues with the lowest basal expression.

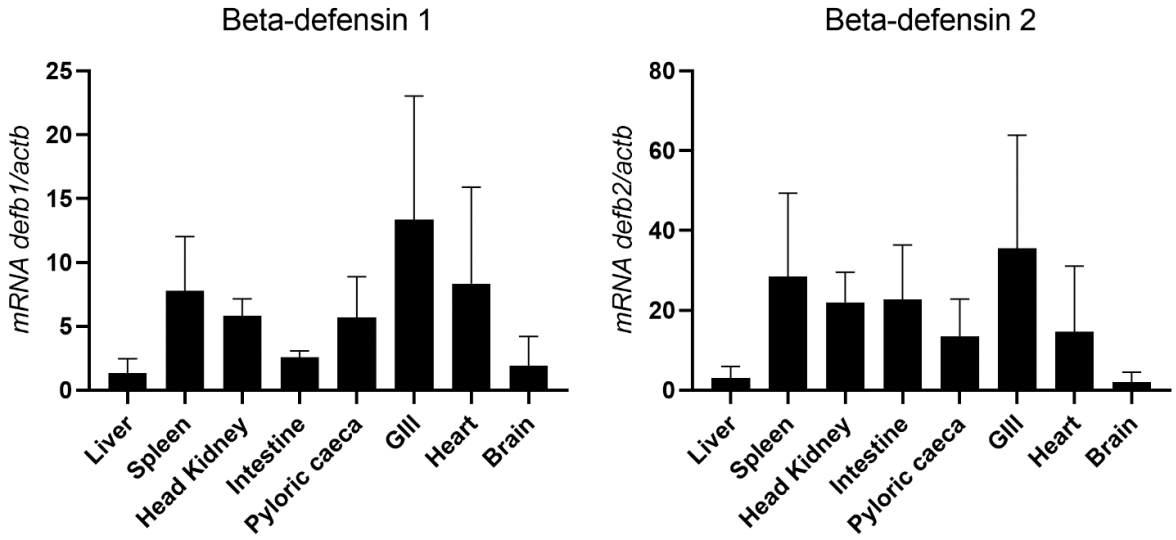

Figure 8. Basal expression of beta-defensin genes in different organs of healthy sea bass, measured by real-time PCR. Each sample was normalized to beta actin (*actb*) calculated by the comparative CT method ($2^{-\Delta\Delta CT}$). Values are presented as means ± standard deviation (S.D.) (n = 5).

Discussion

In the present study, we focused on the molecular characterization of two beta-defensin family members in the European sea bass (*Dicentrarchus labrax*). This fish species presents two different peptides, sharing similar features with other fish beta-defensins, including the six cysteines at conserved positions, folding into three antiparallel beta-sheets, and an overall cationic net charge [19].

Some invertebrate species express a group of peptides called big defensins, formed by a N-terminal hydrophobic domain, mainly constituted by alpha-helices, and a C-terminal

beta-defensin-like domain that includes three disulfide bridges that are linked in the pattern C1–C5, C2–C4 and C3–C6, resulting in three beta-sheets [35–38]. In vertebrate species, three different classes of defensins are found, namely alpha-, beta- and the cyclic theta-defensins, with beta-defensins being found widespread throughout different species [2,8,13,39], while alpha- and theta-defensins are more restricted to mammals [40]. Theta-defensins are even more exclusive, being a result of two truncated alpha-defensins and found only in some primate species, while in humans they became inactivated, due to mutations that resulted in a premature stop codon [10,11,41]. The presence of beta-defensins even in more primitive vertebrates, such as fish or reptiles, may indicate that these particular peptides constitute a more ancient type [40]. Beta-defensins and the C-terminal domain of big defensins share similar features, including the genomic organization, secondary structures and identical cysteine bridges [42]. Thus, beta-defensins likely arose from these big defensins of invertebrate species, through processes of intronization of exonic sequences or exon shuffling, leading to the loss of the amino-terminal domain and the appearance of an ancestral vertebrate beta-defensin, with a two exon/one intron organization [12,42]. Then, species-specific events of intron insertions have led to the appearance of the three exon/two intron structure observed in birds and fish, while mammals retained the original organization [3,12]. Later, after divergence of mammals from other species, extensive events of local duplication originated several clusters of defensins in mammals, and also led to the appearance of alpha- and theta-defensins [13,39,43].

Beta-defensins are present in fish in a varied number and are highly divergent from the mammalian counterparts in terms of sequence and genomic organization. In sea bass and other Perciformes (and also some Pleuronectiformes, Carangiformes or Cichliformes), up to two peptides have been isolated or annotated in genome databases, and belong to beta-defensin types 1 and 2, with no members of other defensin subfamilies so far being isolated in these species. Beta-defensin types 1 and 2 present a low identity, but retain the six cysteines and the triple-stranded beta-sheet structure. However, within each group, beta-defensins share high identity scores, and the genetic loci of these species are well conserved, with many genes surrounding beta-defensins being found in different fish. Other species, particularly the ones belonging to Salmoniformes, express up to seven different defensins [3,12,44]. Sea bass beta-defensins 1 and 2 are more related to beta-defensins 1 and 5 from Atlantic salmon, while the other Salmonid peptides are separated in different branches. Synteny studies also reinforce the relationship between sea bass peptides and these salmonid defensins, as some genes found in the vicinity of sea bass defensins are also found next to Salmonid defensins 1 and 5 [12]. During evolution, Teleost fish have faced three rounds of whole genome duplications (3WGD or fish-specific

WGD), with the salmonid lineage presenting an additional WGD (4WGD or salmonid-specific WGD) [45,46]. These events of genome duplications are often correlated with the diversity of species found among the Teleostean [47]. Many of these duplicated genes were lost during evolutionary processes, while others were retained and even gained novel functions in a lineage-specific manner [48,49]. Beta-defensin genes in salmonids derived from common ancestry and then expanded, after these events of WGDs [12], and diverged from other species, including the European sea bass. While in Salmonid species several beta-defensins can be found, in Perciformes and perhaps other related species, they turned into a more restricted group, with other families of AMPs being more diversified, such as hepcidins or piscidins [50,51].

Three dimensional models of sea bass beta-defensins were predicted using the Swiss model server, with the available NMR or crystal structures of beta-defensins from humans [52,53] and the beta-defensin-like domain of oyster big-defensin [35]. Both sea bass defensins are predicted to fold into three antiparallel beta-sheets, stabilized by the disulphide bonds between cysteines, with beta-defensin 1 presenting an extra alpha-helix at the N-terminal. This alpha-helix is also observed in other fish and non-fish be-ta-defensins. In fish, it seems that the presence of this secondary structure is not exclusive to any beta-defensin type. The alpha-helix was predicted in type 1 beta-defensins of zebrafish, Atlantic cod and blunt snout bream (*Megalobrama amblycephala*) [2,4,28], but it was not found in beta-defensin 1 of Nile tilapia or channel catfish [5,18]. On the contrary, in soiny mullet (*Liza haematocheila*) and turbot (*S. maximus*), the authors describe the presence of an alpha-helix at the N-terminal of beta-defensins characterized in these species, both belonging to the type 2 subfamily [17,54]. In other vertebrate species, a similar folding is observed in different beta-defensins, including human and mouse beta-defensins [52,55–59]. Aside the alpha-helix, the three antiparallel beta-sheets were predicted in sea bass beta-defensin 1, through binding between Cys31–Cys60, Cys38–Cys54 and Cys42–Cys61. For sea bass beta-defensin 2, using the models available in the Swiss model, no alpha-helix was observed. Still, the three antiparallel beta-sheets are present, with the cysteines being linked between Cys30–Cys58, Cys36–Cys52 and Cys40–Cys59. This pattern of cysteine binding is a hallmark of beta-like defensins. Alpha-defensins, on the contrary, are usually smaller and lack the presence of alpha-helices, and, although they also consist in a triple-stranded beta-sheet, their cysteines are connected in the Cys1–Cys6, Cys2–Cys4, Cys3–Cys5 pattern [1,60]. Theta-defensins present a circular structure, without a free N- or C-terminal, as a result of cyclization of two 9-amino-acid segments of alpha-defensin-like molecules [10]. Beta-defensin 2 also presents the CPRRYK basic motif, between Cys4 and Cys5, which is found in all fish beta-defensins type 2 and 3, with the exception of *P. olivaceus*. This motif is also present

in human beta-defensin 2, although in a different loop, between Cys3 and Cys4 [61]. The presence of this conserved motif in phylogenetic distant organisms may suggest a common functional role [2].

The tertiary structure of beta-defensins is similar to what is verified for chemokines, although their amino acid sequences share a reduced identity [60,62]. In fact, human and mice beta-defensins present chemotactic activity towards diverse leukocytes, using different chemokine receptors [29,63]. Fish beta-defensins are also chemotactic for different leukocytes, although the exact mechanism remains undetermined [14,28]. Still, authors observed that head kidney leukocytes were attracted to the recombinant sea-bream defensin 1, but the same was not observed using recombinant human peptides, suggesting a certain degree of selection by fish receptors [14]. Furthermore, the N-terminal portion of blunt snout bream defensin exerts an increased chemotactic activity of head kidney leukocytes, when compared to the C-terminal portion, suggesting that the N-terminal of beta-defensin might be important for this function [28]. Fish beta-defensins are also known to present antibacterial and antiviral activities [4,27,54,64]. The mechanism of action of AMPs relies on an initial interaction between the peptide and bacterial membrane [65]. Particularly for defensins, previous authors suggest that the alpha-helix might be helpful in the interaction of beta-defensin with the bacterial cell wall [66]. Indeed, synthetic and recombinant beta-defensin 2 from large yellow croaker and turbot impaired membrane morphology were recently described, leading to a severe membrane damage [27,54]. Given the similarities between these peptides and sea bass beta-defensins, particularly defensin type 1, we speculate that they may also be involved in such functions, although further studies are necessary to uncover the possible antimicrobial and chemotactic activities of each sea bass defensin.

Sea bass beta-defensins were detected in all tissues analyzed, with an overall predominance of beta-defensin 2. Both genes are highly expressed in the gills, head kidney and spleen, with a low expression in the liver. In other fish species, beta-defensins are also highly expressed in tissues including the skin, gills, head kidney or spleen [5,14,15,20]. On the contrary, in turbot and grouper, beta-defensin 2 shows high basal expression levels in the skin and gills, but also in the liver, with a low expression in the spleen and kidney [26,54]. In salmonids species, the several defensins can present a different pattern of expression, depending on the gene and tissue analyzed, with some defensins being more expressed in other tissues, including the liver [3,12]. In mammals, beta-defensins can also be found in the skin and mucosal surfaces, acting as a first line of defense [67,68]. Assuming the antimicrobial and immunomodulatory roles of beta-defensins, it is not surprising to find a high constitute expression in tissues exposed to the external environment, as well as in fish central immune tissues. Still, beta-defensins show

different patterns of basal expression depending on the species, tissue and gene analyzed.

Conclusions

In summary, we identified two beta-defensins in the European sea bass, with high similarities with other fish peptides in terms of sequence, cysteine position and tertiary structure. Defensins are the most studied group of AMPs, with many fish and non-fish peptides already characterized. However, reports addressing the beta-defensin family in sea bass are still lacking. Further studies are necessary to understand the functions of sea bass beta-defensins, including their antimicrobial and immunomodulatory functions, and how these genes are modulated during infection. It is known that defensins are involved in several immune roles [19], and, as such, an in-depth knowledge of sea bass AMPs might be helpful in the development of novel prophylactic or therapeutic compounds to be used in the production of sea bass.

Materials and Methods

Animals

Healthy European sea bass (*D. labrax*), with an average weight of 30 g, were provided by a commercial fish farm (Sonríonansa, Pesués, Cantabria, Spain) [50]. Prior to the experiments, fish were acclimated for 30 days to the fish-holding facilities of the Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto. Fish were kept in 110 L recirculating sea water (28‰ salinity) tanks at 22 ± 1°C, with a 13 h light/11 h dark cycle and fed daily ad libitum with commercial fish feed. Before each treatment, fish were anesthetized with ethylene glycol monophenyl ether (2-phenoxyethanol, 0.3 mL/L; Merck, Algés, Portugal).

Isolation of Sea Bass Beta-Defensins

Pairs of oligonucleotide PCR primers were designed according to conserved regions of beta-defensins' mRNA sequences from sea bass and other fish species, with sea bass expressed sequence tags (ESTs) and whole-genome shotgun sequences (WGSS) available in the National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov, accessed on April 2021) and Ensembl genome browser 103 (http://www.ensembl.org, accessed on April 2021). cDNA preparations from the whole

intestine, kidney and gill were used in PCR amplifications [50,69]. PCR products were run on 1% agarose gels, and then relevant fragments were purified with the NZYGelpure kit (NZYtech, Lisbon, Portugal), cloned into PCR 2.1-TOPO vectors, propagated in One Shot Mach1-T1R competent cells (Invitrogen, Life Technologies, Carlsbad, CA) and sent for sequencing (GATC, A Eurofins Genomics Company, Ebersberg, Germany). Both strands were sequenced, and chromatograms were analyzed in FinchTV (Geospiza, Seattle, WA, USA) and assembled using Multalin (http://multalin.toulouse.inra.fr/multalin/, accessed on April 2021).

Modeling of Sea Bass Beta-Defensin Peptides

Prediction of the three-dimensional structures of sea bass beta-defensins was performed by protein homology detection/modelling, using the SWISS-MODEL server (https://swissmodel.expasy.org/, accessed on April 2021). Templates were chosen using the best GMQE (Global Model Quality Estimation) and QMEAN (Qualitative Model Energy Analysis) estimations, as well as the highest sequence identity and coverage between templates and sea bass beta-defensins. Human beta-defensin 6 (2lwl.1.A) [52] NMR structure was used as the template for sea bass beta-defensin 1 modeling, while the crystal structure of human beta-defensin 4 (5ki9.1.A) [53] and NMR structure of oyster (*C. gigas*) big defensin (6qbk.1.A) [35] were used for sea bass beta-defensin 2. Molecular graphic images were obtained using the Polyview-3D webserver (http://polyview.cchmc.org/polyview3d.html, accessed on April 2021) [70].

Genomic Organization

Genomic DNA was isolated from sea bass red blood cells, using the NZY Blood gDNA Isolation kit (NZYtech), as previously described [50,69]. Quantification was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and quality was checked by agarose gel electrophoresis. Two micrograms of genomic DNA were amplified by PCR with the primers based on the previously obtained cDNA sequences, with the following cycling profile: 94 °C for 5 min, 30 cycles of 94 °C for 60 s, 59 °C for 60 s, 72 °C for 60 s and a final step of 72 °C for 5 min. Several PCR products were purified, cloned and sent for sequencing. Comparisons were made between genomic DNA and cDNA to evaluate the similarity of the coding regions and to identify intron/exon boundaries. A comparison between the genomic sequences of sea bass beta-defensins with those of other vertebrate species was also made, using the sequences identified with the GenBank and Ensembl accession numbers described in Table S1.

Alignment, Phylogenetic and Syntenic Analysis

Alignments of the amino acid sequences of beta-defensin predicted proteins were performed using MUSCLE from MEGA X [34]. A phylogenetic tree was constructed using the Maximum Likelihood method, with the Jones–Taylor–Thornton (JTT) model, Nearest-Neighbor-Interchange heuristic model, complete deletion of gaps and 1000 bootstrap replications. Sequences used for comparisons and phylogenetic trees and their accession numbers are shown in Table S2.

Synteny analysis between the European sea bass genome and other species was conducted using information available in Ensembl genome browser 103. Orthology/paralogy relationships were derived from the ortholog/paralog prediction function of the Ensembl website. The accession numbers of genes studied are shown in Table S3.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from tissues with the NZY Total RNA Isolation Kit (NZYTech), with the optional on-column DNase treatment, according to the manufacturer's instructions [50,69]. Total RNA quantification was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and quality was assessed by running the samples in an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). For all samples, 2.5 µg of each were converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech), according to the manufacturer's protocol.

Basal Expression of Sea Bass Beta-Defensins

Several tissues from five healthy sea bass were collected for RNA isolation and cDNA synthesis, as previously described [50,69]. Relative levels of beta-defensin mRNAs were quantified by real-time PCR analysis using an CFX96 Real-Time PCR Detection System (Bio-Rad). Pairs of primers used for the reactions were designed according to our beta-defensin sequences and are as follows: defb1-For 5′-ATGGCTTATTATCGTGTGGTTG-3′/defb1-Rev 5′-TCATAGAAAATGAGACA-CACAGC-3′ for beta-defensin 1 and defb2-For 5′-ATGAAGGGACTGAGCTTGGTT-3′/defb2-Rev 5′-CTAAGAACGTGTAGCACAGC-3′ for beta-defensin 2. One µL of each cDNA sample was added to a reaction mix containing 10 µL iTaq Universal SYBR Green Supermix (Bio-Rad), 7 µL of $_{dd}$H20 and 250 nM of each primer, making a total volume of 20 µL per reaction. The cycling profile was as follows: 95 °C for 3.5 min, 40 cycles of 95 °C for 20 s and 59 °C for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reactions. Beta-actin (*actb*) was used as the housekeeping gene (primers actb-For 5′-

CAGAAGGACAGCTACGT-3′/actb-Rev 5′-GTCATCTTCTC-CCTGTTGGC-3′). The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold values was used to analyze gene expression levels. Graphics were generated using GraphPad Prism 9 (GraphPad Software, San Diego CA, USA).

Supplementary Material

Figure S1: Identity scores between vertebrate beta-defensins; Table S1: Accession numbers of vertebrate beta-defensin genes; Table S2: Accession numbers of defensins used in the alignment and phylogenetic analysis; Table S3: Accession numbers of genes used in the synteny analysis of beta-defensins 1 and 2.

Author Contributions

C.B. and J.V.N. conceived and performed the experiments, analyzed data and wrote the original manuscript; P.N.S.R. conceived the experiments; P.C. and J.F.M.G. performed the experiments and analyzed data. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, approved by the Institutional Review Board (or Ethics Committee) of ICBAS (P293/2019/ORBEA, 05/04/2019) and conducted by experienced and trained Federation of European Laboratory Animal Science Associations Category investigators.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

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Suplemmentary Material

	D. labrax β-defensin1	*D. labrax* β-defensin2	*S. aurata* β-defensin	*S. aurata* β-defensin-like2	*L. calcarifer* β-defensin-like1	*L. calcarifer* β-defensin-like2	*E. coioides* β-defensin	*O. niloticus* β-defensin1	*A. regius* β-defensin	*L. crocea* β-defensin-like1	*L. crocea* β-defensin-like2	*D. rerio* β-defensin-like1	*D. rerio* β-defensin-like2	*D. rerio* β-defensin-like3	*G. morhua* β-defensin	*S. salar* β-defensin1a	*S. salar* β-defensin2	*S. salar* β-defensin3	*S. salar* β-defensin4	*S. salar* β-defensin5a	*T. nigroviridis* β-defensin-like1	*T. nigroviridis* β-defensin-like2	*S. maximus* β-defensin-like1	*S. maximus* β-defensin2	*P. olivaceus* β-defensin1	*P. olivaceus* β-defensin3	*P. olivaceus* β-defensin4	*I. punctatus* β-defensin	*S. chuatsi* β-defensin	*O. latipes* β-defensin	*L. haematochelia* β-defensin	*A. dabryanus* β-defensin	*S. lalandi* β-defensin-like1	*S. lalandi* β-defensin-like2	*M. musculus* β-defensin1	*H. sapiens* β-defensin2	*O. aries* β-defensin1	*G. gallus* β-defensin1	*B. jararaca* β-defensin2
D. labrax β-defensin1	100%																																							
D. labrax β-defensin2	46%	100%																																							
S. aurata β-defensin	97%	46%	100%																																						
S. aurata β-defensin-like2	47%	99%	47%	100%																																					
L. calcarifer β-defensin-like1	97%	46%	100%	47%	100%																																				
L. calcarifer β-defensin-like2	46%	99%	46%	98%	46%	100%																																			
E. coioides β-defensin	46%	98%	46%	97%	46%	98%	100%																																		
O. niloticus β-defensin1	92%	44%	94%	46%	94%	44%	44%	100%																																	
A. regius β-defensin	44%	98%	44%	97%	44%	98%	98%	43%	100%																																
L. crocea β-defensin-like1	97%	46%	98%	47%	98%	46%	46%	93%	44%	100%																															
L. crocea β-defensin-like2	48%	92%	48%	91%	48%	92%	92%	47%	92%	48%	100%																														
D. rerio β-defensin-like1	80%	41%	80%	41%	80%	41%	41%	78%	40%	79%	43%	100%																													
D. rerio β-defensin-like2	39%	69%	39%	68%	39%	69%	70%	40%	70%	39%	69%	39%	100%																												
D. rerio β-defensin-like3	41%	61%	41%	62%	41%	61%	60%	42%	60%	40%	60%	37%	54%	100%																											
G. morhua β-defensin	83%	43%	87%	44%	87%	43%	43%	81%	42%	84%	46%	76%	40%	41%	100%																										
S. salar β-defensin1a	87%	46%	89%	47%	89%	46%	46%	83%	44%	87%	48%	76%	38%	40%	83%	100%																									
S. salar β-defensin2	49%	74%	49%	76%	49%	74%	73%	49%	73%	48%	74%	43%	61%	74%	47%	49%	100%																								
S. salar β-defensin3	41%	47%	41%	47%	41%	47%	47%	41%	46%	41%	46%	46%	41%	39%	37%	40%	42%	47%	100%																						
S. salar β-defensin4	57%	48%	57%	47%	57%	48%	48%	56%	48%	58%	47%	56%	42%	33%	57%	53%	44%	43%	100%																						
S. salar β-defensin5a	41%	84%	41%	83%	41%	84%	84%	42%	86%	41%	82%	40%	72%	58%	39%	41%	70%	41%	43%	100%																					
T. nigroviridis β-defensin-like1	88%	42%	87%	43%	87%	42%	42%	84%	41%	87%	44%	38%	40%	76%	42%	44%	74%	48%	39%	54%	39%	100%																			
T. nigroviridis β-defensin-like2	44%	93%	44%	92%	44%	92%	91%	46%	91%	44%	88%	41%	69%	61%	42%	44%	74%	47%	49%	84%	42%	100%																			
S. maximus β-defensin-like1	90%	42%	93%	43%	93%	42%	42%	90%	41%	91%	44%	77%	40%	41%	83%	84%	47%	38%	53%	41%	82%	43%	100%																		
S. maximus β-defensin2	46%	96%	46%	94%	46%	97%	94%	94%	94%	46%	91%	41%	70%	61%	43%	46%	74%	46%	47%	87%	42%	90%	42%	100%																	
P. olivaceus β-defensin1	58%	41%	60%	42%	60%	40%	40%	59%	39%	59%	42%	48%	36%	37%	56%	54%	42%	41%	40%	37%	53%	43%	59%	40%	100%																
P. olivaceus β-defensin3	50%	39%	52%	40%	52%	38%	38%	51%	38%	51%	38%	40%	33%	32%	48%	47%	38%	34%	36%	36%	36%	46%	40%	51%	38%	91%	100%														
P. olivaceus β-defensin4	46%	33%	49%	34%	49%	32%	32%	48%	32%	48%	32%	36%	27%	27%	46%	43%	33%	30%	34%	28%	41%	34%	48%	32%	79%	81%	100%														
I. punctatus β-defensin	66%	49%	66%	48%	66%	49%	49%	67%	49%	66%	48%	71%	44%	36%	60%	63%	46%	44%	64%	46%	46%	62%	49%	62%	48%	40%	36%	31%	100%												
S. chuatsi β-defensin	46%	99%	46%	98%	46%	100%	98%	44%	98%	46%	92%	41%	69%	61%	43%	46%	74%	47%	48%	84%	42%	92%	42%	92%	97%	40%	38%	32%	49%	100%											
O. latipes β-defensin	46%	90%	46%	89%	46%	91%	89%	46%	89%	46%	86%	42%	69%	64%	43%	46%	74%	48%	47%	83%	43%	88%	43%	91%	42%	41%	36%	50%	91%	100%											
L. haematochelia β-defensin	44%	96%	44%	94%	44%	96%	96%	46%	96%	44%	90%	41%	71%	60%	42%	44%	72%	47%	48%	84%	42%	91%	43%	92%	41%	39%	33%	50%	96%	88%	100%										
A. dabryanus β-defensin	47%	57%	47%	58%	47%	57%	56%	46%	56%	47%	59%	44%	53%	56%	48%	46%	59%	48%	38%	53%	47%	57%	47%	57%	41%	33%	28%	38%	57%	56%	56%	100%									
S. lalandi β-defensin-like1	91%	43%	94%	44%	94%	43%	43%	89%	42%	92%	46%	77%	41%	42%	87%	84%	48%	39%	54%	39%	82%	42%	93%	43%	59%	51%	48%	62%	43%	43%	42%	47%	100%								
S. lalandi β-defensin-like2	47%	98%	47%	99%	47%	98%	98%	46%	92%	47%	92%	41%	69%	62%	44%	47%	76%	47%	47%	84%	43%	91%	43%	94%	41%	39%	33%	48%	98%	89%	96%	58%	44%	100%							
M. musculus β-defensin1	28%	34%	28%	33%	28%	34%	34%	28%	34%	29%	33%	27%	30%	23%	28%	28%	31%	32%	34%	32%	27%	34%	28%	34%	30%	30%	27%	33%	34%	33%	34%	27%	28%	33%	100%						
H. sapiens β-defensin2	36%	42%	36%	43%	36%	42%	42%	37%	42%	37%	40%	34%	37%	33%	36%	34%	40%	39%	39%	39%	34%	41%	36%	42%	34%	32%	28%	40%	42%	41%	42%	33%	36%	43%	100%						
O. aries β-defensin1	36%	41%	36%	42%	36%	41%	41%	37%	41%	36%	40%	34%	37%	31%	37%	37%	39%	36%	39%	39%	36%	41%	38%	42%	41%	40%	33%	44%	41%	41%	42%	34%	36%	42%	46%	100%					
G. gallus β-defensin1	43%	44%	43%	46%	43%	44%	44%	43%	44%	42%	43%	41%	38%	36%	40%	42%	43%	41%	40%	40%	41%	43%	41%	44%	40%	37%	30%	43%	44%	43%	44%	42%	46%	47%	46%	46%	100%				
B. jararaca β-defensin2	41%	43%	41%	42%	41%	43%	43%	40%	42%	41%	42%	42%	39%	38%	40%	41%	44%	39%	39%	41%	39%	42%	41%	43%	37%	31%	26%	43%	43%	44%	43%	36%	41%	42%	40%	51%	44%	49%	100%		
	D. labrax β-defensin1	*D. labrax* β-defensin2	*S. aurata* β-defensin	*S. aurata* β-defensin-like2	*L. calcarifer* β-defensin-like1	*L. calcarifer* β-defensin-like2	*E. coioides* β-defensin	*O. niloticus* β-defensin1	*A. regius* β-defensin	*L. crocea* β-defensin-like1	*L. crocea* β-defensin-like2	*D. rerio* β-defensin-like1	*D. rerio* β-defensin-like2	*D. rerio* β-defensin-like3	*G. morhua* β-defensin	*S. salar* β-defensin1a	*S. salar* β-defensin2	*S. salar* β-defensin3	*S. salar* β-defensin4	*S. salar* β-defensin5a	*T. nigroviridis* β-defensin-like1	*T. nigroviridis* β-defensin-like2	*S. maximus* β-defensin-like1	*S. maximus* β-defensin2	*P. olivaceus* β-defensin1	*P. olivaceus* β-defensin3	*P. olivaceus* β-defensin4	*I. punctatus* β-defensin	*S. chuatsi* β-defensin	*O. latipes* β-defensin	*L. haematochelia* β-defensin	*A. dabryanus* β-defensin	*S. lalandi* β-defensin-like1	*S. lalandi* β-defensin-like2	*M. musculus* β-defensin1	*H. sapiens* β-defensin2	*O. aries* β-defensin1	*G. gallus* β-defensin1	*B. jararaca* β-defensin2

Supplementary Figure 1. Identity scores between vertebrate beta-defensins. Percentages were determined using the SIAS (Sequence Identity And Similarity) server (http://imed.med.ucm.es/Tools/sias.html), with BLOSUM62 scoring matrix and considering the length of multiple sequence alignment.

Supplementary Table 1. Accession numbers of vertebrate beta-defensin genes.

Species	Order	Gene	Accession number
Dicentrarchus labrax	Perciformes	Beta-defensin 1	CBXY010003646
Dicentrarchus labrax	Perciformes	Beta-defensin 2	CBXY010003659
Sparus aurata	Perciformes	Beta-defensin-like 2	ENSSAUG00010026539
Epinephelus coioides	Perciformes	Beta-defensin	JN698964
Oreochromis niloticus	Cichliformes	Beta-defensin 1	KJ577575
Paramisgurnus dabryanus	Cypriniformes	Beta-defensin 1	KC494302
Paramisgurnus dabryanus	Cypriniformes	Beta-defensin 2	KC261302
Tetraodon nigroviridis	Tetraodontiformes	Beta-defensin-like 1	BN000873
Tetraodon nigroviridis	Tetraodontiformes	Beta-defensin-like 2	BN000874
Onchorhynchus mykiss	Salmoniformes	Beta-defensin 1	FN545575
Onchorhynchus mykiss	Salmoniformes	Beta-defensin 2	FN545576
Onchorhynchus mykiss	Salmoniformes	Beta-defensin 3	FN545577
Homo sapiens	Primates	Beta-defensin 2	AF040153
Mus musculus	Rodentia	Beta-defensin 4	AF288371
Ovis aires	Artiodactyla	Beta-defensin 1	BK010312
Gallus gallus	Galliformes	Beta-defensin 1	AY621316
Bothrops jararaca	Squamata	Beta-defensin	KC117163

Supplementary Table 2. Accession numbers of defensins used in the alignment and phylogenetic analysis.

Species		Gene	Accession number
Sparus aurata	Perciformes	Beta-defensin	FM158209
Sparus aurata	Perciformes	Beta-defensin-like 2	ENSSAUT00010069764.1
Epinephelus coioides	Perciformes	Beta-defensin	AET25528
Argyrosomus regius	Perciformes	Beta-defensin	ASW20415
Lates calcarifer	Perciformes	Beta-defensin-like 1	ENSLCAT00010056645.1
Lates calcarifer	Perciformes	Beta-defensin-like 2	ENSLCAT00010013452.1
Larimichthys crocea	Perciformes	Beta-defensin-like 1	ENSLCRT00005005151.1
Larimichthys crocea	Perciformes	Beta-defensin-like 2	ENSLCRT00005035395.1
Oreochromis niloticus	Cichliformes	Beta-defensin 1	AGW83444
Paramisgurnus dabryanus	Cypriniformes	Beta-defensin 1	AGK65596
Paramisgurnus dabryanus	Cypriniformes	Beta-defensin 2	AGH10110
Cyprinus carpio L.	Cypriniformes	Beta-defensin 3	AGZ03658
Danio rerio	Cypriniformes	Beta-defensin-like 1	NP_001075022
Danio rerio	Cypriniformes	Beta-defensin-like 2	NP_001075023
Danio rerio	Cypriniformes	Beta-defensin-like 3	NP_001075024
Ctenopharyngodon idella	Cypriniformes	Beta-defensin 3	AQY18997
Carassius auratus	Cypriniformes	Beta-defensin-like 3	ENSCART00000074063.1
Gadus morhua	Gadiformes	Beta-defensin	AEB69787
Salmo salar	Salmoniformes	Beta-defensin 1a	QIX04703
Salmo salar	Salmoniformes	Beta-defensin 2	QIX04705
Salmo salar	Salmoniformes	Beta-defensin 3	QIX04706
Salmo salar	Salmoniformes	Beta-defensin 4	QIX04707
Salmo salar	Salmoniformes	Beta-defensin 5a	QIX04708
Oncorhynchus mykiss	Salmoniformes	Beta-defensin 1	CAK54950
Oncorhynchus mykiss	Salmoniformes	Beta-defensin 2	CAR82090
Oncorhynchus mykiss	Salmoniformes	Beta-defensin 3	CAR82091
Oncorhynchus mykiss	Salmoniformes	Beta-defensin 4	CAR82092
Takifugu rubripes	Tetraodontiformes	Beta-defensin-like 1	CAJ57646
Tetraodon nigroviridis	Tetraodontiformes	Beta-defensin-like 1	CAJ57644
Tetraodon nigroviridis	Tetraodontiformes	Beta-defensin-like 2	CAJ57645
Paralichthys olivaceus	Pleuronectiformes	Beta-defensin 1	ADA84138
Paralichthys olivaceus	Pleuronectiformes	Beta-defensin 3	ADA84140
Paralichthys olivaceus	Pleuronectiformes	Beta-defensin 4	ADA84141

Supplementary Table 2. *Cont.*

Species		Gene	Accession number
Scophthalmus maximus	Pleuronectiformes	Beta-defensin-like 1	ENSSMAT00000025713.1
Scophthalmus maximus	Pleuronectiformes	Beta-defensin 2	MW648585
Ictalurus punctatus	Siluriformes	Beta-defensin	APU66342
Oplegnathus fasciatus	Centrarchiformes	Beta-defensin	AJA33388
Siniperca chuatsi	Centrarchiformes	Beta-defensin	ACO88907
Oryzias latipes	Beloniformes	Beta-defensin	ACG55699
Liza haematocheila	Mugiliformes	Beta-defensin	AIK66783
Acipenser dabryanus	Acipenseriformes	Beta-defensin	QBJ27760
Seriola lalandi	Carangiformes	Beta-defensin-like 1	ENSSLDT00000023067.1
Seriola lalandi	Carangiformes	Beta-defensin-like 2	ENSSLDT00000026660.1
Mus musculus	Rodentia	Beta-defensin 1	AAB72003
Mus musculus	Rodentia	Beta-defensin 4	NP_062702
Homo sapiens	Primates	Beta-defensin 1	AAB49758
Homo sapiens	Primates	Beta-defensin 2	AAC33549
Ovis aries	Artiodactyla	Beta-defensin 1	DAB41723
Gallus gallus	Galliformes	Beta-defensin 1	AAT48925
Bothrops jararaca	Squamata	Beta-defensin 2	AGF25387
Crassostrea gigas	Ostreida	Beta-defensin 1	AEE92768
Homo sapiens	Primates	Alpha-defensin 1	NP_004075
Mus musculus	Rodentia	Alpha-defensin 1	NP_034161
Papio anubis	Primates	Theta-defensin a	ACJ12913

Supplementary Table 3. Accession numbers of genes used in the synteny analysis of beta-defensins 1 and 2.

	Species				
Genes	***D. labrax***	***L. calcarifer***	***S. aurata***	***S. lalandi*[b]**	***S. maximus*[b]**
defb1	ENSDLAG00005026052	ENSLCAG00010025743[a]	ENSSAUG00010014090	ENSSLDG00000017411[a]	ENSSMAG00000015557[a]
defb2	ENSDLAG00005001996	ENSLCAG00010006231	ENSSAUG00010026539	ENSSLDG00000020114	ENSSMAG00000013172
atp1b2b	ENSDLAG00005026068	ENSLCAG00010025763	ENSSAUG00010015305	ENSSLDG00000017566	ENSSMAG00000015277
CAPN6	ENSDLAG00005001788	ENSLCAG00010006146	ENSSAUG00010025836	ENSSLDG00000020262	ENSSMAG00000013231
chrdl2	ENSDLAG00005002011	ENSLCAG00010006252	ENSSAUG00010026542	ENSSLDG00000020064	ENSSMAG00000013155
cnksr2b	ENSDLAG00005025976	ENSLCAG00010025729	ENSSAUG00010014051	ENSSLDG00000017251	ENSSMAG00000015588
il13ra2	ENSDLAG00005001759	ENSLCAG00010006126	ENSSAUG00010025832	ENSSLDG00000020312	ENSSMAG00000013307
KCNAB3	ENSDLAG00005002038	ENSLCAG00010006338	ENSSAUG00010026552	ENSSLDG00000020006	ENSSMAG00000013080
lrch2	ENSDLAG00005001681	ENSLCAG00010006112	ENSSAUG00010025830	ENSSLDG00000020331	ENSSMAG00000013376
mepce	ENSDLAG00005001988	ENSLCAG00010006223	ENSSAUG00010026538	ENSSLDG00000020120	ENSSMAG00000013182
mogat3b	ENSDLAG00005026062	ENSLCAG00010025759	ENSSAUG00010015302	ENSSLDG00000017526	ENSSMAG00000015327
neu3.1	ENSDLAG00005002025	ENSLCAG00010006274	ENSSAUG00010026547	ENSSLDG00000020043	ENSSMAG00000013071
nyap1	ENSDLAG00005026065[a]	ENSLCAG00010025761[a]	ENSSAUG00010015303	ENSSLDG00000017545	ENSSMAG00000015310[a]
or121	ENSDLAG00005026051	ENSLCAG00010025739	ENSSAUG00010014085[a]	ENSSLDG00000017386[a]	ENSSMAG00000015567[a]
PAK3	ENSDLAG00005001894	ENSLCAG00010006195	ENSSAUG00010025886	ENSSLDG00000020143	ENSSMAG00000013188
rnf121	ENSDLAG00005025916	ENSLCAG00010025715	ENSSAUG00010014027	ENSSLDG00000017174	ENSSMAG00000015786
rnf167	ENSDLAG00005026054	ENSLCAG00010025746	ENSSAUG00010014093	ENSSLDG00000017455	ENSSMAG00000015502
rrm1	ENSDLAG00005025894	ENSLCAG00010025699	ENSSAUG00010013817	ENSSLDG00000017093	ENSSMAG00000015872
Spag7	ENSDLAG00005002002	ENSLCAG00010006239	ENSSAUG00010026541	ENSSLDG00000020103	ENSSMAG00000013168
srrt	ENSDLAG00005026057	ENSLCAG00010025754	ENSSAUG00010014122	ENSSLDG00000017477	ENSSLDG00000017477
trpc2b	ENSDLAG00005025880	ENSLCAG00010025683	ENSSAUG00010013753	ENSSLDG00000017035	ENSSMAG00000015927
xrra1	ENSDLAG00005002022	ENSLCAG00010006262	ENSSAUG00010026544	ENSSLDG00000020051c	ENSSMAG00000013136

defb: beta-defensin; *atp1b2b*: ATPase Na+/K+ transporting subunit beta 2b/ Sodium/ potassium-transporting ATPase subunit beta-2; *CAPN6*: Calpain 6; *chrdl2*: chordin 2; *cnksr2b*: Connector enhancer of kinase suppressor of Ras 2b; *il13ra2*: Interleukin 13 receptor, alpha 2; *KCNAB3*: Potassium voltage-gated channel subfamily A regulatory beta subunit 3; *lrch2*: Leucine-rich repeats and calponin homology (CH) domain containing 2; *mepce*: Methylphosphate capping enzyme/ 7SK snRNA methylphosphate capping enzyme; *mogat3b*: Monoacylglycerol O-acyltransferase 3b/ Diacylglycerol O-acyltransferase 2; *neu3.1*: Sialidase 3 (membrane sialidase), tandem duplicate 1/ Neuraminidase 3; *nyap1*: Neuronal Tyrosine Phosphorylated Phosphoinositide-3-Kinase Adaptor 1; *or121*: Odorant receptor, family E, subfamily 121, member 1; *PAK3*: p21 (RAC1) activated kinase 3/ Serine/threonine-protein kinase PAK 3; *rnf121*: Ring finger protein 121; *rnf167*: Ring finger protein 167/ E3 ubiquitin-protein ligase RNF167; *rrm1*: Ribonucleotide reductase M1 polypeptide/ Ribonucleoside-diphosphate reductase large subunit; *spag7*: Sperm associated antigen 7; *srrt*: Serrate RNA effector molecule homolog; *trpc2b*: Transient receptor potential cation channel subfamily C member 2b/ Short transient receptor potential channel 2-like; *xrra1*: X-ray radiation resistance associated 1. [a] Predicted gene; [b] Reverse strand; [c] Incomplete gene.

Chapter III

The Era of Antimicrobial Peptides: use of hepcidins to prevent or treat bacterial infections and iron disorders

Barroso C, Carvalho P, Nunes M, Gonçalves JFM, Rodrigues PNS* and Neves, JV* (2021). The Era of Antimicrobial Peptides: use of hepcidins to prevent or treat bacterial infections and iron disorders. *Frontiers in Immunology* 12:754437. (*These authors contributed equally to this work)

Abstract

The current treatments applied in aquaculture to limit disease dissemination are mostly based on the use of antibiotics, either as prophylactic or therapeutic agents, with vaccines being available for a limited number of fish species and pathogens. Antimicrobial peptides are considered as promising novel substances to be used in aquaculture, due to their antimicrobial and immunomodulatory activities. Hepcidin, the major iron metabolism regulator, is found as a single gene in most mammals, but in certain fish species, including the European sea bass (*Dicentrarchus labrax*), two different hepcidin types are found, with specialized roles: the single type 1 hepcidin is involved in iron homeostasis trough the regulation of ferroportin, the only known iron exporter; and the various type 2 hepcidins present antimicrobial activity against a number of different pathogens. In this study, we tested the administration of sea bass derived hepcidins in models of infection and iron overload. Administration with hamp2 substantially reduced fish mortalities and bacterial loads, presenting itself as a viable alternative to the use of antibiotics. On the other hand, hamp1 seems to attenuate the effects of iron overload. Further studies are necessary to test the potential protective effects of hamp2 against other pathogens, as well as to understand how hamp2 stimulate the inflammatory responses, leading to an increased fish survival upon infection.

Introduction

During the last decades, aquaculture became the fastest growing food production sector, with nearly half of fishes consumed worldwide being raised on fish farms [1]. However, fish species are produced under intensive aquaculture practices, leading to the appearance of disease outbreaks, mostly caused by bacteria or viruses [2], associated with high mortalities and production losses [3]. Vaccination to prevent disease in aquaculture is being routinely used in certain fish species, mostly salmonids, but efficient vaccines for other fishes and pathogens are still lacking. As such, fish farmers rely on the use of antibiotics, for prophylactic and therapeutic purposes [4,5]. However, the misuse of antibiotics in animal production, which led to the emergence of antibiotic-resistant microorganisms, with serious public health implications, as well as the inability of antibiotics in treating viral diseases, urgently presses for the development of alternatives to these drugs [4,6]. Antimicrobial peptides (AMPs) are considered as promising novel compounds to be used in aquaculture industry, due to their antimicrobial properties, immunomodulatory roles and reduced probability to develop bacterial resistance [7–9].

Fish present an extraordinary repertoire of AMPs, including the major groups of peptides, such as hepcidins, beta-defensins, cathelicidins and the fish specific piscidins [10,11]**.**

Hepcidin is a small cysteine rich peptide, first described in mammals by Krause et al. (2000) and named LEAP-1 (liver-expressed antimicrobial peptide) [12]. Later, Park et al. [13] isolated the same peptide and named hepcidin due to its hepatic expression and bacterial killing *in vitro*. However, the major role of hepcidin is the regulation of iron metabolism, by inhibiting post-translationally the iron exporter ferroportin [14,15]. Hepcidin is induced by iron overload and infection or inflammation, and inhibited by iron deficiency and hypoxia [16,17]. During an inflammatory stimulus, hepcidin is induced by inflammatory cytokines, leading to a decrease of iron release from hepatocytes, macrophages and enterocytes, trough ferroportin internalization and degradation. As a consequence, circulating iron is limited, as well as it availability for pathogens. However, as a long term effect, this also limits iron availability for erythropoiesis, leading to a condition known as anemia of inflammation or anemia of chronic disease [16,18].

Although most mammals present a single hepcidin (with the mouse being an exception [19,20]), with a dual function as an iron regulator and antimicrobial molecule, genome duplications and positive selection led to the appearance of multiple copies of hepcidin in certain fish species [21–25]. In the European sea bass (*Dicentrarchus labrax*), two different hepcidin types were described, with specialized roles: the single type 1 hepcidin (hamp1) is homologous to the mammalian counterpart, with a preponderant role on iron metabolism; and the various type 2 hepcidins (hamp2) show a direct activity against different bacteria [26]. Teleost fish presenting two hepcidin types show a considerable degree of subfunctionalization, with hamp1 having a conserved inhibitory function on ferroportin, while the multiple hamp2 mostly performs antimicrobial roles [26,27]. Thus, while the antimicrobial role of hepcidin in mammals is limited, the presence of several type 2 hepcidins in some fish species indicates a more significant role of hepcidin as an antimicrobial molecule in fish [26].

Several studies have shown the activity of hepcidin against an array of pathogens *in vitro* [28–32]. *In vivo*, treatment with hepcidin resulted in an increased fish survival and reduced bacterial or viral loads [28,33–35]. In sea bass, only one report addressed the effects of hepcidin administration in infected fish with *Vibrio anguillarum*, with sea bass presenting a higher resistance and reduced mortalities [36]. These studies show the potential of hepcidin to be used as an alternative to the antimicrobial treatments currently applied in aquaculture. However, information concerning the effects of hepcidin administration in fish is still very scarce, particularly the use of hepcidin type 1 in models of iron disorders.

In this study, we tested the administration of hamp1 or hamp2 in our experimental models of infection with *Photobacterium damselae* spp. *piscicida* and iron overload. Our results

demonstrate a clear beneficial effect of hamp2 in infected animals, as this molecule is capable of controlling bacterial infections and reducing fish mortalities, without interfering with iron metabolism. On the other hand, hamp1 administration seems to attenuate the effects of experimental iron overload. As such, fish hepcidins can be differentially applied in the treatment or prevention of infections and iron disorders. Further studies are necessary to test the potential protective effect of hamp2 against other pathogens, as well as to understand how hamp2 stimulate fish immune responses, leading to a higher fish resistance to infection.

Results

Hamp1, but not hamp2, has a significant impact on the iron status of sea bass

Before we could evaluate the prophylactic or therapeutic potential of either hamp1 or hamp2 to treat infectious diseases and iron disorders, we would need to understand their impact on the iron status of healthy animals. To assess that effect, we administered either hamp1 or hamp2 alone, and then evaluated blood parameters that are influenced by iron availability, such as hematocrit and red blood cells number, iron mobilization by looking into serum iron levels, and also the expression of various iron related genes, such as hepcidin itself, its target, the sole known iron exporter ferroportin, as well as the iron storage protein ferritin and the intestinal divalent metal transporter slc11a2alpha.

Administration of hamp1 to healthy sea bass led to a drastic decrease in both the hematocrit (Figure 1A) and number of red blood cells (RBC) (Figure 1B), effectively leading to a condition of anemia. These decreases occurred very steeply up to day 4 post-administration, after which a gradual recovery could be seen, returning to near normal levels after 21 days. This was also accompanied by a gradual decrease in serum iron levels (Figure 1C), and an increase in iron accumulation in the liver (Figure 1D), with both parameters peaking at day 7 followed by steady returns to normal levels. Administration of hamp2 had no significant effects on any of these parameters (Figure 1A-D).

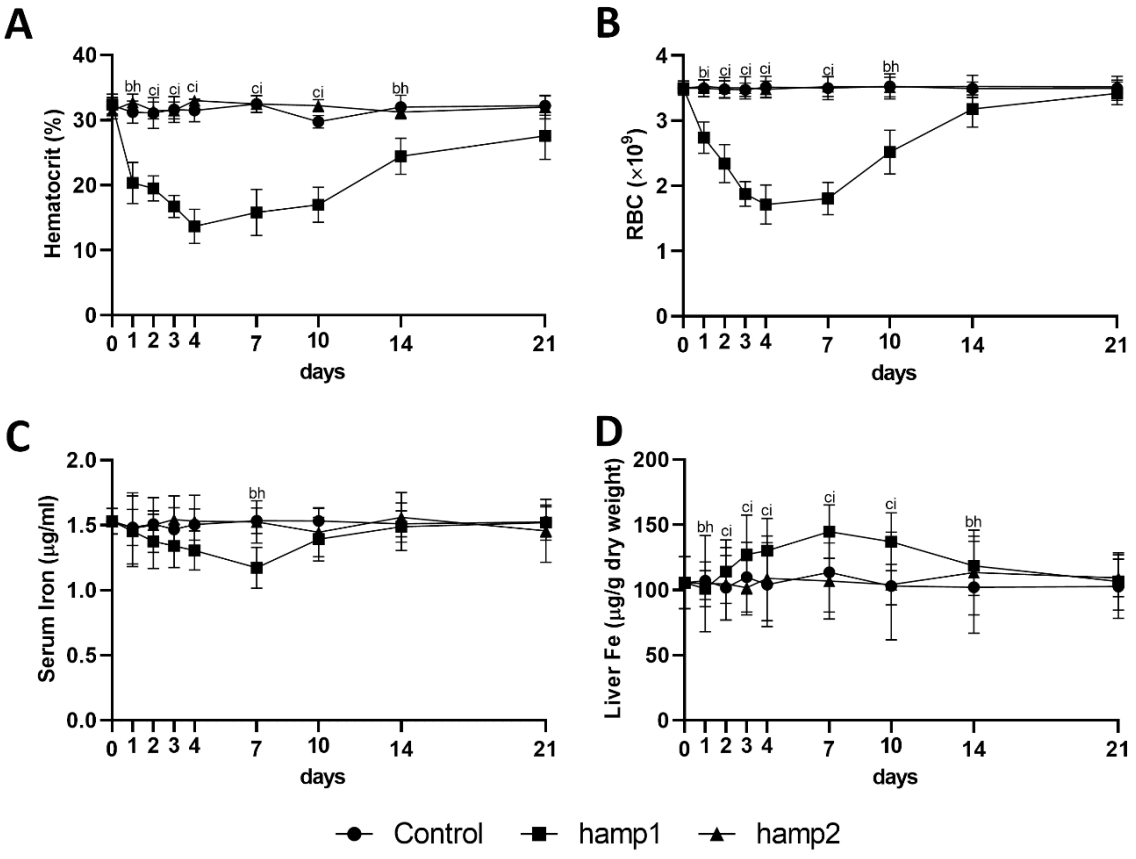

Figure 1. Hematological and serological parameters and tissue iron content in sea bass administered hamp1 or hamp2 peptides. (A) hematocrit; (B) red blood cell (RBC) number; (C) serum iron; (D) liver iron. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and hamp1, **d**, **e**, **f** between control and hamp2 and **g**, **h**, **i** between hamp1 and hamp2.

When looking at gene expression in the liver, administration of hamp1 led to early increases in both *hamp1* and *hamp2* expression (Figure 2A-B). It also led to a very significant reduction in *fpn1* expression (Figure 2C), as early as day 1 post-administration, followed by a slow but gradual recovery up to 21 days. Ferritin expression levels were found to be increased at day 7 (coinciding with the peak of iron accumulation in the liver), followed by a recovery towards day 21, but still above normal levels at day 10 (Figure 2D). In the intestine, *fpn1* was similarly downregulated (Figure 2E), although with a slightly faster recovery to normal levels than in the liver (day 10 vs. day 14). This was accompanied by significant increases in *fth* expression at days 4 and 7 (Figure 2F), and

decreases in the expression of slc11a2alpha at days 7 and 10 (Figure 2G). Administration of hamp2 also led to significant early increases in the expression of both *hamp1* and *hamp2* in the liver (Figure 2A-B), but had no impact in the expression of any other tested genes, either in the liver or intestine (Figure 2C-G).

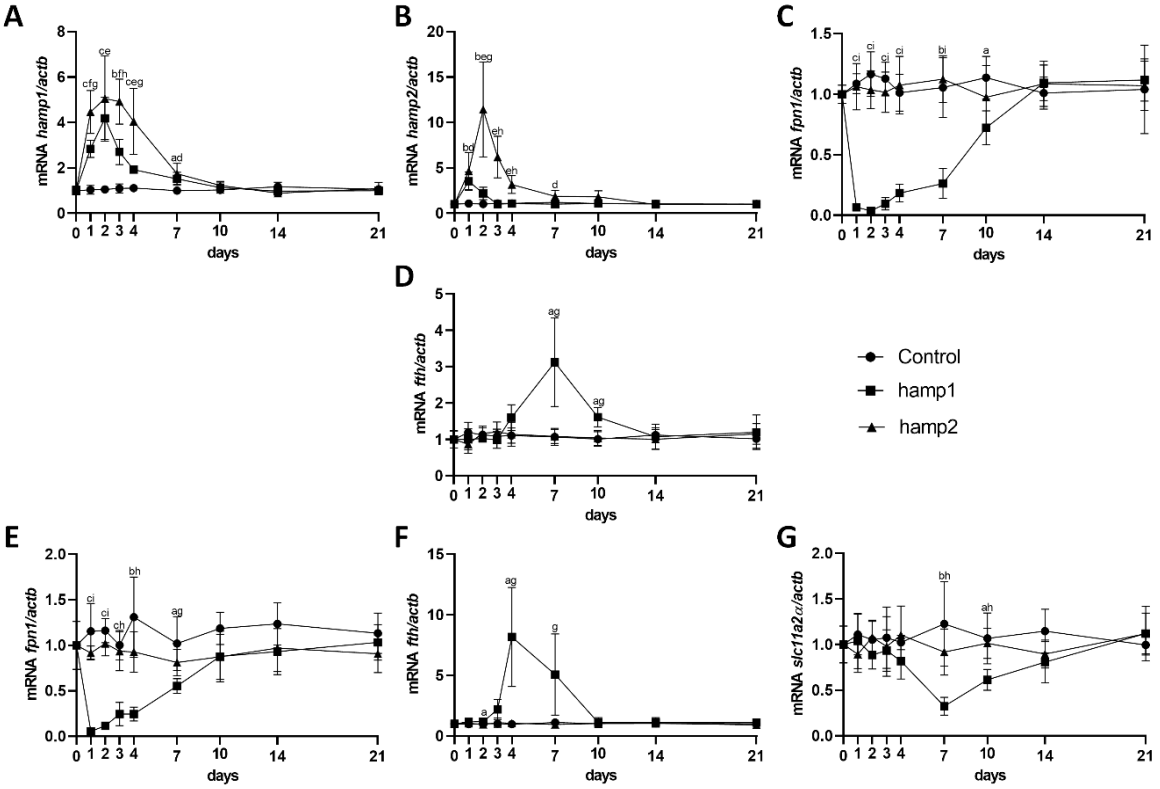

Figure 2. Gene expression in the liver and intestine at 1, 2, 3, 4, 7, 10, 14 and 21 days after hamp1 or hamp2 peptide administration. (A) *hamp1*, (B) *hamp2*, (C) *fpn1*, (D) *fth* expression in the liver; (E) *fpn1*, (F) *fth,* (G) *slc11a2alpha* expression in the intestine of peptide administered (hamp1/2) sea bass. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and hamp1, **d**, **e**, **f** between control and hamp2 and **g**, **h**, **i** between hamp1 and hamp2.

Hamp1 both attenuates and potentiates the various effects of iron overload

To test the potential of hepcidin to treat iron disorders, we performed a simple model of iron overload (to serve as a baseline for comparison), as well as a model of iron overload

followed by administration of either hamp1 or hamp2 (despite earlier results suggesting a limited involvement of hamp2 in iron metabolism). Furthermore, since hamp1 has a significant impact on the iron status of healthy animals, leading to anemia, a model of peptide administration followed by iron overload was tested but not pursued, as the animals would be in a debilitated state and subsequent iron overload was found to cause further damaging effects.

Iron overload alone led to increases in hematocrit, RBC numbers, liver iron accumulation and serum iron levels (Figure 3), with all parameters peaking mostly at 7 days post-overload (barring serum iron, that peaked at 4 days), followed by gradual decreases towards normal levels up to day 21, but with some parameters still slightly elevated at that time point (namely, hematocrit and liver iron). Iron overload followed by hamp2 administration had comparable effects to iron overload alone in all of these parameters, not differing significantly in any of them.

However, when hamp1 is administered after iron overload, no significant increases are observed in either hematocrit or RBC numbers. However, there are still increases in serum iron, although slightly lower than in the other experimental groups, and in liver iron content, in this case significantly higher than in the other experimental groups (Figure 3).

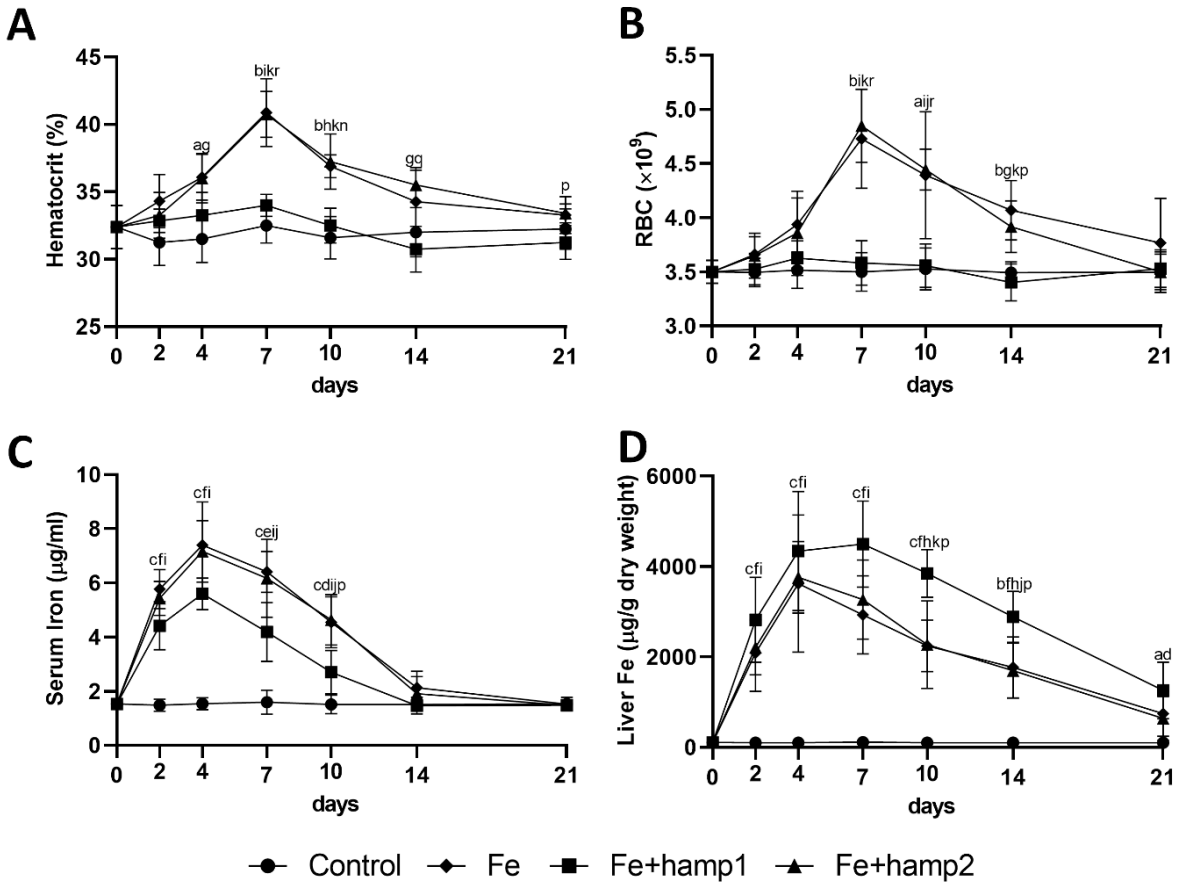

Figure 3. Hematological and serological parameters and tissue iron content in sea bass after experimental iron overload (Fe) or iron overload and peptide administration (Fe+hamp1/2). (A) hematocrit; (B) red blood cell (RBC) number; (C) serum iron; (D) liver iron. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and Fe animals, **d**, **e**, **f** between control and Fe+hamp1, **g**, **h**, **i** between control and Fe+hamp2, **j, k, l** between Fe and Fe+hamp1, **m, n, o** between Fe and Fe+hamp2 and **p, q, r** between Fe+hamp1 and Fe+hamp2.

Both iron overload alone and iron overload followed by hamp2 administration mostly caused similar effects on gene expression. We observed a high increase in *hamp1* in both experimental groups (Figure 4A), but only administration of hamp2 caused a further increase in *hamp2* expression in the liver (Figure 4B). Similar increased patterns of expression were observed for *fth* in the liver (Figure 4D), with no changes in the intestine (Figure 4F), as well as of decreased expression of *fpn1* both in the liver and intestine (Figure 4C,E), and *slc11a2alpha* in the intestine (Figure 4G). Once again, iron overload followed by administration of hamp1 had different effects, causing either dissimilar or more pronounced changes in gene expression. Only a minor increase in *hamp1* expression was observed, with no significant changes in *hamp2* (Figure 4A-B). Stronger decreases in *fpn1* (Figure 4C,E) and *slc11a2alpha* (Figure 4G) expressions were also observed, as well as higher increases in *fth* expression (Figure 4D,F), both in the liver and intestine, possibly indicating a much more pronounced limitation in iron release and absorption.

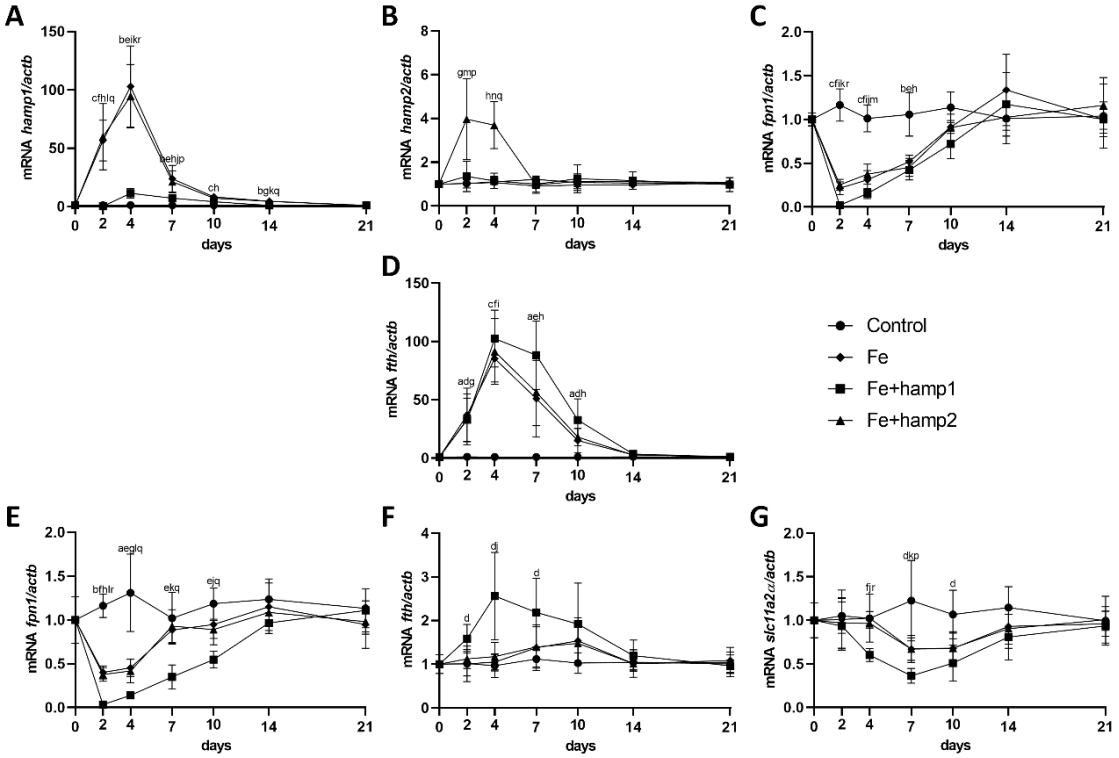

Figure 4. Gene expression in the liver and intestine at 2, 4, 7, 10, 14 and 21 days after experimental iron overload and peptide administration. (A) *hamp1*, (B) *hamp2*, (C) *fpn1*, (D) *fth* expression in the liver; (E) *fpn1*, (F) *fth*, (G) *slc11a2alpha* expression in the intestine of iron overload (Fe) and iron overload and peptide administered (Fe+hamp1/2) sea bass. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and Fe animals, **d**, **e**, **f** between control and Fe+hamp1, **g**, **h**, **i** between control and Fe+hamp2, **j, k, l** between Fe and Fe+hamp1, **m, n, o** between Fe and Fe+hamp2 and **p, q, r** between Fe+hamp1 and Fe+hamp2.

Hamp2 has a significant protective effect against infection with *P. damselae*

In order to investigate the potential of hepcidin to prevent or treat bacterial diseases, we made experimental infections with the Gram-negative bacteria *Photobacterium damselae* spp. *piscicida*. Two major models were performed, one of infection followed by peptide administration (infected+hamp1/2 - therapeutic potential) and the other of peptide administration followed by infection (hamp1/2+infected - prophylactic potential), as well as a simple model of infection without any kind of peptide administration (as a baseline for comparison). Although with some differences, both models demonstrated that hamp2 is

highly effective against *P. damselae*, whereas hamp1 is not only ineffective, but even slightly more deleterious.

In the model of infection followed by peptide administration, we could see a clear reduction in fish mortality derived from hamp2 administration, reduced from around 55% in the control infection to less than 17% in hamp2 administered animals (Figure 5A). Hamp1 on the other hand, not only it did not reduce mortality but in fact slightly increased it, to around 59%. Similar results can be seen for the CFU counts in both the spleen and head kidney, with much reduced bacterial loads in hamp2 administered animals, whereas no differences were observed between infected and infected+hamp1 animals (Figure 5B-C).

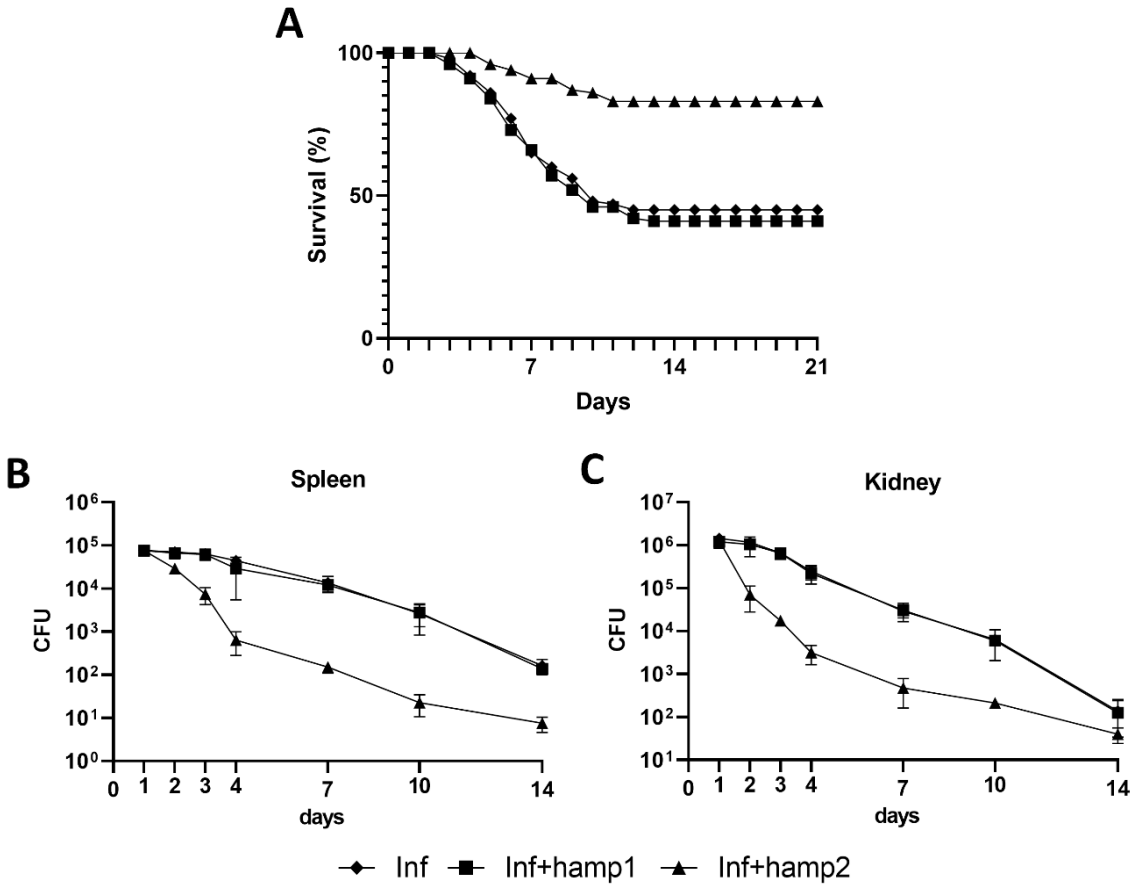

Figure 5. Survival curves and CFU counts in sea bass administered hepcidin 24 hours after experimental infection (post-infection). Mortality was assessed during 21 days of infection with *P. damselae*, followed by administration of either hamp1 or hamp2. Colony forming unit (CFU) values are expressed as means ± standard deviation (n=5).

Infection alone led to significant decreases in all hematological and serological parameters, up to 4 days post-infection, with gradual recovery to normal levels from day 7 forward (Figure 6A-C). These decreases were less pronounced with hamp2 administration, with a faster recovery to normal levels, but more pronounced with hamp1 administration, with higher decreases of the hematocrit, RBC numbers and serum iron levels, when compared with control infection. No significant changes were observed for liver iron content in any of the experimental groups, although a slight tendency for increase could be seen in solely infected animals (Figure 6D).

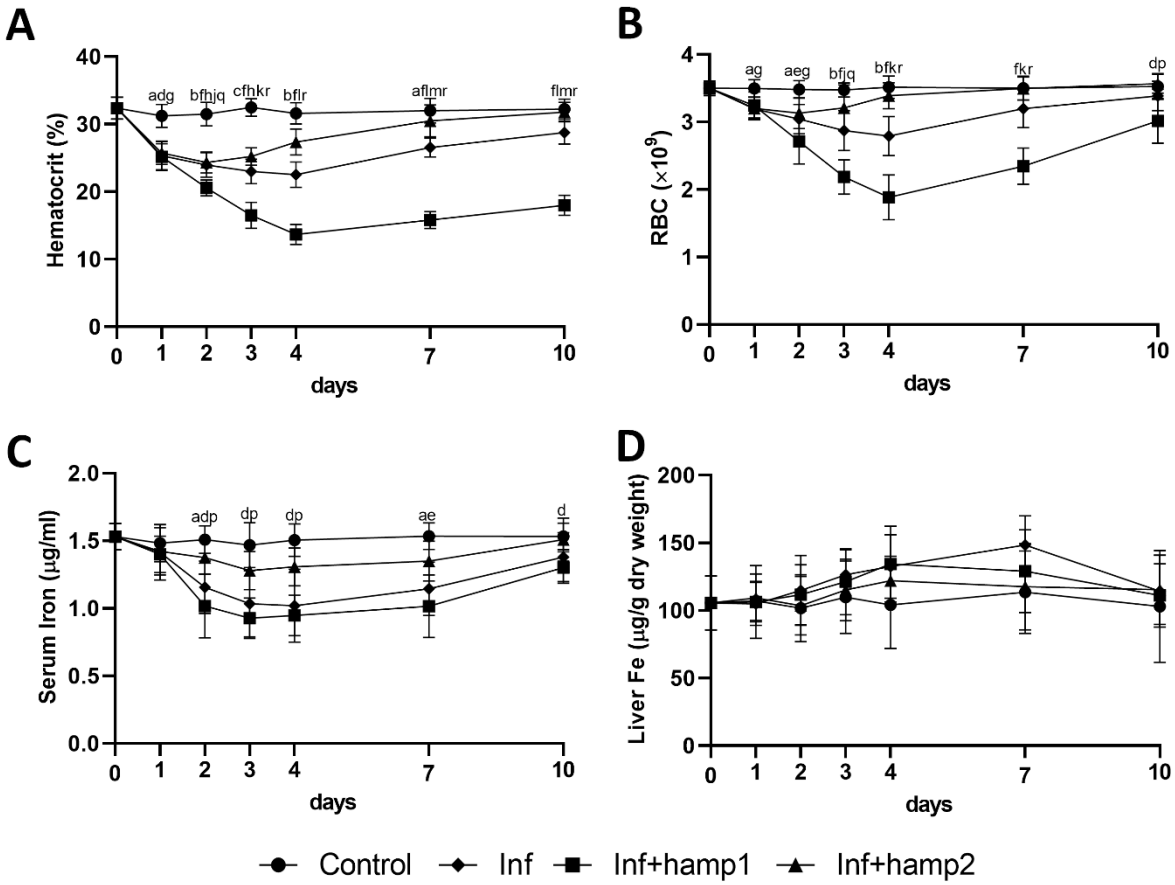

Figure 6. Hematological and serological parameters and tissue iron content in sea bass administered hepcidin 24 hours after experimental infection (post-infection). (A) hematocrit; (B) red blood cell (RBC) number; (C) serum iron; (D) liver iron. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and infected (Inf) animals, **d**, **e**, **f** between control and infected+hamp1 (Inf+hamp1), **g**, **h**, **i** between control and infected+hamp2 (Inf+hamp2), **j,**

k, l between Inf and Inf+hamp1, **m, n, o** between Inf and Inf+hamp2 and **p, q, r** between Inf+hamp1 and Inf+hamp2.

Looking at gene expression, in the liver of infected animals a significant increase in *hamp1* expression is observed at day 1, quickly reverting to near normal levels at day 2 (Figure 7A). *Hamp2* also sees the highest increase at day 1, and gradually decreases up to day 10, but still kept slightly overexpressed (Figure 7B). Increases in *fth* can also be been, peaking at 3 days post-infection, whereas *fpn1* gradually decreases up to 2 days, then slowly returns to normal levels (Figure 7C-D). In the intestine, no significant changes could be seen in the expression of any of the tested genes (Figure 7E-G). In infected+hamp1 animals, similar patterns of expression can be observed in the liver (Figure 7A-D), with similar increases in *hamp1*, *hamp2* and *fth* expression, although *hamp1* levels are kept elevated up to day 2, rather than just day 1. *Fpn1* suffers an even more pronounced downregulation, reaching lower levels and recovering slower. However, in the intestine, significant changes can be observed for all genes (Figure 7E-G), with a downregulation of both *fpn1* and *slc11a2alpha*, in the earlier and late days of infection, respectively, and an up regulation of *fth*. Lastly, in infected+hamp2 animals, patterns of gene expression in both the liver and intestine were similar to the ones observed for infected animals, with one notable exception: *hamp2* levels in the liver still peaked at day 1 post-infection but decreased more rapidly at day 2 (around 5 times lower than infected and infected+hamp1) (Figure 7B).

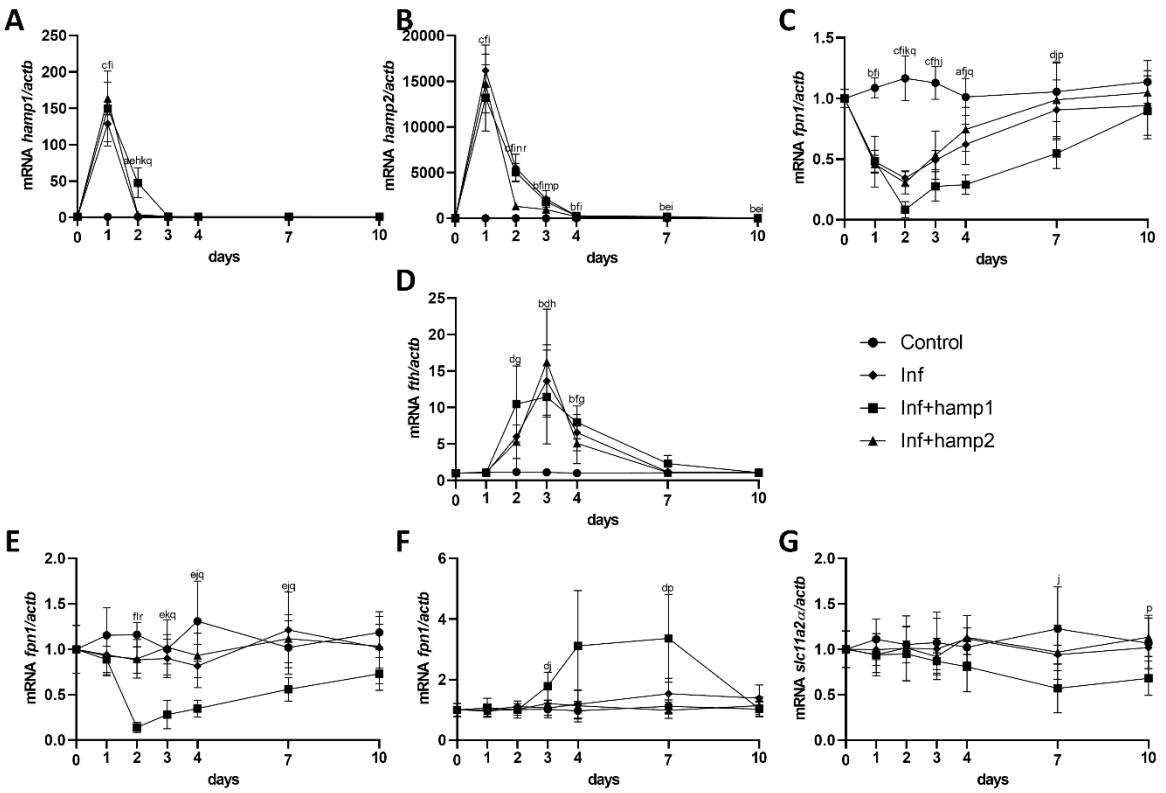

Figure 7. Gene expression in the liver and intestine at 1, 2, 3, 4, 7 and 10 days, in sea bass administered hepcidin 24 hours after experimental infection (post-infection). (A) *hamp1*, (B) *hamp2*, (C) *fpn1*, (D) *fth* expression in the liver; (E) *fpn1*, (F) *fth*, (G) *slc11a2alpha* expression in the intestine of infected (Inf) and infected and peptide administered (Inf+hamp1/2) sea bass. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and Inf animals, **d**, **e**, **f** between control and Inf+hamp1, **g**, **h**, **i** between control and Inf+hamp2, **j, k, l** between Inf and Inf+hamp1, **m, n, o** between Inf and Inf+hamp2 and **p, q, r** between Inf+hamp1 and Inf+hamp2.

In our final experimental model, of peptide administration followed by infection, mortality was greatly reduced by the pre-administration of hamp2, from the 55% of the control infection to a mere 6% (Figure 8A). Once again, hamp1 not only did not reduce mortality but increased it slightly more, to around 60%. Results for the CFU counts were even more expressive, with rapidly decreasing bacterial loads in hamp2 administered animals, to the point where no CFUs could be counted after 7 and 10 days of infection in the spleen and

head kidney, respectively (Figure 8B-C). Again, no significant differences were observed between infected and hamp1+infected animals.

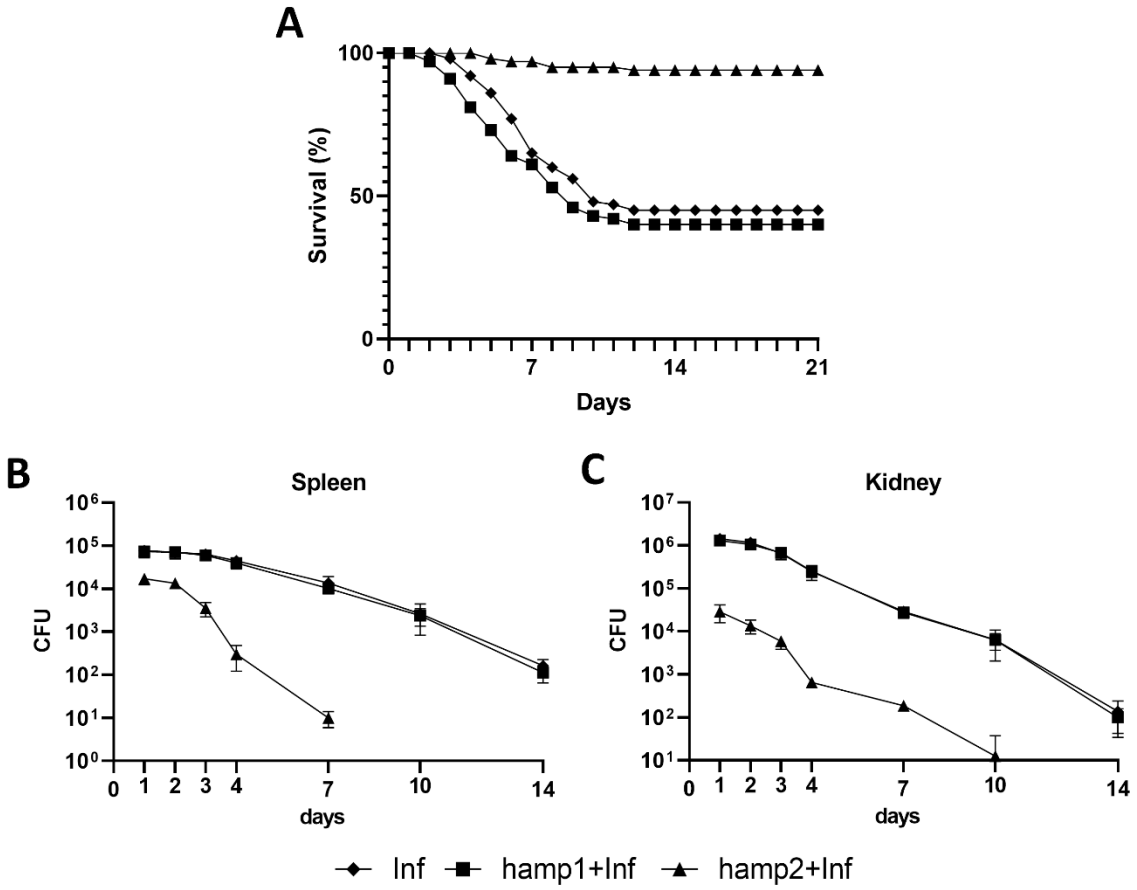

Figure 8. Survival curves and CFU counts of sea bass administered hepcidin 24 hours before experimental infection (pre-infection). Mortality was assessed during 21 days of infection with *P. damselae*, preceded by administration of either hamp1 or hamp2. Colony forming unit (CFU) values are expressed as means ± standard deviation (n=5).

Variations in hematological and serological parameters were mostly comparable to the previous experimental model of infection, but with a clear difference at day 0, where hematocrit and RBC numbers were already significantly reduced in hamp1 administered animals. This is not unexpected since in this experiment this day would be equivalent to day 1 post-peptide administration (Figure 1). Decreases were observed up to 4 days post-infection and gradual recovery to normal levels from day 7 forward (Figure 9A-C). Again, these decreases were more pronounced with hamp1 administration and less pronounced

with hamp2 administration, when compared with control infection, and no significant changes were observed for liver iron content (Figure 9D).

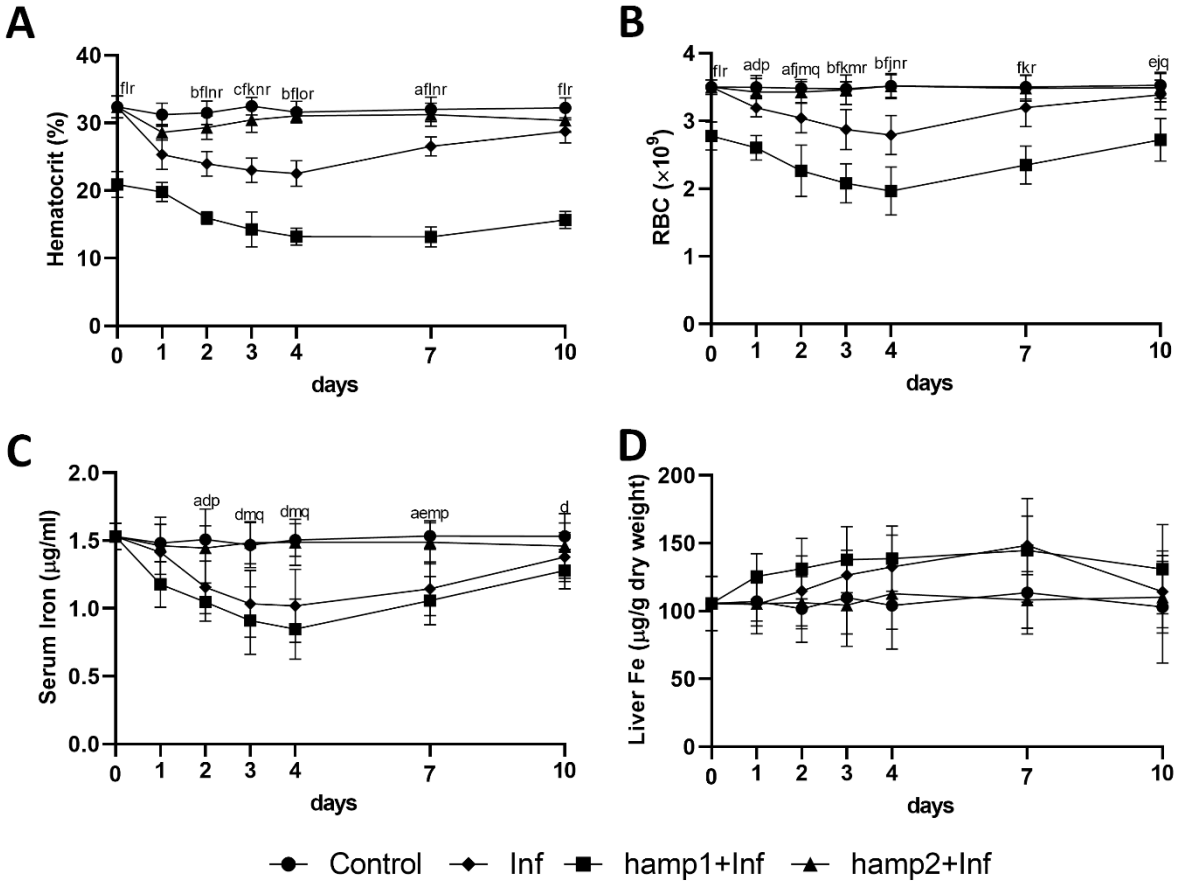

Figure 9. Hematological, serological and tissue iron content in sea bass administered hepcidin 24 hours before experimental infection (pre-infection). (A) hematocrit; (B) red blood cell (RBC) number; (C) serum iron; (D) liver iron. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c** between control and Inf animals, **d**, **e**, **f** between control and hamp1+Inf, **g**, **h**, **i** between control and hamp2+Inf, **j, k, l** between Inf and hamp1+Inf, **m, n, o** between Inf and hamp2+Inf and **p, q, r** between hamp1+Inf and hamp2+Inf.

Gene expression patterns were also very similar to the ones observed for the previous infection model, but nevertheless with several significant differences. *Hamp1* expression was found to be increased in response to both hamp1 and hamp2 administration, but with

a much more limited increase in response to hamp1 (Figure 10A). Similarly, *hamp2* also responded to the administration of either peptide, but to a much lesser extent to hamp2 (Figure 10B). Also, at day 0, both hepcidin types are already slightly overexpressed in the peptide administered groups, when compared with infection alone, again reminiscent of day 1 of the peptide administration experiment (Figure 2). Similarly, *fpn1* levels were already much lower than normal at day 0, both in the liver and intestine, but followed a similar pattern of decline and subsequent recovery (Figure 10C,E). *Fth* levels were found to be elevated in both the liver and intestine (Figure 10D,F), whereas *slc11a2alpha* levels in the intestine starting decreasing significantly at day 4 post-infection, followed by a recovery towards normal levels, but still under expressed after 10 days (Figure 10G).

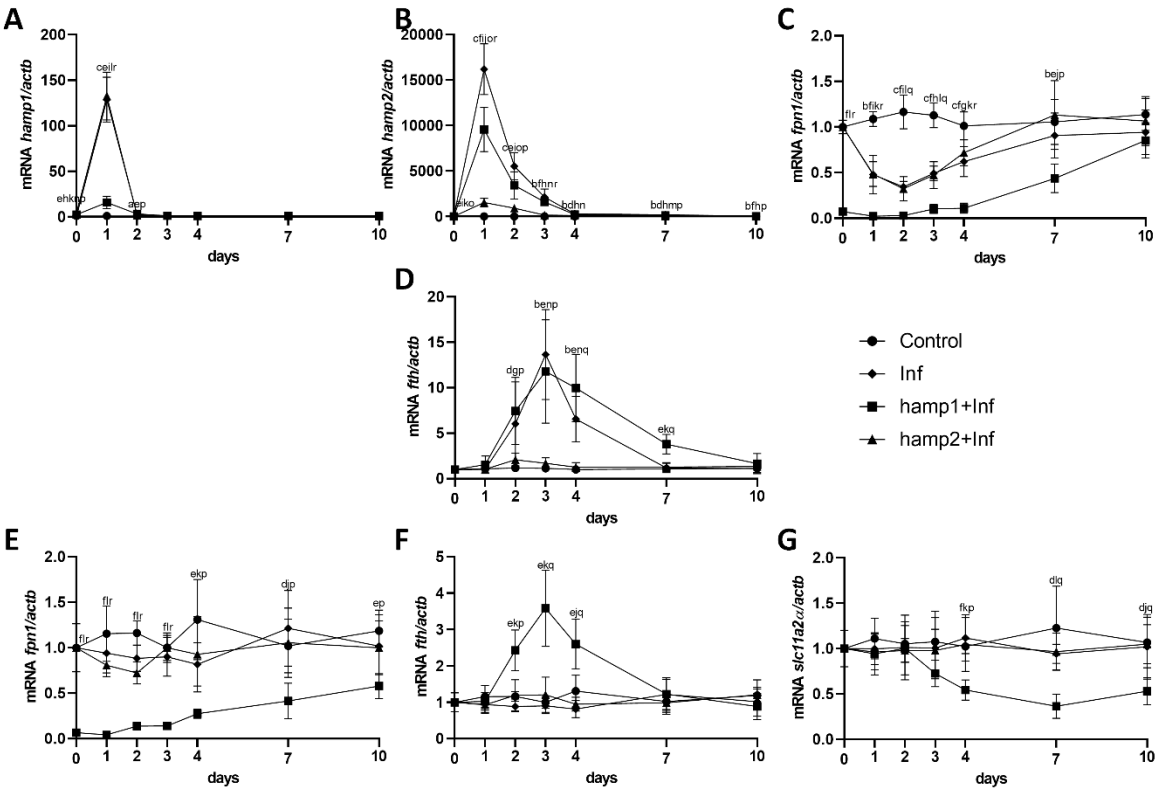

Figure 10. Gene expression in the liver and intestine at 1, 2, 3, 4, 7 and 10 days, in sea bass administered hepcidin 24 hours before experimental infection (pre-infection). (A) *hamp1*, (B) *hamp2*, (C) *fpn1*, (D) *fth* expression in the liver; (E) *slc11a2alpha*, (F) *fpn1*, (G) *fth* expression in the intestine of infected (Inf) and peptide administered and infected (hamp1/2+Inf) sea bass. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters **a**, **b**, **c**

between control and Inf animals, **d**, **e**, **f** between control and hamp1+Inf, **g**, **h**, **i** between control and hamp2+Inf, **j, k, l** between Inf and hamp1+Inf, **m, n, o** between Inf and hamp2+Inf and **p, q, r** between hamp1+Inf and hamp2+Inf.

Discussion

Diseases are a major problem in aquaculture, every year causing significant production and economic losses. Some preventive measures can be taken, mostly reliant on vaccination strategies, but there is a lack of effective commercial vaccines for a large number of pathogens. Therapeutic measures can also be applied, but usually too late and are mostly based on antibiotics, the use of which is becoming increasingly problematic, due to increased pathogen multiresistance, toxicity, inability to treat viral diseases and the possibility of entering the human food chain [37,38]. As such, it has become crucial to find efficient therapeutic alternatives to replace or reduce the use of antibiotics. Antimicrobial peptides are a promising alternative to the use of antibiotics and other chemical compounds [7,9,39,40] presenting several advantages, such as limited toxic effects and broad spectrum of antimicrobial properties against bacteria, as well as viruses, fungi, parasites and even anomalous cells. They are also less prone to cause bacterial resistance, but still not totally impervious to that effect [41–44]. Among antimicrobial peptides, hepcidin is of particular interest, especially in teleost fish. In mammals, hepcidin is present as a single gene (with the mouse being the sole exception, so far) [12,13,19,20], and although originally characterized as an antimicrobial peptide, that function was quickly relegated to the background. Currently, hepcidin is considered the *de facto* key regulator of iron metabolism, due to its interaction with the only known iron exporter, ferroportin [15,45–47]. This effectively limits the applications of mammalian hepcidin mostly to the treatment of iron disorders, by targeting the hepcidin/ferroportin axis, since its use in infections would have a severe impact in several iron related parameters, with negative consequences to the host. However, many teleost fish [23,24,48,49], sea bass included [26], present two different types of hepcidin, with different functions: type 1 hepcidin, very similar to mammalian hepcidin and also with a role in iron metabolism, and one to several type 2 hepcidins, with almost exclusively antimicrobial roles. As such, contrary to mammalian hepcidin, fish hepcidins have the potential to be differentially applied in the treatment or prevention of iron disorders and infections. Although some studies exist on the antimicrobial potential of fish derived hepcidins, information about their possible applications in iron disorders is lacking.

In order to determine the potential applications of sea bass derived hepcidins, in this study, we have performed several experimental models of both iron overload and infection, where we administered two different peptides, hamp1 and hamp2, and evaluated their effects on several parameters, including iron levels, hematological parameters and expression of iron metabolism-related relevant genes.

The impact of administration of hepcidin in healthy animals has shown that hamp1 had a significant impact on several hematological parameters, causing decreases in both hematocrit and red blood cell numbers, and thus leading to a condition of severe anemia. The role of type 1 hepcidins is well established, with its major target being the iron exporter ferroportin [27,45]. When hepcidin levels are increased, it binds to ferroportin, causing its internalization and degradation in various cell types, most relevant of all the hepatocytes, the major place for iron storage, the reticuloendothelial macrophages, deeply involved in hemocateresis and the intestinal enterocytes, responsible for iron absorption, effectively blocking any kind of iron release from these cells. A biological increase in hamp1 levels is known to occur in response to conditions of iron overload, to signal the body to limit further iron absorption [16,17,50], as well as during various infectious diseases, as a mechanism to limit pathogen proliferation [26,51–53]. An increase in the expression of *hamp1* has an inhibitory effect on ferroportin, limiting its expression and membrane presence, and thus limiting iron release from hepatocytes, macrophages and enterocytes. Furthermore, the accumulation of iron in the intestinal enterocytes, in the form of ferritin, will also lead to a suppression of slc11a2-mediated iron uptake, decreasing iron absorption [54–56]. During infections, this mechanism is responsible for limiting iron availability for pathogens, effectively starving them of this essential nutrient. However, this is a double edged sword, since iron is not only unavailable for pathogens, but also for several of the host's processes, including erythropoiesis. This means that, during prolonged infections, erythrocytes are still being recycled, but since there is no iron available, erythropoiesis comes to a standstill, thus leading to a gradual decrease in the number of red blood cells and hematocrit, causing a condition commonly referred to as anemia of inflammation or anemia of chronic disease. When administered to healthy animals, it simply leads to anemia and to a possible debilitative state (while not causing mortality, the animals were generally more lethargic and less prone to feed). On the other hand, administration of hamp2 to healthy animals had no visible effects on any of the evaluated parameters, once again opening the door to the differential application of these peptides.

Following these observations, we then introduced a new variable, iron overload. Iron overload alone led to alterations in the various measured hematological parameters, with significant increases in red blood cell numbers and hematocrit, as well as circulating iron

levels and liver iron accumulation. It is well known that iron deficiency or anemia lead to a decrease in hematological parameters and as such, it is frequently assumed that iron overload would lead to a reverse situation, of increased hematological parameters. This is not so straightforward, as in many cases iron overload can also cause no changes or even lead to anemia (such as some forms of beta thalassemia on hypochromic microcytic anemia [57,58]), but we have previously observed that in sea bass, during iron overload and despite the associated increase in *hamp1* expression, hematological parameters are often increased [56,59], and that can also be seen here. It is likely an additional mechanism is triggered to cope with the excess of iron, by using more in the production of hemoglobin and red blood cells and thus, keeping it in a non-free, non-toxic form, but why and how exactly this occurs remains unknown. Similarly, gene expression profile is in accordance with an attempt to increase iron storage and limit iron release and absorption, denoted by the decreased *fpn1* and *slc11a2alpha* expression, and further increased *hamp1* expression, as well as of *fth*. Since we had already observed that hamp2 has no effect on iron metabolism [26], we expected a similar pattern of changes in animals administered both iron dextran and the hamp2 peptide, which was what we observed. The only significant difference when compared with iron overloaded animals was an increase in *hamp2* expression, likely induced by hamp2 itself, as neither iron or hamp1 administration seem to produce a similar effect.

However, iron overload followed by administration of hamp1 had a significantly different outcome. There were no discernible changes in either hematological parameter, circulating serum iron levels were slightly less elevated and liver iron accumulation significantly higher throughout the duration of the experiment, which seems to indicate a higher rate of iron retention, limiting the increased erythropoiesis that occurs in iron overloaded animals. Gene expression also reflects this, with an even more aggressive suppression of *fpn1* and *slc11a2alpha* expression, as well as a higher *fth* expression. Contrary to hamp2, which itself induced an increase in its own expression, administration of hamp1 limited *hamp1* expression when compared to iron overload, or iron overload and hamp2 administered animals, indicating that the administered hamp1 levels were already high enough to deal with the increased iron levels. Taken together, all this data points towards a possible application of hamp1 in the treatment of iron overload, similar to mammalian hamp1 [60,61]. Sea bass hamp1 can even be used as a substitute to mammalian hepcidins, since it is also known to be able to regulate mammalian ferroportin [27]. The potential to prevent iron overload is less encouraging, with some preliminary tests showing that animals pre-treated with hamp1 before iron overload seemed to be become somewhat more susceptible, to point of having some mortality not seen in animals with only iron overload. It seems that any benefit derived from iron withholding is

quickly undermined by the debilitative state derived from anemia, and probably leads to a less than ideal coping with iron toxicity, but this will require further studies.

Having explored the possible impact of hepcidin on iron metabolism, we then moved on to its other major function, the role during infection. Here, things are a little bit different between mammals and teleost fish. Although hepcidin was originally characterized in mammals as an antimicrobial peptide [12,13], that function is now considered very minor, with hepcidin assuming the role of key regulator of iron metabolism [14,16,17]. During infection, its major function is to lead to iron withholding, to limit pathogen proliferation, with seemingly minimal antimicrobial activity. However, in teleost fish presenting two hepcidin types, these functions seem to be separated. Fewer studies exist pertaining the role of fish hepcidins in iron metabolism, but there are some reports showing that type 1 hepcidins (characterized by the presence of the hypothetical iron regulatory sequence Q-S/I-H-L/I-S/A-L in the N-terminal region) seem to be mostly involved in iron regulation, likely limiting iron availability for pathogens [26,27,49,62,63]. On the other hand, type 2 hepcidins (lacking the iron regulatory sequence) are highly diverse and are considered to have a mostly antimicrobial activity, responding to a wide variety of pathogens, including bacteria, fungi, viruses and parasites [26,28–30,33,64–67], and even to anomalous cells [68,69]. In both cases we say mostly because, due the huge diversity in teleost fish hepcidins, there is the occasional report indicating some potential antimicrobial activity for hamp1 or a small role in iron regulation for hamp2, as well as reports where hepcidins were wrongly characterized as a different type, and as such, attributed an erroneous function. To complicate matters even more, in teleost fish with a single hepcidin (type 1, such as Cyprinids and Salmonids), the antimicrobial activity can be very significant, asides from its role in iron metabolism [35,70–74]. Nevertheless, there are various functional studies testing the effects of hepcidin peptides on a diversity of pathogens, mostly in *in vitro* conditions [28–32,75–78], but a few also *in vivo* [28,33,34,36], with most results pointing towards a much higher diversity (in amino acid composition and cysteine number) and antimicrobial activity of type 2 hepcidins. We have also previously characterized both hamp1 and various hamp2 in sea bass, and tested them in *in vitro* conditions, with hamp1 has showing little to no antimicrobial activity, whereas the various hamp2 have shown differential activity against Gram-negative and Gram-positive bacteria [26], and based on those results, we have selected the most promising hamp2, the one with highest antimicrobial activity, for further testing in the *in vivo* experimental infections of this work.

We took two different approaches to infection: a therapeutic study, where we administered hepcidin after infection, and a prophylactic study, where we administered hepcidin before infection, to evaluate both the potential to treat and prevent bacterial diseases, in this case pasteurelosis, caused by *Photobacterium damsela* spp. *piscicida*. The outcomes of both

experiments were very similar, with clear conclusions. First, that hamp1 does not seem to help in the prevention or treatment of pasteurelosis, and may actually make it worse as evidenced by the slight increase in animal mortality. Again, this is likely derived from the significant impact that hamp1 has on several hematological and iron parameters, leading to a condition of anemia that introduces a debilitative state and makes the animals more susceptible to infection. However, we should not completely exclude the usefulness of hamp1, even more so because we could observe a biological response of *hamp1* to infection [26]. More in depth studies using lower doses of hamp1, which would have a more limited impact on iron metabolism, would have to be performed in order to evaluate its real potential, either alone or together with hamp2. The second conclusion is that hamp2 is highly effective against pasteurelosis, even more so when administered before infection, as indicated by the lower mortality and bacterial loads observed. These findings are also in agreement with a recent study from Álvarez et al. [36], that has shown that pre-administration of a type 2 hepcidin in sea bass can limit mortality caused by *Vibrio anguillarum*, reducing it from around 72% to less than 24%. Although seemingly not as effective in the prevention of vibriosis, when compared with pasteurelosis, those results are nevertheless very promising, if we take into consideration that *V. anguillarum* seems to be much more resistant to type 2 hepcidins than *P. damselae* [26]. Additionally, as expected from previous results, hamp2 had no significant impact on iron metabolism and also hampered the development of anemia of inflammation, contributing for a better health status of the animals.

In summary, we have shown that the administration of the two sea bass hepcidins types elicit different responses, with hamp1 impacting in the regulation of iron metabolism and hamp2 having a very significant protective activity against bacterial infections. Hamp2 apart from having a direct antimicrobial activity, may also be involved in immunomodulatory processes [79–81] and the inflammatory response, but further studies will be required to address this matter. Nevertheless, the doors are clearly open for the potential application of sea bass derived hepcidins in the treatment of iron disorders and, more importantly, as viable substitutes for the use of antibiotics in the prevention and treatment of infections, if we can overcome some of the current limitations for a wide use of antimicrobial peptides, such as costs and more effective ways of administration.

Materials and Methods

Animals

Healthy European sea bass (*Dicentrarchus labrax*), with an average weight of 50 g, were provided by a commercial fish farm in the north of Spain (Sonríonansa S.L., Pesués, Cantabria, Spain). Prior to the experiments, fish were acclimated for 30 days to the fish holding facilities of the Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto. Fish were kept in 110 liters recirculating sea water (28±1 ‰ salinity) tanks at 22±1 ºC, with a 13/11-hour light/dark cycle and fed daily *ad libitum* with commercial fish feed with an iron content of approximately 200 mg iron/kg feed. Before each treatment, fish were anaesthetized with ethylene glycol monophenyl ether (2-phenoxyethanol, 3 mL/10 L, Merck, Algés, Portugal). All animal experiments were carried out in strict compliance with national and international animal use ethics guidelines (including ARRIVE guidelines), approved by the animal welfare and ethic committees of ICBAS (permit P293/2019/ORBEA, 05/04/2019) and conducted by experienced and trained FELASA Function A+B+D investigators.

In vivo experimental models

To evaluate the effects of hamp1 or hamp2 administration in different conditions, several experimental models were established (Figure 11). Five fish from each experimental group were collected at the various time points after treatments, euthanized with an overdose of anesthetic, blood and serum collected for hematological and serological parameters determination, dissected, and tissues excised and snap frozen in liquid nitrogen and stored at -80 °C, for further use in tissue iron content determination and gene expression. Mortality was assessed during the experimental infections. Colony forming units (CFU) counts in the spleen and head kidney of infected fish were performed. Briefly, spleen and head kidney were aseptically collected, homogenized in tryptic soy broth (TSB) 1% NaCl, serially diluted, plated on tryptic soy agar (TSA) 1% NaCl and incubated at 25ºC for 24-48 hours. No animals were excluded in any of the experiments.

Peptide administration. Fish were intraperitoneally injected with commercially synthesized sea bass hepcidin peptides, either with 100 µl of a 50 µM solution of hamp1 (QSHLSLCRWCCNCCRGNKGCGFCCKF), or hamp2 (HSSPGGCRFCCNCCPNMSGC-GVCCRF) (Bachem AG, Bubendorf, Switzerland), diluted in sterile PBS. Samples were collected after 1, 2, 3, 4, 7, 10, 14, and 21 days post-peptide administration.

Iron overload. To induce iron overload, fish were intraperitoneally injected with 100 µl iron dextran (5 mg) (Sigma-Aldrich, St. Louis MO, USA) diluted in sterile PBS to a final concentration of 50 mg/ml. Samples were collected after 2, 4, 7, 10, 14, and 21 days post-iron administration.

Iron overload and peptide administration. Fish were first injected with iron dextran, followed by administration of either hamp1 or hamp2 peptides 24 hours later, as previously described. Samples were collected after 2, 4, 7, 10, 14, and 21 days post-iron administration.

Infection. Photobacterium damselae spp. *piscicida* strain PP3 was cultured to midlogarithmic growth in tryptic soy broth (TSB) growth medium, supplemented with 1% NaCl. After measuring absorbance at 600 nm, bacteria were washed and resuspended in sterile PBS to a final concentration of 10^6 CFU/ml. Fish were then intraperitoneally injected with 100 µl (10^5 CFU) of bacterial suspension. Samples were collected after 1, 2, 3, 4, 7 and 10 days post-infection.

Infection and peptide administration. Fish were first infected with *P. damselae*, followed by administration of either hamp1 or hamp2 peptides 24 hours later, as previously described. Samples were collected after 1, 2, 3, 4, 7 and 10 days post-infection.

Peptide administration and infection. Fish were first injected with either hamp1 or hamp2 peptides, followed by infection with *P. damselae* 24 hours later, as previously described. Samples were collected after 1, 2, 3, 4, 7 and 10 days post-infection.

Controls. Fish were intraperitoneally injected with 100 µl of sterile PBS.

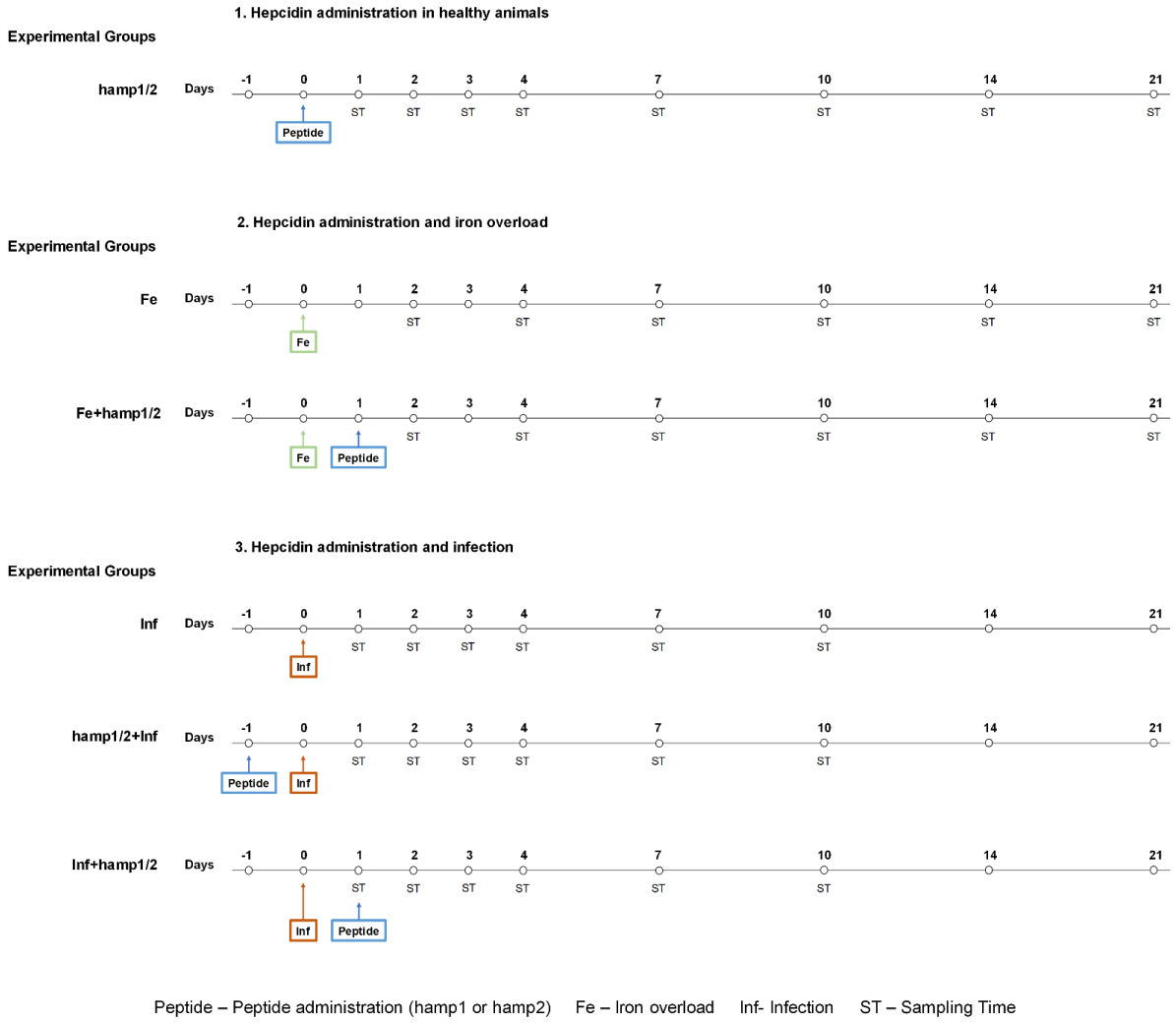

Figure 11. Experimental design. Healthy sea bass were first treated with hamp1 or hamp2. Then, different models were established: Iron overload (5mg of iron dextran/ fish); iron overload followed by hamp1 or hamp2 administration, 24 hours later; Infection (10^5 CFU of *phdp* PP3/ fish); Pre-treatment with hamp1 or hamp2 followed by infection with PP3, 24 hours later; Post-treatment with hamp1 or hamp2, 24 hours after infection with PP3. Samples from fish were collected after 1, 2, 3, 4, 7, 10, 14 and 21 days of experiment.

Hematological parameters and tissue iron content

For determination of hematological parameters, 100 µl of blood were used in a 1:1 dilution with EDTA (1:10 diluted in sterile PBS) (BD Biosciences, San Jose CA, USA). For determination of serum parameters, non-heparinized blood was transferred into 1.5 ml microcentrifuge tubes, allowed to clot for 4 h at 4 ºC, and centrifuged at 16000 × g until a clear serum was obtained. Hematocrit was determined with microcappilaries, red blood

cells were counted with an automated cell counter (Countess Automated Cell Counter, Invitrogen) and manually confirmed, and serum iron was blindly determined by a certified laboratory (CoreLab, Centro Hospitalar do Porto, Portugal). Non-heme iron was measured in livers by the bathophenanthroline method [82]. Briefly, liver samples with an average weight of 100 mg were placed in iron-free Teflon vessels (ACV-Advanced Composite Vessel, CEM Corporation, Matthews NC, USA) and dried in a microwave oven (MDS 2000, CEM Corporation). Subsequently, dry tissue weights were determined and samples digested in an acid mixture (30% hydrochloric acid and 10% trichloroacetic acid) for 20 h at 65 °C. After digestion, a chromogen reagent (5 volumes of deionised water, 5 volumes of saturated sodium acetate and 1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycollic acid) was added to the samples in order to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The extinction coefficient for bathophenanthroline is 22.14 $mM^{-1}cm^{-1}$.

RNA isolation and cDNA synthesis

Total RNA was isolated from tissues with the NZY Total RNA Isolation kit protocol for tissue samples (NZYtech, Lisboa, Portugal) with the optional on-column DNase treatment, according to the manufacturer's instructions. Total RNA quantification was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and quality was assessed by running the samples in an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). For all samples, 2.5 µg of each were converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech) according to the manufacturer's protocol.

Gene expression analysis

Relative levels of *hamp1*, *hamp2*, *fpn1*, *fth* and s*lc11a2alpha* RNA were quantified by real-time PCR analysis using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). A total of 1 µL of each cDNA sample was added to a reaction mix containing 7.5 µL iTaq Universal SYBR Green Supermix (Bio-Rad), 5 µL double distilled H_2O, and 250 nM of each primer (Table 1), making a total volume of 15 µL per reaction. A non-template control was included for each set of primers. The cycling profile was the following: 95 °C for 3.5 min, 40 cycles of 95 °C for 20 s and 59 °C for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reactions. Beta-actin (*actb*) was used as the housekeeping gene (M-value 0.177) (selected as the most stable gene among a suite of 5 candidates using the Delta CT method, Normfinder and Genorm, through RefFinder, http://blooge.cn/RefFinder/) [83]. The comparative CT

method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold values was used to analyze gene expression levels.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software Inc, San Jose CA, USA). Multiple comparisons were performed with One-way ANOVA and *post hoc* Student Newman-Keuls test. A p value <0.05 was considered statistically significant.

Ethics Statement

All animal experiments were carried out in strict compliance with national and international animal use ethics guidelines (including ARRIVE guidelines), approved by the animal welfare and ethic committees of ICBAS (permit P293/2019/ORBEA, 05/04/2019) and conducted by experienced and trained FELASA Function A+B+D investigators.

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Author Contributions

C.B. designed and conducted experiments, analyzed data and wrote the paper; J.V.N. designed, conducted and supervised the experiments, analyzed data and wrote the paper; P.N.S.R. designed and supervised the experiments; P.C., M.N. and J.F.M.G. conducted experiments; J.V.N. had primary responsibility for final content. All authors read and approved the final manuscript.

Table 1. Primers used for gene expression analysis.

		FOR (5' → 3')	REV (5' → 3')
Actin, beta	*actb*	CAGAAGGACAGCTACGT	GTCATCTTCTCCCTGTTGGC
Hepcidin 1	*hamp1*	CATTGCAGTTGCAGTGACACT	CAGCCCTTGTTGCCTCTG
Hepcidin 2	*hamp2*	CTGCTGTCCCAGTCACTGA	ACCACATCCGCTCATATTAGG
Ferroportin	*fpn1*	GGCCTACTACAACCAGAACAT	AGGCCGCACTTCTTGCGAA
Ferritin H	*fth*	AACCATGAGTTCTCAGGTGAG	TTAGCTGCTCTCTTTGCCCAG
Solute Carrier Family 11 Member 2, alpha	*slc11a2alpha*	CGCGTTCAACCTCCTCTCCTCT	AGCCCTCGCAGTACGGCACA

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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Chapter IV

The diverse piscidin repertoire of the European Sea bass (*Dicentrarchus labrax*): molecular characterization and antimicrobial activities

Barroso C, Carvalho P, Carvalho C, Santarém N, Gonçalves JFM, Rodrigues PNS* and Neves JV* (2020). The diverse piscidin repertoire of the European sea bass (*Dicentrarchus labrax*): molecular characterization and antimicrobial activities. *International Journal of Molecular Sciences*, 21 (13), 4613. (*These authors contributed equally to this work)

Abstract

Fish rely on their innate immune responses to cope with the challenging aquatic environment, with antimicrobial peptides (AMPs) being one of the first line of defenses. Piscidins are a group of fish specific AMPs isolated in several species. However, in the European sea bass (*Dicentrarchus labrax*), the piscidin family remains poorly understood. We identified six different piscidins in sea bass, performed an in-depth molecular characterization and evaluated their antimicrobial activities against several bacterial and parasitic pathogens. Sea bass piscidins present variable amino acid sequences and antimicrobial activities, and can be divided in different sub groups: group 1, formed by piscidins 1 and 4; group 2, constituted by piscidins 2 and 5, and group 3, formed by piscidins 6 and 7. Additionally, we demonstrate that piscidins 1 to 5 possess a broad effect on multiple microorganisms, including mammalian parasites, while piscidins 6 and 7 have poor antibacterial and antiparasitic activities. These results raise questions on the functions of these peptides, particularly piscidins 6 and 7. Considering their limited antimicrobial activity, these piscidins might have other functional roles, but further studies are necessary to better understand what roles might those be.

Introduction

Fish are surrounded by a hostile milieu that contains a variety of microorganisms, with many of them pathogenic. Under normal conditions, fish are capable to cope with these potential invaders using a system of non-specific immune responses that confers the initial protection against pathogens [1]. Included in the repertoire of innate defenses are the antimicrobial peptides (AMPs). AMPs are found widespread throughout nature and possess strong antimicrobial and immunomodulatory activities. Due to their unique properties, AMPs have been the focus of a growing body of interest and multiple peptides are currently well characterized in several fish species [2–4].

Some fish species possess a particular group of amphipathic and alpha-helical AMPs. The first peptide belonging to this family was found in the skin secretions of the winter flounder (*Pleuronectes americanus*) and named pleurocidin [5]. Later, an amphipathic alpha-helical peptide was also found in the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) [6]. With the increasing interest in these fish specific AMPs, it is now known that these molecules are present in several teleost species from different families [7–10] and are generally called piscidins. Piscidins constitute a diverse group of AMPs with distinct amino acid sequences. They are produced as a prepropeptide that undergoes

proteolytic cleavage to remove the signal peptide and the prodomain, resulting in a small mature peptide, usually with 18 to 26 amino acids [11–16]. However, there are reports in several species, including the white bass (*Morone chrysops*), striped bass (*Morone saxatilis*) and hybrid striped bass, of piscidins with mature peptides that range from 22 to 55 amino acids [17–19]. This diversity suggests that piscidins are undergoing a positive Darwinian selection and gene duplications [20,21], supporting the variety of peptides and reduced sequence identity among the piscidin family members.

The basal expression of piscidin genes is found to vary within and between fish species. These genes are constitutively expressed in tissues including the gills, skin, intestine, head kidney or spleen [8,9,16,22,23]. Several reports have shown a modulation of piscidin gene expression after infection with different pathogens [10,24,25]. Furthermore, piscidin peptides can be found in mucosal tissues at such concentrations that are lethal for pathogens [26,27]. In vitro, the capacity of these AMPs to kill different fish and mammalian pathogens has been tested, and results demonstrate that piscidins are, in fact, active against them [17,28–32]. Together, these evidences support the role of piscidins as peptides involved directly in the immune response against infection.

In the European sea bass (*Dicentrarchus labrax*, Moronidae), a commercially important species in aquaculture, a detailed study of the piscidin family is missing, although a 22 amino acid piscidin like peptide has been isolated and called dicentracin, and shares a high similarity with the white bass and striped bass moronecidin [33]. Furthermore, peptides identified as piscidins were detected in sea bass, using different techniques, including Western blot, ELISA, or immunohistochemistry, in tissues such as the intestine and gills [27,34]. However, while the characterization of piscidins in other fish species demonstrates the diversity and antimicrobial role of these peptides, in sea bass, the identification of the several piscidin types and a detailed study of their biological roles remains poorly explored.

In this study, we thoroughly characterize the piscidin family in sea bass, at the genomic and protein levels. The basal expression of these genes was evaluated, as well as the antimicrobial activity of piscidin mature peptides against a wide range of pathogens. Similarly to other species from the Moronidae family, sea bass piscidins are divided into different sub-groups, presenting a diverse amino acid sequence and antimicrobial activities [19]. Furthermore, sea bass piscidin mature peptides are generally larger in length (ranging from 44 to 65 amino acids), when compared to the typical piscidins (ranging from 18 to 22 amino acids), showing a degree of conservation in this family of fishes. However, further studies will be required to better understand the specific functions of these peptides, and to bring new insights about the potential of these molecules in pathogen clearance, immune defenses and possibly other functions.

Results

Molecular Characterization of Sea Bass Piscidins

Six different piscidin genes were obtained by PCR amplifications and 5'/3' RACE using liver, intestine, gill and pyloric caeca cDNA. Piscidin genes were deposited on GenBank under accession numbers MT066191 to MT066196. Potential cleavage of sites of signal peptides and prodomains were determined using SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) and alignment of sea bass piscidins with peptides isolated in previous studies [17,19,33]. Molecular weights (Da) were determined, as well as the isoelectric points (pI) and net charges at pH 7. Results are shown in Table 1 and Figure 1.

Table 1. Characterization of sea bass piscidin genes and peptides.

	Piscidin1	**Piscidin2**	**Piscidin4**	**Piscidin5**	**Piscidin6**	**Piscidin7**
Accession number	MT066191	MT066192	MT066193	MT066194	MT066195	MT066196
ORF (bp)[1]	240	213	204	246	279	240
5' UTR (bp)[2]	80	111	81	109	-	-
3' UTR (bp)[2]	134	38	438	97	-	120
Prepropeptide (aa)[3]	79	70	67	81	92	79
Signal peptide (aa)	22	22	22	22	22	22
Mature peptide (aa)	22	44	22	46	65	55
Prodomain (aa)	35	4	23	13	5	2
Molecular weight (Da)	2571	5362	2454	5406	7182	6174
Isoelectric point (pI)	14.00	11.82	14.00	12.03	10.34	10.52
Net charge (at pH 7)	4.4	7.1	4.2	12.2	5.0	6.0

[1] Open reading frame; [2] 5' and 3' untranslated regions; [3] Piscidin full length amino acid (aa) sequence.

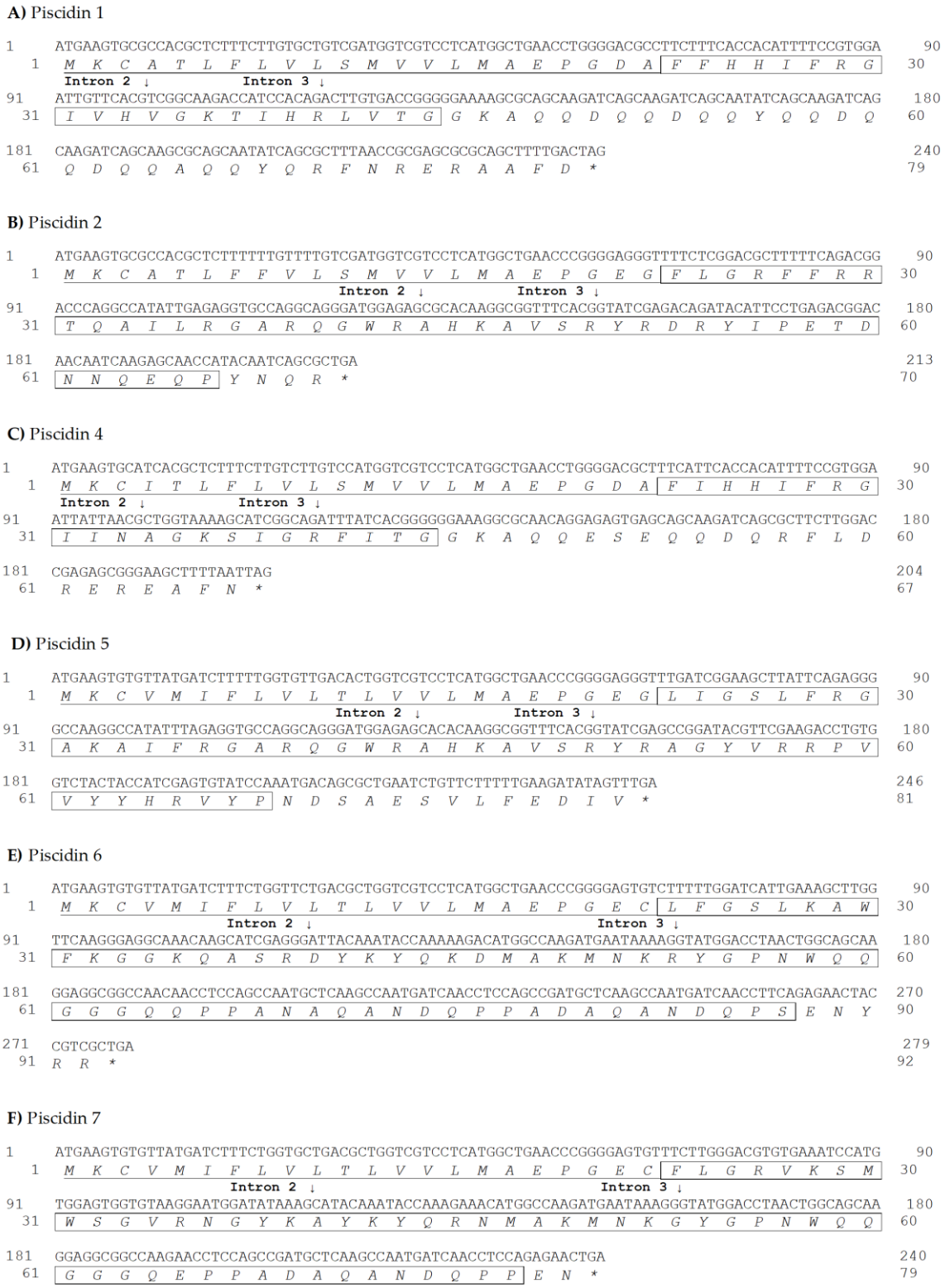


Figure 1. Sea bass piscidins coding DNA and amino acid sequences. Piscidin 1 (**A**), piscidin 2 (**B**), piscidin 4 (**C**), piscidin 5 (**D**), piscidin 6 (**E**) and piscidin 7 (**F**). Nucleotides are indicated in the upper row, and amino acids are indicated in italic in the lower row.

Signal peptides are underlined and mature peptides are boxed. Intron positions are indicated by arrows.

Alignment of sea bass piscidins shows a low degree of identity of the mature peptides and prodomains, with different sizes and amino acid compositions, and a highly similar signal peptide (Figure 2). Identity scores between the different sea bass piscidins are shown in Table 2. Piscidin 1 shares a high identity with piscidin 4 (67.3%), piscidin 2 shares the highest percentage of identity with piscidin 5 (58.2%) and piscidins 6 and 7 share an identity of 64.3%.

```
                                 |
Piscidin 1   MKCATLFLVLSMVVLMAEPGDAFF-------HHIFRGIVHVGK------TIHRLVTGGKAQQDQQDQQYQQDQQDQQAQQYQRFNRERAAFD------ 79
Piscidin 2   MKCATLFFVLSMVVLMAEPGEGFLGRFFRRTQAILRGARQGWRAHK---AVSRYRDRYIPE----------TDN-NQEQPYN--QR------------ 70
Piscidin 4   MKCITLFLVLSMVVLMAEPGDAFI-------HHIFRGIINAGK------SIGRFITGGKAQ----------QESEQQDQRFL--DREREAFN------ 67
Piscidin 5   MKCVMIFLVLTLVVLMAEPGEGLIGSLFRGAKAIFRGARQGWRAHK---AVSRYRAGYVRRPVVYYHRVYPNDS-AESVLFE--DIV----------- 81
Piscidin 6   MKCVMIFLVLTLVVLMAEPGECLFGSL----KAWFKGGKQASRDYKYQKDMAKMNKRYGPNWQQGGGQQPPANAQANDQPPA--DAQANDQPSENYRR 92
Piscidin 7   MKCVMIFLVLTLVVLMAEPGECFLGRV----KSMWSGVRNGYKAYKYQRNMAKMNKGYGPNWQQGGGQEPPADAQANDQPPE--N------------ 79
Consensus    ***  :*:**::********: ::           *  :  :       : :       .
```

Figure 2. Alignment of sea bass piscidins. Signal peptides are shaded gray and predicted cleavage sites are underlined. Identical residues are denoted by (*), conserved substitutions by (:) and semi-conserved substitutions by (.).

Table 2. Identity scores of full length sea bass piscidin amino acid sequences.

	Piscidin1	**Piscidin2**	**Piscidin4**	**Piscidin5**	**Piscidin6**	**Piscidin7**
Piscidin1		37.8%	67.3%	30.6%	25.5%	33.7%
Piscidin2	37.8%		48.0%	58.2%	28.6%	43.9%
Piscidin4	67.3%	48.0%		36.7%	27.6%	34.7%
Piscidin5	30.6%	58.2%	36.7%		38.8%	48.0%
Piscidin6	25.5%	28.6%	27.6%	38.8%		64.3%
Piscidin7	33.7%	43.9%	34.7%	48.0%	64.3%	

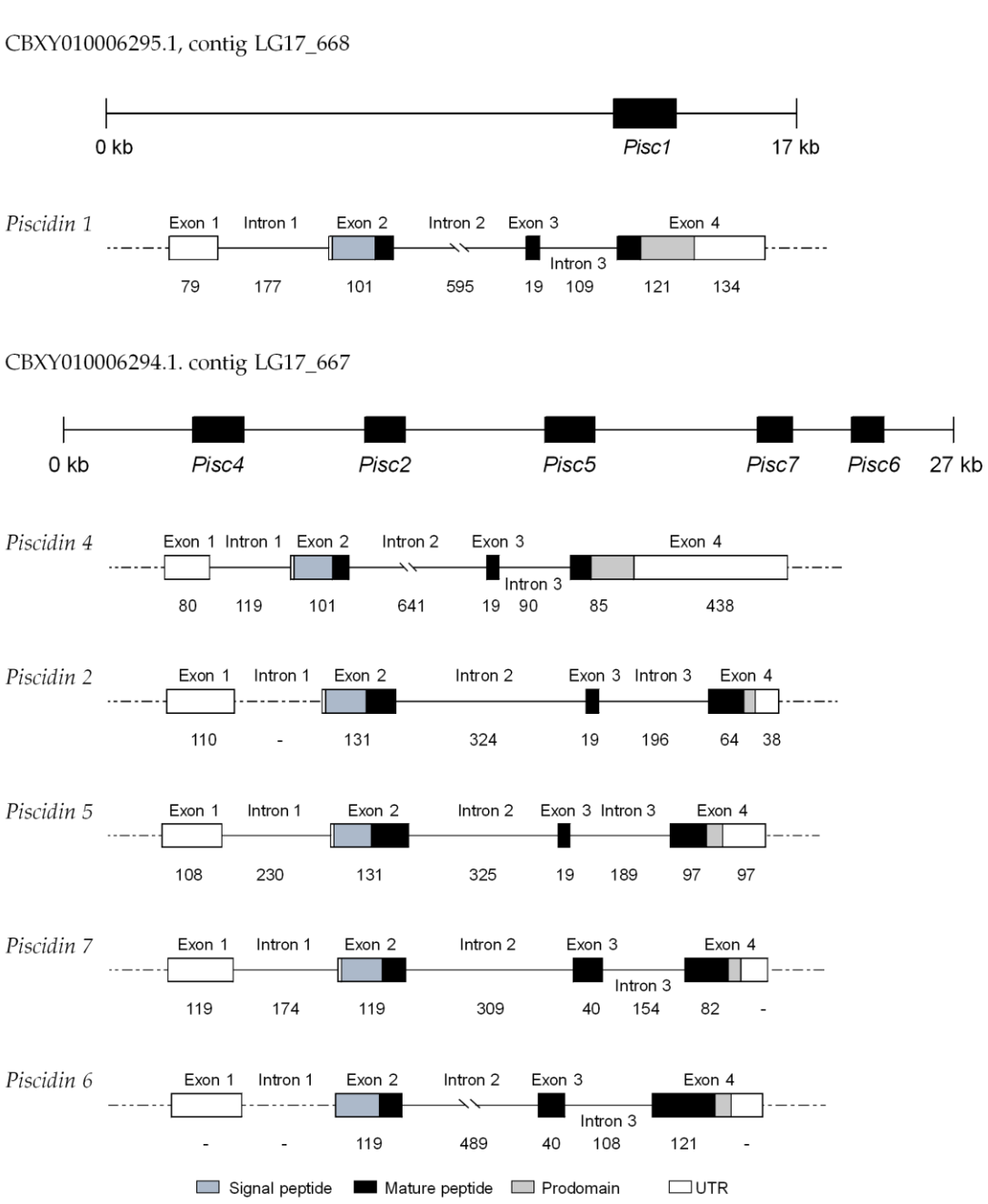
Identity scores were determined using the SIAS software, with BLOSUM62 scoring matrix and considering the length of multiple sequence alignment.

Genomic Organization

All piscidin genes were found to have a four exon/three intron structure (Figure 3A). The first exon is formed by a 5' UTR that extends until the first nucleotide of exon 2. The signal peptides are encoded by exon 2 and the mature peptides are encoded by exons 2, 3 and 4. The prodomains are encoded by exon 4, followed by the 3' UTR. All exon–intron boundaries follow the classical splicing motifs (GT/intron/AG). Piscidin genes show variable-sized exons, mostly the exon 4. Piscidins 1 and 4 share the same-sized exon 2 and 3 (101 and 19 bp), as well as piscidins 2 and 5 (131 and 19 bp). Piscidins 6 and 7 also show second and third exons with similar sizes (119 and 40 bp) however, the third exon is bigger when compared to the other piscidins. Comparisons with contigs available in gene databases have also shown that these genes likely share the same genetic locus, as piscidins 2, 4, 5, 6 and 7 were all found in the same contig, (CBXY010006294), with piscidin 1 in a separate contig (CBXY010006295). However, it is also likely that piscidin 1 is located upstream of piscidin 4, in total forming three clusters of 2 piscidins each, sharing higher degrees of similarity among them (Piscidin1/Piscidin4, Piscidin2/Piscidin5, and Piscidin6/Piscidin7), a hypothesis further reinforced by sequence comparisons, phylogenetic analysis and molecular modeling.

Comparison between sea bass piscidin genes and those of other fish species shows a high similarity in terms of gene structure, with the usual organization of four exons/three introns of variable sizes (Figure 3B). However, variations from the common organization occur, particularly in Nile tilapia (*Oreochromis niloticus*) and orange spotted-grouper (*Epinephelus coioides*), that show a genomic structure comprised by three exons/two introns and five exons/four introns, respectively. Similarities in the third exon are observed between species, being the smaller exon found in piscidin genes, with sizes that range from 19 to 49 bp. The second intron is the largest among the different fish species, with variable sizes (126 to 1654 bp), with the exception of the Atlantic cod (*Gadus morhua*) gaduscidins.

A)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Pisc1
17 kb
Piscidin 1
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
Intron 3
Exon 4
79
177
101
595
19
109
121
134
CBXY010006294.1. contig LG17_667
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Pisc2
Pisc5
Pisc7
Pisc6
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19
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85
438
Piscidin 2
110
-
131
324
19
196
64
38
Piscidin 5
108
230
131
325
19
189
97
97
Piscidin 7
119
174
119
309
40
154
82
-
Piscidin 6
-
-
119
489
40
108
121
-
Signal peptide
Mature peptide
Prodomain
UTR

B)

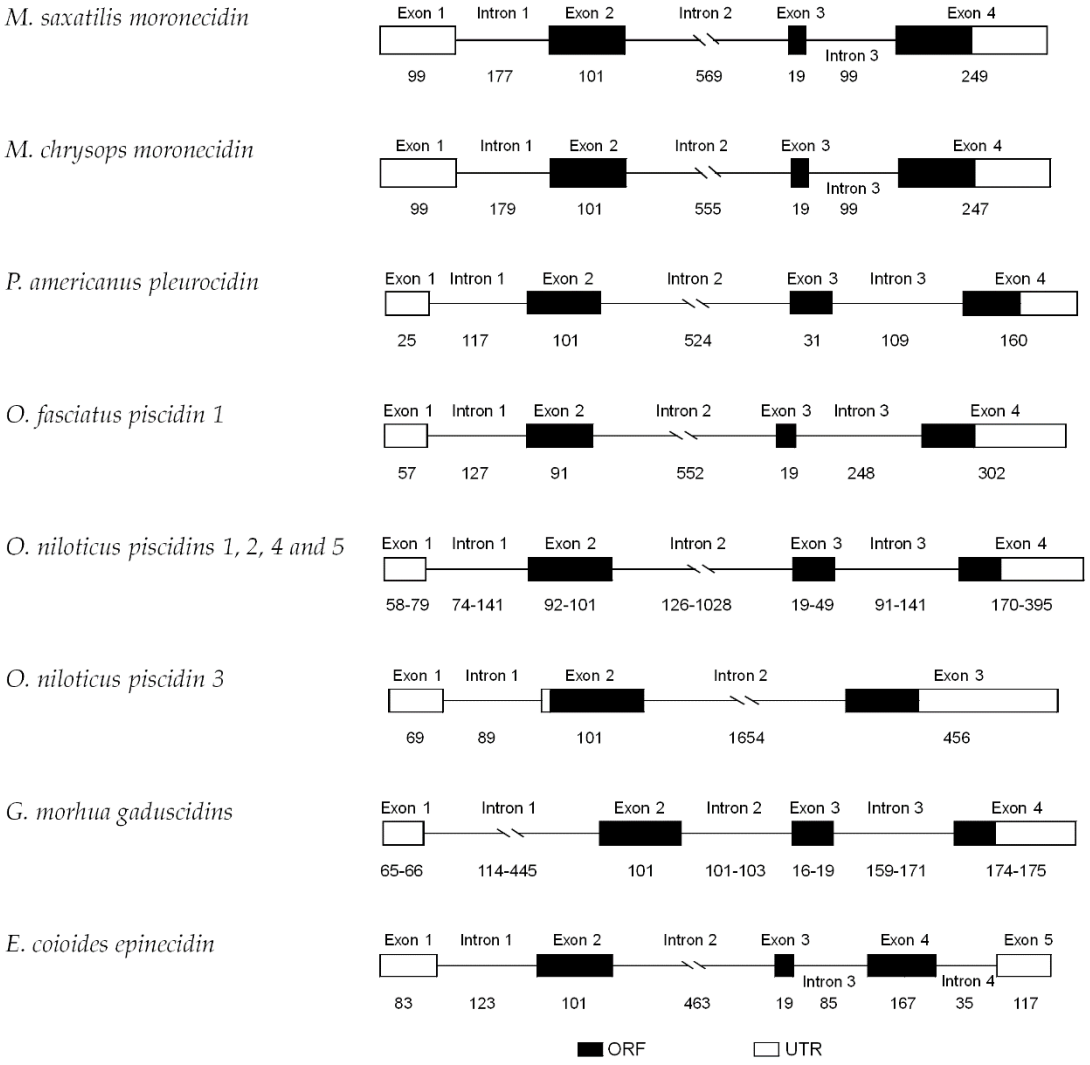

Figure 3. Genomic organization of sea bass piscidin genes. (**A**) Exon/intron diagram of sea bass piscidin genes and their position in the two different contigs of whole genome shotgun sequences. (**B**) Comparative view with other fish species. Exons are shown as boxes and introns as solid lines, with sizes in base pairs indicated below. Regions with unknown sizes are shown as dashed lines.

Sequence Comparison and Phylogenetic Analysis

Comparison between sea bass piscidins and peptides from other fish species shows a low degree of similarity in the mature peptides and prodomains, with the exception of piscidins that belong to other species from the Moronidae family, namely striped bass, white bass and hybrid striped bass (Figure 4). Piscidin 1 shares 97.3% of homology with dicentracin and identities between 41.6% and 97.3% with other piscidins; piscidin 2 between 41.6% and 82.3%; piscidin 4 between 45.1% and 92.9%; piscidin 5 between 36.3% and 86.7%; piscidin 6 between 23.9% and 78.8% and piscidin 7 between 37.2% and 89.4% (Table S1).

Phylogenetic analysis clusters piscidins and cecropin separated from sea bass hepcidins, another family of antimicrobial peptides (Figure 5). Among piscidins, two big clusters are evident. The first one includes a more diverse set of piscidins and piscidin-like peptides, such as moronecidin, pleurocidin, gaduscidin, epinecidin and cecropin, as well as sea bass piscidins 1 and 4. The second cluster is solely comprised of piscidins, and can be further sub-divided in two groups, separating piscidins 2 and 5 from piscidins 6 and 7. As such, we can consider that sea bass piscidins are divided into three sub-groups: piscidins 1 and 4 clusters with sea bass dicentracin, moronecidins and piscidins 3 from white (*M. saxatilis*) and striped (*M. chrysops*) basses; piscidins 2 and 5 are positioned in the same cluster with white (*M. saxatilis*), striped (*M. chrysops*), and hybrid striped (*M. saxatilis* x *M. chrysops*) basses piscidins 4 and 5; and finally, piscidins 6 and 7 are included in the same group of white (*M. saxatilis*) and striped (*M. chrysops*) basses piscidins 6 and 7. Among the two big clusters, analysis separate piscidins belonging to Moronidae species from the Atlantic cod (*G. morhua*) gaduscidins, pleurocidins from winter flounder (*P. americanus*) and American plaice (*Hippoglossoides platessoides*), cecropin A from cecropia moth (*Hyalophora cecropia*) and tilapia piscidins (*O. niloticus*).

```
D.labrax Piscidin1          MKCATLFL----VLSMVVLMAEPGDAF-FHH----------IFRGIVHVGKTIH-RLVTGGKAQDQDQQY----------Q-QDQQDQQAQQYQRFNRERAAFD----------
D.labrax Piscidin2          MKCATLFF----VLSMVVLMAEPGEGF-LGR--FFRRTQAILRG-ARQGWRAH-KAVSRYRDRYI----------------PETDNNQEQFYNQR----------------
D.labrax Piscidin4          MKCITLFL----VLSMVVLMAEPGDAF-IHH----------IFRGIINAGKSIG-RFITGGKAQQE----------------SEQQDQRFLDRERAFN----------
D.labrax Piscidin5          MKCVMIFL----VLTLVVLMAEPGEGL-IGS---LFRGAKAIFRG-ARQGWRAH-KAVSRYRAGYVRRPVVY----------------YHRVYPNDSAESVLFEDIV----------
D.labrax Piscidin6          MKCVMIFL----VLTLVVLMAEPGECL-FGS----------LKAWFKG-GKQASRDY-KYQKDMAKMNKRYGPNW----------QQGGGQQPPANAQANDQPPADAQANDQPSENYRR
D.labrax Piscidin7          MKCVMIFL----VLTLVVLMAEPGECF-LGR----------VKSMWSG-VRNGYKAY-KYQRNMAKMNKGYGPNW----------QQGGGQEPPADAQANDQPPEN----------
D.labrax Dicentracin        MKCATLFL----VLSMVVLMAEPGDAF-FHH----------IFRGIVHVGKSIH-KLVTGGKAQDQDQQDQQY----------Q-QDQQDQQAQEQYQRFNRERAAFD----------
M.chrysops Moronecidin      MKCATLSL----VLSMVVLMAEPGDAF-FHH----------IFRGIVHVGKTIH-KLVTGGKAEQDQDQQDQQY----------Q-QDQQDQQAQQYQRFNRERAAFD----------
M.chrysops Piscidin3        MRCITLFL----VLSMVVLMAEPGDAF-IHH----------IFRGIVHAGRSIG-RFLTGGKAQQE----------------REQQDQRFMDRERDAFN----------
M.chrysops Piscidin4        MKCVMIFL----VLTLVVLMAEPGEGF-FRH---LFRGAKVLFQG-ARQGWRAH-KAVSRYRNRYV----------------PETDNNQEQFYNQR----------------
M.chrysops Piscidin5        MKCVMIFL----VLTLVVLMAEPGEGL-IGS---LFRGAKAIFRG-ARQGWRSH-KAVSRYRARYVRRPVIY----------------YHRVYPNEER----------------
M.chrysops Piscidin6        MKCVMIFL----VLTLVVLMAEPGECL-FGS----------VKAWFKG-AKKGFQDY-RYQKDMAKMNKRYGPNW----------QQRGGQEPPADAQANDQPPENYRR----------
M.saxatilis Moronecidin     MKCATLFL----VLSMVVLMAEPGDAF-FHH----------IFRGIVHVGKTIH-RLVTGGKAEQDQDQQDQQY----------Q-QEQQEQQAQQYQRFNRERAAFD----------
M.saxatilis Piscidin3       MRCITLFL----VLSMVVLMAEPGDAF-IHH----------IFRGIVHAGRSIG-RFLTGGKAQQE----------------REQQDQRFMDRERDAFN----------
M.saxatilis Piscidin4       MKCVMIFL----VLTLVVLMAEPGEGF-FRH---LFRGAKAIFRG-ARQGWRAH-KVVSRYRNRDV----------------PETDNNQEEPYNQR----------------
M.saxatilis Piscidin6       MKCVMIFL----VLTLVVLMAEPGECL-FGS----------VKAWFKG-GKKGFQDY-RYQKDMAKMNKRYGPNW----------QQRGGQEPPADAQANDQPPENYRR----------
M.saxatilis Piscidin7       MKCVMIFL----VLTLVVLMAEPGECF-FGR----------LKSMWRG-ARGGLKAY-RYQKDMAKMNKRYGPNW----------QQGGGQEPPADAQANDQPPENYRR----------
M.chrysops x M.saxatilis Piscidin4
                            MKCVMIFL----VLTLVVLMAEPGEGF-FRH---LFRGAKAIFRG-ARQGWRAH-KVVSRYRNRDV----------------PETDNNQEEPYNQR----------------
M.chrysops x M.saxatilis Piscidin5
                            MKCTALFL----VLSLVVLMAEPGDAI-FHH----------IFKGIVHVGKTIH-RLVTGGQNMKD----------------QQKLEQRSFDQERAAFD----------
O.niloticus Piscidin1       MKSAVIFL----ALFMVFMAAEPGECFDWDS----------VLKG---------VE-GFVRGYFGKEKAKELVK----------SLKADFQNYKHLRQREFD----------
O.niloticus Piscidin2       MKCAAVFL----MLSMVILMAEPGECI-WDA----------IFHGAKHF---LH-RLVNGPFFLRQFLQVVF----------VLVVFLVELDKRAISYHPRRLNFD----------
O.niloticus Piscidin3       MKCTMLFL----VLSMVVLMAEPGEAF-IHH----------IIGGLFSVGKHIH-SLIHGHGNVKQQQQQ----------QEQLNQRSFNREQFKRERAAFN----------
O.niloticus Piscidin4       MKCTILFL----VLSMVVLMAEPGEGF-IHH----------IIGGLFSAGKAIH-RLIRRRRRGELQLEQELQQQLEQLEKLQ-QQEKLNQRFNREQLKRERVAFN----------
O.niloticus Piscidin5       MKSAIIFL----VFTLVLFMADPAELQ----------LQGKQVSGEVVQ-KVLQELIQSVAKPGP----------------KDVNFNEFEDAVMS----------
G.morhua Gaduscidin1        MRYIVLLV----VVLLLAMMVQPADCF-IHH----------IIGWISH-GVRAIHRAIH----------------GEKAEEYIMVD----------
G.morhua Gaduscidin2        MRCIFLLF----VVLLLAMMVLPAEGF-LHH----------IVGLIHHGLSLF-G----------------DRADKAEEYIAVD----------
E.coioides Epinecidin1      MRCIALFL----VLSLVVLMAEPGEGF-IFH----------IIKGLFHAGKMIH-GLVTRRRHGVEE----------LQDLDQRAFEREKAFA----------
E.coioides Piscidin2        MRCIALFL----VLSLVVLMAEPGEGF-FFH----------IVKGLFHAGRMIH-GLVNRRRHRHGMEELDL----------DQRAFEREKAFA----------
E.coioides Piscidin3        MKCTVVFL----VLSMVVFMAEPGECI-FGL----------LLHGAIHVGKLIH-GLVRRHGEEQLDD----------------LEQLDKRALDYNPG---RPGFD----------
E.coioides Piscidin4        MKFVMVFL----VLSLVVLMAEPGEGF-FRHIKSFWKGAKAIFRG-ARQGWREH-RALSKQRKMDQGGG----------------GNEVDNGTPPYWQK----------
P.americanus Pleurocidin    MKFTAIFL----MMAIFVLMVEPGECG-WGS----------FFKKAAHVGKHVG-KAALTHYLGD----------------KQELNKRAVDEDPNVIVFE----------
L.crocea Piscidin-like      MKCTAVFL----VLFMVVLMAEPGECI-WGL----------IAHGVGHVGRLIH-GLIRGHGAEEQ----------------HVQLDKRSLSYDPPKKLQWRED----------
L.crocea Piscidin5-like     MKGVMIFL----VLTLVVLMA--GECI-GGD----------NYGTFDNSNGNNFQHGSNSNNQNNGGYPWYGARW----------QRSLPGSEDDGSK----------
A.regius Piscidin           MKFTAVFL----VLFMVVLMAEPGECI-WGL----------IAHGVAHVGSMIH-GLIHNHGAEEQ----------------QEQLDKRSLSYDPPKKLQWRD----------
O.fasciatus Piscidin1       MKCIVIFL----VLSMVVLMAEPGEGF-LGM----------LLHG---VGHAIH-GLIHGKQNVEEQQQQ----------QEQLDKRSVDYNPGQPNLD----------
O.fasciatus Piscidin6       MKCVMIFL----VLTLVVLMAEPGECF-FQE----------LKEGWKN-IKKAWKEE-AFQKDLKKMAKKYGPDW----------L-QKIGVTTTPP----------------
O.fasciatus Piscidin7       MKYVMIFL----VLTLVVLMAEPGECF-FGR----------LKAVFRG-ARQGWKEH-RYHRDMRKMAERYGPNW----------QQKLGGNANFQPEENAEDAPAYQR----------
H.platessoides Pleurocidin-like AP1
                            MKFTATFL----MLFIFVLMVEPGECG-WKS----------VFRKAKKVGKTVG-GLALDHYLGE----------------QQELDKRAVDEDPSAIVFD----------
H.cecropia Cecropin A       MNFSRIFFFVFACLTALAMVNAAAPEPK-WK----------LFKKIEKVGQNIRDGIIKAGPAVAV----------------VGQATQIAKG----------
Consensus                   *.    .   . .:: :
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Figure 4. Alignment of sea bass piscidins with peptides from other fish species and with cecropin A from cecropia moth. Identical residues are denoted by (*), conserved substitutions by (:) and semi-conserved substitutions by (.).

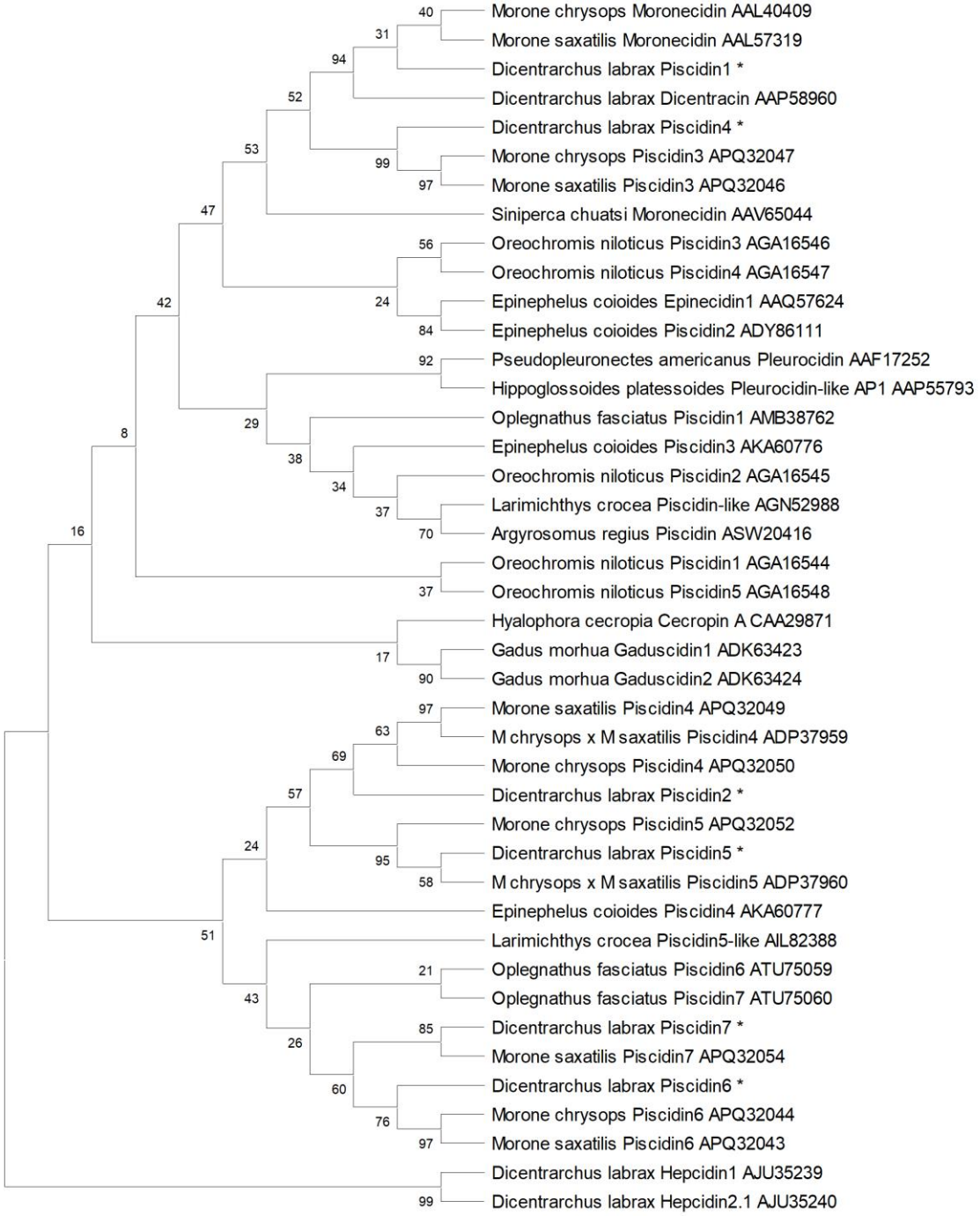

Figure 5. Phylogenetic analysis of piscidin peptides. The evolutionary history was inferred by using the Maximum Likelihood method and Jones–Taylor–Thornton (JTT) matrix-based model [35]. The bootstrap consensus tree inferred from 10000 replicates is taken to represent the evolutionary history of the taxa analyzed [36]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches [36]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a

matrix of pairwise distances estimated using a JTT model. This analysis involved 42 amino acid sequences. All positions with less than 95% site coverage were eliminated. There were a total of 56 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [37].

Basal Expression of Sea Bass Piscidins

Constitutive expression of piscidin genes was evaluated in different tissues of healthy sea bass, namely liver, spleen, head kidney, intestine, pyloric caeca, gill, heart and brain (Figure 6). The highest expression of most piscidin genes is observed in the intestine and pyloric caeca, with a moderate expression in the gill, heart and brain, and a low expression in the liver, spleen and head kidney. Piscidin 2, however, has a different pattern of expression, with highest expression in the spleen, gill and heart, moderate expression in the liver, head kidney, pyloric caeca and brain, and lowest expression in the intestine.

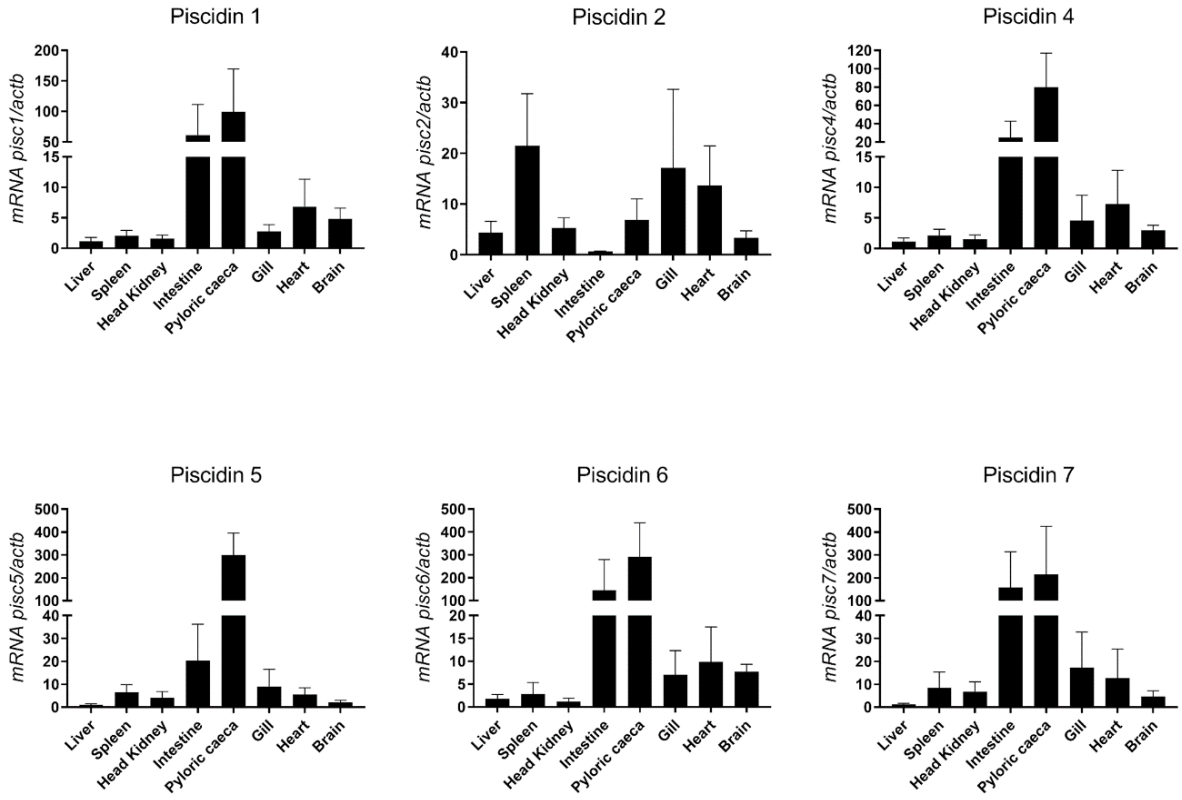

Figure 6. Basal expression of piscidin genes in different organs of healthy sea bass, measured by real-time PCR. Each sample was normalized to beta actin (*actb*) calculated by the comparative CT method ($2^{-\Delta\Delta CT}$). Values are presented as means ± standard deviation (S.D.) (n = 5).

Modeling of Sea Bass Piscidins

Schiffer–Edmundson helical wheel modeling was used to predict the hydrophobic and hydrophilic regions in the secondary structure of sea bass piscidin mature peptides (Figure 7). All piscidins are predicted to form an amphipathic alpha-helix, with piscidin 1 and piscidin 4 showing a clear hydrophobic slant to one side, while piscidins 2, 5, 6 and 7 present most of the hydrophobic residues along one side of the helix and the hydrophilic residues along the other. Piscidin 6 and piscidin 7 likely have a more limited amphipathicity, since some hydrophilic residues might interfere with the hydrophobic side.

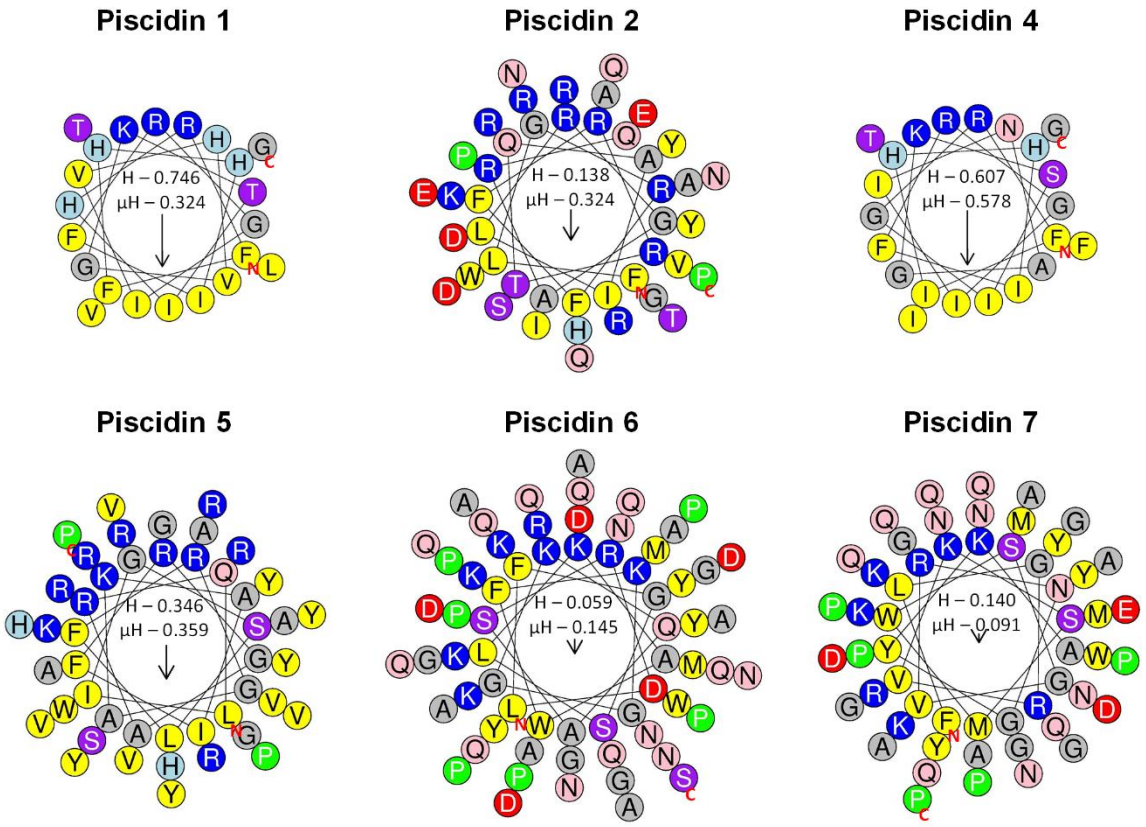

Figure 7. Schiffer–Edmundson helical wheel diagrams of sea bass piscidins. Piscidin 1, piscidin 2, piscidin 4, piscidin 5, piscidin 6 and piscidin 7. Positively charged residues are represented in blue circles, the negatively charged in red, the hydrophobic in yellow, the hydrophilic in purple, the amide in pink and the small residues in grey. Arrows indicate the direction of the hydrophobic moments. The red N and C represent the N-terminal and C-terminal of the peptide sequence. H represents peptide hydrophobicity, and μH represents the hydrophobic moment, which is a quantitative measure of amphipathicity.

Antibacterial Activity of Sea Bass Piscidins

The antibacterial activity of piscidin mature peptides was evaluated using different Gram-negative and Gram-positive bacteria, known to cause severe diseases in aquaculture (Table 3). Piscidins 1 and 5 are highly active against almost all the bacteria tested and show the lowest MIC values that range from 2.4 ± 0.8 to 64.2 ± 2.1 µM and 1.3 ± 1.2 to 47.9 ± 6.1 µM, respectively. Piscidins 2 and 4 show a more moderate degree of activity. Piscidin 2 is capable of inhibiting the growth of many bacterial strains, but MIC values are higher than the ones observed for piscidins 1 and 5 (6.9 ± 2.8 to 146.1 ± 21.4 µM). Piscidin 4 is active only against *Photobacterium damselae* subsp. *piscicida*, *P. damselae* subsp. *damselae*, *Lactococcus garviae*, and *Streptococcus parauberis*, with values that range from 1.8 ± 1.8 to 81.3 ± 9.5 µM. On the contrary, piscidins 6 and 7 show little or no antibacterial activity against these bacteria. Nevertheless, piscidin 6 is capable of inhibiting *S. parauberis* growth (87.7 ± 123.0 µM). None of the peptides had an effect on *Aeromonas hydrophila* and *Edwardsiella tarda* growth, even at the highest concentration levels tested.

Anti-parasitic Activity of Sea Bass Piscidins

The anti-parasitic activity of sea bass piscidin mature peptides was determined using two different parasites, namely the bloodstream forms of *Trypanosoma brucei brucei* and *Leishmania infantum* promastigotes (Table 4). *T. brucei* is more susceptible to the action of the peptides, when compared to *L. infantum*. Piscidins 1, 2, 4, 5, and 7 show high activity against *T. brucei*, with low inhibitory concentration values, that range from 1.74 ± 0.13 to 7.30 ± 0.32 µM, with piscidin 2 being the most active; on the contrary, piscidin 6 is less active, presenting a IC_{50} of 56.50 ± 1.61 µM. *L. infantum* growth is also inhibited by the different piscidins, with the exception of piscidin 6. Inhibitory concentrations determined range from 5.20 ± 0.03 to 66.77 ± 0.08 µM, with piscidin 1 being the most active and piscidin 7 the less active.

Table 3. Antibacterial activity of sea bass synthetic piscidins.

Bacteria	**MIC (μM)**					
Gram-negative	**Piscidin1**	**Piscidin2**	**Piscidin4**	**Piscidin5**	**Piscidin6**	**Piscidin7**
P. damselae subsp. *piscicida*	7.4 ± 1.6	9.1 ± 1.7	29.1 ± 12.0	3.3 ± 0.1	N.A.	N.A.
P. damselae subsp. *damselae*	15.0 ± 0.2	105.8 ± 22.1	81.3 ± 9.5	14.8 ± 2.0	N.A.	N.A.
V. anguillarum	16.3 ± 0.6	146.1 ± 21.4	N.A.	10.1 ± 2.7	N.A.	N.A.
V. alginolyticus	34.8 ± 0.3	66.2 ± 2.0	N.A.	36.9 ± 2.3	N.A.	N.A.
A. salmonicida subsp. *salmonicida*	64.2 ± 2.1	67.7 ± 0.2	N.A.	47.9 ± 6.1	N.A.	N.A.
A. hydrophila subsp. *hydrophila*	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Y. ruckeri	48.4 ± 3.8	137.6 ± 3.1	N.A.	25.4 ± 1.8	N.A.	N.A.
E. tarda	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Gram-positive						
L. garviae	33.9 ± 3.8	N.A.	48.8 ± 40.1	42.3 ± 16.4	N.A.	N.A.
S. parauberis	2.4 ± 0.8	6.9 ± 2.8	1.8 ± 1.8	1.3 ± 1.2	87.7 ± 123.0	N.A.

Growth inhibition of *P. damselae* spp. *piscicida*, *P. damselae* spp. *damselae*, *V. anguillarum*, *V. alginolyticus*, *A. salmonicida*, *A. hydrophila*, *Y. ruckeri*, *E. tarda*, *L. garviae* and *S. parauberis* incubated for 24h with serial dilutions of the synthetic piscidin putative mature peptides (up to 200 μM). The MIC was determined as the lowest concentration of piscidin that reduced 50% of bacterial growth when compared to the controls. Results are representative of two separate experiments and shown as means ± SD. N.A. indicates that piscidin was not active against the bacteria tested at the highest concentration tested.

Table 4. Antiparasitic activity of sea bass synthetic piscidins.

	IC_{50} (µM)					
	Piscidin1	**Piscidin2**	**Piscidin4**	**Piscidin5**	**Piscidin6**	**Piscidin7**
T. brucei brucei	3.88 ± 0.11	1.74 ± 0.13	3.24 ± 0.16	4.42 ± 0.12	56.50 ± 1.61	7.30 ± 0.32
L. infantum	5.20 ± 0.03	5.52 ± 0.26	8.34 ± 0.81	7.24 ± 0.61	N.A.	66.77 ± 0.08

T. brucei brucei and *L. infantum* were incubated for 72h with serial dilutions of the synthetic piscidin putative mature peptides (up to 100 µM). The IC_{50} was determined as the lowest concentration of piscidin that inhibited 50% of parasite growth when compared to the controls. Results are representative of two separate experiments and shown as means ± SD. N.A. indicates that piscidin was not active against the parasites tested at the highest concentration tested.

Discussion

Piscidins, a fish specific antimicrobial peptide family, has been widely studied in various fish species, with proved involvement in different roles, including a direct pathogen killing action and an immunomodulatory activity. In sea bass, only a single member of this family, dicentracin, was characterized in terms of sequence and tissue expression [33]. Later, studies regarding the modulation of dicentracin expression after different stimuli became available, demonstrating that this antimicrobial peptide is triggered under harmful conditions, being an indicator of fish health [38,39]. Although previous reports already demonstrated that there are different piscidins in sea bass [27,33,34,40], the studies on the characterization and gene expression of piscidins in this fish species were focused on a single AMP, with no previous reports that describe in detail the diversity of the piscidin family in sea bass. In this work, we were able to identify six different piscidins in sea bass and expanded the piscidin coding and amino acid sequences characterized in this fish species. After comparing piscidin 1 with dicentracin, differences were observed in two amino acids of the mature peptides (Thr37/Ser37; Arg40/Lys40) and in one residue of the prodomains (Gln66/Glu66). In fact, multiple isoforms of piscidin 1 were isolated in sea bass, with differences in these particular residues (results not shown). Piscidin 1 and dicentracin share a high percentage of identity with white bass and striped bass moronecidins. Lauth et al. studied these two isoforms in the hybrid striped bass and showed the high similarities between both peptides [41]. Likewise, replacements in piscidin 1 and dicentracin mature peptides were with amino acids with similar properties, suggesting that these isoforms maintain its conformation and function [33]. All sea bass piscidins possess a similar peptide structure, formed by a signal peptide, a mature peptide and prodomain. However, while the signal peptide maintains a high degree of similarity between them, the prodomains and the active mature peptides are strikingly different. Molecular characterization, sequence alignment and phylogenetic analysis show a relationship between the genera *Dicentrarchus* and *Morone* [6,17–19], with respect to the number and amino acid compositions of piscine AMPs.

Piscidin family members diverge in their number and sequences. The general genomic features of these genes include a first exon constituted by a 5'UTR, a translation initiation site positioned at exon 2 and a small sized exon 3 [9,11,14,15,42]. Previous reports showed the existence of genome clusters composed by different piscidin genes. These genes are formed by the usual four exon/three intron structure but encode different putative antimicrobial peptides, with the exception of Nile tilapia (*O. niloticus*) and grouper (*E. coioides*), that present piscidin genes with three exons/two introns and five exons/four introns, respectively [7,9,43]. During the evolutionary process, teleost fish have suffered

whole genome and/or segmental duplications, leading to multiple copies of several gene families and to an enormous diversification and adaptation of species found among the Teleostean [44]. Furthermore, several genes in fish, including the ones that encodes for AMPs, are evolving rapidly and events of positive selection were already demonstrated, with a high rate of amino acid substitutions, particularly in the mature peptides [20,21,45,46]. Piscidins are no exception; in sea bass, these AMPs possess several differences, but the general structure of piscidins genes is similar among them, being found tandemly in the sea bass genome. It is possible that piscidin genes diverged from a common ancestral and, during evolution, duplications and different degrees of mutations led to the existence of multiple piscidin orthologues in sea bass.

Comparisons between sea bass and other fish species piscidins show a conserved signal peptide in terms of amino acid composition and cleavage site, with the mature peptides and prodomains sharing reduced similarities. Sea bass piscidins were also found to be formed by a high number of positive and hydrophobic residues, supporting the amphipathic nature of these AMPs. Sea bass piscidins can be divided into different sub-groups: group 1, formed by piscidins 1 and 4, both with a mature peptide of 22-aa; group 2, formed by piscidins 2 and 5, with 44- and 46-aa, respectively; and group 3, constituted by piscidins 6 and 7, with the biggest mature peptides in length, 65- and 55-aa, respectively. Salger et al. classified piscidins from white bass, striped bass and hybrid striped bass in a similar fashion: the Class I piscidins are constituted by the smaller piscidins (22-aa mature peptide), with a broad activity against different bacteria and ciliated protozoans; the Class II piscidins are formed by 44- and 46-aa mature peptides, also with varied antimicrobial activities against bacteria and parasites; and finally, the Class III piscidins are constituted by the biggest piscidins (55-aa mature peptides), showing mostly anti-protozoal activity and a reduced antibacterial activity [19]. Considering the similarities between these species, a division into different groups can also be established for sea bass peptides. The genomic structure of piscidin genes may also support the proposed division of this family, since genes from each group share the same sized exons 2 and 3.

The overall basal expression of piscidins in sea bass resembles what was described for other teleost species. Piscidin genes and peptides are usually detected not only in sites with potential for pathogen entry, but also in immune related tissues of fish, including the intestine, spleen, head kidney, gills and skin. They can also be detected more broadly in organs such as the liver, heart or brain, depending on the piscidin and fish species [8,16,25,27,42]. Sea bass piscidin genes were detected in the organs above mentioned, being the gut the tissue with the highest basal expression levels. Assuming the antimicrobial role of piscidins, it is expected to find such peptides in mucosal tissues, such

as the gastrointestinal tract and gills. Piscidin 2, homologous of white bass and striped bass piscidin 4, presents a unique pattern of expression, with the spleen and gills assuming the more abundant expression. Salger et al. also observed different levels of expression of piscidins 4 and 5 in the hybrid striped bass, being the first more abundant in the gill and the second in the intestine, showing a tissue-specific profile for these two piscidins [18,19]. Dicentracin was detected in circulating, peritoneal and head kidney leukocytes [33]. Considering the overall low constitutive expression of piscidin 1 observed in the head kidney, a hematopoietic organ in fish, it seems that this is not the main tissue presenting piscidin 1 expressing cells. Nevertheless, some kidney resident cells may express this particular piscidin, and thus it being detected by *in situ* hybridization techniques [33].

The amphipathic alpha-helical secondary structure presented by several AMPs is crucial for peptide activity. Some reports already explored piscidin conformation and activity, as well as their mode of action on pathogen membranes, using artificial membranes that mimic the natural ones and can elucidate the mechanisms underlying the effects observed on pathogenic cells [47–49]. In addition, Schiffer–Edmundson helical wheel diagrams are often used to predict the hydrophobic and hydrophilic regions in the secondary structure of these peptides. Using these helical wheel diagrams, other authors were able to predict the amphipathic alpha-helical conformation of piscidins 1 and 3 from hybrid striped bass [6,41], that was later confirmed in different studies. Piscidin 1 and 3 adopt an amphipathic alpha-helical structure in the presence of membrane-mimicking environments, allowing peptides to be oriented parallel to membrane surface and form pores, possibly through toroidal or in-plane diffusion mechanisms [50–52]. In this study, we predicted the amphipathicity of the secondary structure of sea bass piscidins. Piscidins 1 and 4, homologous of striped/white bass piscidins 1 and 3, fold into the typical structure for these peptides, constituted by an alpha-helix where hydrophobic residues clearly stay together in one side of the structure, in opposition to the hydrophilic ones. However, these two mature peptides share an identity of 63.6% and the most significant differences are a histidine substituted for an asparagine at position 11 and a glycine substituted for a histidine at position 17, that would probably result in different antimicrobial activities. This is in accordance to the results of Silphaduang and Noga, that evaluated protein sequences and antimicrobial capacities of piscidins 1 and 3 and observed a higher activity of piscidin 1 against a wide range of pathogens, when compared to piscidin 3 [6]. Likewise, sea bass piscidin 4 is less active against pathogens and, thus, is in accordance with previous findings.

The structure and mode of action of white/striped bass piscidin 4 was also addressed and authors determined that this antimicrobial peptide presents an amphipathic alpha-helical

N-terminal, while the C-terminal folds into random coils and sheets [17,18,53]. Nevertheless, this peptide may act on pathogens in a similar manner than the smaller piscidins, through binding of the N-terminal to cell membranes, then forming pores compatible to an in-plane diffusion mechanism [53]. Moreover, due to the particular structure of these two similar peptides, authors suggested that additional functions might be associated to piscidins 4 and 5 [17,19]. More specifically, piscidin 5 present a β-sheet region that is similar to known pattern recognition receptors, namely carbohydrate and lipopolysaccharide binding motifs [19]. Sea bass piscidins 6 and 7 are also predicted to be amphipathic in nature, with a reduced amphipathicity, when compared to the other piscidin family members. Salger et al. predicted a different secondary structure for these piscidins, consisting in a coil-β-sheet-coil-helix organization and showing a degree of amphipathicity [19]. However, the structure and potential roles of these atypical piscidins are not well understood.

Although there are evident similarities between fish species that belong to the *Morone* and *Dicentrarchus* genera, specific differences between each species are also observed, with sea bass being no exception. Comparison between piscidins from *D. labrax* with *Morone* species shows variances in specific residues, that can translate into diverse antimicrobial activities. However, the main differences are observed in piscidins 5, 6 and 7. Piscidin 5 is expressed in white bass, while it was only isolated as a non-functional pseudogene in striped bass. Likewise, piscidin 7 is present in striped bass, but the authors were unable to detect it in the white bass genome [19]. In sea bass, both piscidins were isolated and detected in different tissues, with sea bass piscidin 5 presenting a prodomain constituted by 13 amino acids, contrary to what is observed for white bass. Moreover, despite similarities, sea bass piscidin 6 mature peptide shows unique features, namely a different amino acid composition between positions 14 and 17 (QARS in sea bass, KGFQ in white and striped basses), and a 10 amino acid repetition in the C-terminal of the mature peptide (ADAQANDQPS). Thus, while piscidin 6 in *Morone* species is constituted by a 55 amino acid mature peptide, in sea bass, this present an active peptide with 65 amino acids. This goes in accordance with previous findings, showing that smaller sized piscidins are usually more conserved sequence wise, whereas larger sized piscidins become much more diversified. Many duplicated genes continue under selective pressure, and while several of the duplicates usually retain their original functions, other genes are retained owing to different processes, particularly subfunctionalization, when the functions of the ancestral gene are divided among the duplicated genes, or neofunctionalization, by gaining or accumulating a novel function. Some duplicates may even become nonfunctional due to the accumulation of deleterious mutations [46]. As suggested by Salger et al., piscidins could be subjected to these evolutionary processes,

resulting in a diversified family with different anti-bacterial and anti-protozoan properties [19].

As expected, sea bass piscidins exhibited multiple antibacterial activities, depending on the pathogen and peptide. Results of the present study show the diversity of piscidin activities, even within each sub-group. Sea bass piscidins 1 and 5, positioned into different sub groups, are the most active peptides, inhibiting the growth of almost all pathogens analyzed and presenting the lowest inhibitory concentrations. The MICs and minimal bactericidal concentrations (MBCs) of piscidins 1/moronecidins and piscidin 3 from white, striped and hybrid striped bass were previously addressed, with different outcomes. Silphaduang and Noga observed different inhibitory and bactericidal concentrations for *A. salmonicida* and *A. hydrophila*, with the first one presenting a higher resistance [6]. Furthermore, Lauth et al. observed a resistance of *A. hydrophila* when incubated with a synthetic and amidated white bass moronecidin [41]. In this study, we observed that *A. hydrophila* growth was not inhibited by any of the peptides tested, even at the highest concentration levels. Furthermore, sea bass piscidins 1, 2 and 5 presented intermediate levels of *A. salmonicida* inhibition, with no effect of piscidins 4, 6 and 7. The other bacteria tested show a diverse degree of susceptibility against synthetic piscidin peptides. *Photobacterium damselae* subsp. *piscicida* and *S. parauberis* are the most susceptible strains. On the contrary, *E. tarda* is resistant to all piscidins. A similar outcome is observed in other studies, with piscidins presenting a weak activity against *E. tarda* [25,54]. As for *P. damselae* subsp. *piscicida*, a previous study shows varied activities of hybrid striped bass piscidin 4 against different strains [17]. These differences observed between the several strains may be related with specific variations between them, translating into a diverse activity by piscidins against these bacteria [14]. The action of AMPs relies on an initial binding between them and cell membranes, through hydrophobic and electrostatic interactions. Secondary structure, charge, hydrophobicity, and amphipathic character are of most importance for peptide activity [55]. Thus, the reduced hydrophobicity and amphipathicity of piscidins 6 and 7 may also explain why these peptides present such a poor activity against the tested bacteria, when compared to the other piscidins.

The effects of piscidins on mammalian bacteria, virus and fungi are well demonstrated [30,41,56,57]. However, the antimicrobial activity against mammalian parasites is not well explored. Thus, the anti-parasitic activity of sea bass piscidins was evaluated against *T. brucei brucei* and *L. infantum*, parasites known to infect mammalian species, including humans, mainly in developing tropical countries [58]. To our knowledge, this is the first report that explores the action of fish antimicrobial molecules against these particular parasites. We demonstrate that piscidins 1 to 5 are highly effective in inhibiting the growth

of both parasites in vitro, with piscidin 6 and 7 presenting the lowest anti-parasitic capacity. According to the findings of Salger et al., Class III piscidins present a reduced action against bacteria, but a strong anti-protozoal activity [19]. In this study, this was not observed, with the smaller peptides being more active against *T. brucei* and *L. infantum* when compared to sea bass piscidins 6 and 7. Still, the action of these synthetic piscidins was not tested on fish parasites, and their antiparasitical activity may be more effective on these microorganisms, while the other smaller peptides have a broader effect on different pathogens.

Conclusions

We characterized in sea bass a diverse group of piscidins, closely related with species from the genus *Morone*. This may suggest that piscidins belonging to the Moronidae family have evolved to a specialized family of antimicrobial molecules, perhaps with distinct functions, asides the wide range of activity against several pathogens. Thus, further studies are required to understand the roles of these molecules, particularly piscidins 6 and 7. Although described as piscidins, these two peptides present a limited antimicrobial activity, which opens the possibility of them being involved in other immune or non-immune mechanisms, which would not be uncommon, as there are other antimicrobial peptides that present several functions.

Materials and Methods

Animals

European sea bass (*Dicentrarchus labrax*), with an average weight of 30 g, were provided by a commercial fish farm (Sonríonansa S.L., Pesués, Cantabria, Spain). Fish were kept at the fish holding facilities of the Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto, Portugal, in 110-L recirculating sea water (28‰ salinity) tanks at 23 °C, with a 13 h/11 h light-dark cycle, and fed daily to satiation with commercial fish feed. Before each treatment, fish were anesthetized with ethylene glycol monophenyl ether (2-phenoxyethanol, 0.3 mL/L; Merck, Algés, Portugal). All animal experiments were carried out in strict compliance with national and international animal use ethics guidelines, approved by the animal welfare and ethic committees of ICBAS (P293/2019/ORBEA, 05/04/2019), and conducted by experienced and trained Federation of European Laboratory Animal Science Associations Category C investigators.

Isolation of Sea Bass Piscidins

Pairs of oligonucleotide PCR primers were designed according to conserved regions of dicentracin mRNA sequence from sea bass and piscidins from other fish species, sea bass expressed sequence tags (ESTs) and whole-genome shotgun sequences (WGSS) available in the National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov). cDNA preparations from whole intestine, liver, gill and pyloric caeca were used in PCR amplifications. PCR products were run on 1.2% agarose gels, and relevant fragments purified with the NZYGelpure kit (NZYtech, Lisbon, Portugal), cloned into pCR™2.1-TOPO® vectors, propagated in One Shot® Mach1™-T1R competent cells (Invitrogen, Life Technologies, Carlsbad, CA), and sent for sequencing (GATC, A Eurofins Genomics Company, Ebersberg, Germany). Both strands were sequenced, and chromatograms were analyzed in FinchTV (Geospiza, Seattle, WA) and assembled using Multalin (http://multalin.toulouse.inra.fr/multalin/). The Schiffer–Edmundson helical wheel diagrams, hydrophobicity and hydrophobic moment were determined using HeliQuest (http://heliquest.ipmc.cnrs.fr/) [59].

Genomic Organization

Genomic DNA was isolated from sea bass red blood cells, using the NZY Blood gDNA Isolation kit (NZYtech, Lisbon, Portugal). Quantification was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and quality was checked by agarose gel electrophoresis. Two micrograms of genomic DNA were amplified by PCR with the primers based on the previously obtained cDNA sequences, with the following cycling profile: 94 °C for 5 min, 30 cycles of 94 °C for 60 s, 59 °C for 60 s, 72 °C for 60 s and a final step of 72 °C for 5 min. Several PCR products were purified, cloned, and sent for sequencing. Comparisons were made between cDNA and genomic DNA to assess the similarity of the coding regions and to identify intron/exon boundaries. A comparison between the genomic sequences of sea bass piscidins with those of other species was also made, using the sequences identified with the following GenBank accession numbers and previous studies: *M. chrysops* moronecidin (AF394243), *M. saxatilis* moronecidin (AF394244); *Pseudopleuronectes americanus* pleurocidin (AF210241); *Oplegnathus fasciatus* piscidin-1 (KT354978); *Oreochromis niloticus* piscidin1 to 5 [9]; *Gadus morhua* gaduscidins/piscidins [42]; *Epinephelus coioides* epinecidin-1 [7].

Amplification of 5’ and 3’ Flanking Regions

The 5’ and 3’ RACE were carried out using the 5’/3’ RACE Kit, 2nd Generation (Roche Applied Science, Amadora, Portugal) according to the manufacturer’s instructions.

Conditions for PCR were as follows: 94 °C for 2 min, 94 °C for 15 s, 59 °C for 30 s, 72 °C for 40 s, for 10 cycles; 94 °C for 15 s, 59 °C for 30 s, 72 °C for 40 s (plus 20 s/cycle), for 25 cycles, with a final elongation at 72 °C for 7 min. When necessary, a second PCR amplification was performed using the same conditions for an additional 30 cycles. To increase sequence coverage and obtain possible promoter regions, amplifications of genomic DNA were performed using the Universal Genome Walker Kit (Clontech, MountainView, CA), according to the manufacturer's instructions. Amplification products were run on agarose gels, relevant fragments purified, cloned, and sequenced as previously described.

Alignment and Phylogenetic Analysis

Alignments of the amino acid sequences of the piscidin predicted proteins were performed using MUSCLE from MEGA X [37]. A phylogenetic tree was constructed using the Maximum Likelihood method, with the Jones–Taylor–Thornton (JTT) model [35], Nearest-Neighbor-Interchange heuristic model, partial deletion of gaps, and 10000 bootstrap replications. Sequences used for comparisons and phylogenetic trees and their accession numbers were as follows: *D. labrax* WGSS (CBXY010006294 and CBXY010006295); *D. labrax* dicentracin (AAP58960); *Siniperca chuatsi* moronecidin (AAV65044); *Oreochromis niloticus* piscidins 1 to 5 (AGA16544, AGA16545, AGA16546, AGA16547 and AGA16548); *Gadus morhua* gaduscidin 1 and 2 (ADK63423 and ADK63424); *Epinephelus coioides* epinecidin 1 (AAQ57624); *E. coioides* piscidins 2, 3 and 4 (ADY86111, AKA60776 and AKA60777); *Pseudopleuronectes americanus* pleurocidin (AAF17252); *M. chrysops* moronecidin (AAL40409); *M. chrysops* piscidins 3 to 6 (APQ32047, APQ32050, APQ32052 and APQ32044); *M. saxatilis* moronecidin (AAL57319); *M. saxatilis* piscidins 3, 4, 6 and 7 (APQ32046, APQ32049, APQ32043 and APQ32054); *M. chrysops* × *M. saxatilis* piscidins 4 and 5 (ADP37959 and ADP37960); *Larimichthys crocea* piscidin-like (AGN52988); *L. crocea* piscidin5-like (AIL82388); *Argyrosomus regius* piscidin (ASW20416); *Oplegnathus fasciatus* piscidins 1, 6 and 7 (AMB38762, ATU75059 and ATU75060); *Hippoglossoides platessoides* pleurocidin-like AP1 (AAP55793); *Hyalophora cecropia* cecropin A (CAA29871); *D. labrax* hepcidin 1 (AJU35239); *D. labrax* hepcidin 2 variant 1 (AJU35240). Identity scores between the different piscidin peptides were determined using Sequence Identity And Similarity (SIAS) software (http://imed.med.ucm.es/Tools/sias.html).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from tissues with the NZY Total RNA Isolation Kit (NZYTech, Lisboa, Portugal), according to the manufacturer's instructions. Total RNA quantification

was performed using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and quality was assessed by running the samples in an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). For all samples, 1.25 µg of each were converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisboa, Portugal), according to the manufacturer's protocol.

Basal Expression of Sea Bass Piscidins

Several tissues from five healthy sea bass were collected for RNA isolation and cDNA synthesis, as previously described. Relative levels of piscidin mRNAs were quantified by real-time PCR analysis using an CFX96™ Real-Time PCR Detection System (Bio-Rad). One microliter of each cDNA sample was added to a reaction mix containing 10 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 7 µL of $_{dd}$H20, and 250 nM of each primer (Table S2), making a total volume of 20 µL per reaction. The cycling profile was as follows: 95 °C for 3.5 min, 40 cycles of 95 °C for 20 s, and 59 °C for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reactions. Beta actin (*actb*) was used as the housekeeping gene. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold values was used to analyze gene expression levels.

Antibacterial Activity of Sea Bass Piscidin Peptides

The biological activity of sea bass piscidin mature peptides was studied by determining their antimicrobial properties. Synthetic piscidin peptides were based on the predicted coding sequences and were commercially produced (NZYtech, Lisbon, Portugal) with an additional C-terminal amidation (Table S3). Peptides were incubated in serial dilutions with ten bacterial strains, known to cause severe diseases in aquaculture: *Photobacterium damselae* subsp. *piscicida* (DSM 22834), *P. damselae* subsp. *damselae* (DSM 7482), *Vibro anguillarum* (DSM 21597), *V. alginolyticus* (DSM 2171), *Aeromonas salmonicida* subsp. *salmonicida* (DSM 19634), *A. hydrophila* subsp. *hydrophila* (DSM 30187), *Yersinia ruckeri* (DSM 18506), *Edwardsiella tarda* (DSM 30052), *Lactococcus garviae* (DSM 20684) and *Streptococcus parauberis* (DSM 6631). In short, 1×10^8 bacteria per milliliter were incubated in optimal growth conditions with the peptide in flat-bottom 96-well plates, in a final volume of 100 µL, and OD was read at 600 nm in a plate reader after 24 h of incubation. Wells with no added peptide were used as controls, and wells without bacteria were used as blanks. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of piscidin that reduced 50% of bacterial growth when compared to

the controls. The MICs reported correspond to representative results from two independent experiments.

Antiparasitic Activity of Sea Bass Piscidin Peptides

The anti-protozoal activity of sea bass piscidins was evaluated using two parasites: *Trypanosoma brucei brucei* bloodstream forms (strain Lister 427) and *Leishmania infantum* promastigotes (strain MHOM/MA/67/ITMAP-263). Parasites were incubated in optimal growth conditions with serial dilutions of the different peptides in flat-bottom 96-well plates, in a final volume of 200 µL, during 72 h. Cell metabolic activity was measured by resazurin reduction. Briefly, 20 µL of resazurin (50 µM) were added and incubated for 4 h. The fluorescence of resorufin, resulting from resazurin reduction by metabolically active cells, was measured at λ_{ex} = 544 nm and λ_{em} = 590 nm in Synergy 2 (BioTek, Winooski, Vermont, USA). Cultures with no added peptide were used as negative controls and cultures with the reference drugs, namely pentamidine (10 nM) and miltefosine (40 µM) were used as positive controls for *T. brucei* and *L. infantum* inhibition assays, respectively. Wells with culture medium were used as blanks. Anti-parasitic effect was evaluated by the determination of IC_{50} value (concentration required to inhibit growth in 50%) and calculated by non-linear regression analysis using GraphPad Prism version 8.1.1 for Windows (GraphPad Software, San Diego California, USA). The IC_{50} reported correspond to representative results from two independent experiments.

Supplementary Material

Table S1. Identity scores of full length sea bass piscidin amino acid sequences with peptides from other fish species; Table S2. Primers used for gene expression analysis; Table S3. Synthetic piscidin mature peptides.

Author Contributions

C.B. and J.V.N. conceived and performed the experiments, analyzed data and wrote the original manuscript; P.N.S.R. conceived the experiments and analyzed data; P.C., C.C., N.S., J.F.M.G. conceived and performed the experiments and analyzed data. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

Supplementary Table 1. Identity scores of full length sea bass piscidin amino acid sequences with peptides from other fish species.

Family	Species	Piscidin	Sea bass piscidins					
			Piscidin 1	Piscidin 2	Piscidin 4	Piscidin 5	Piscidin 6	Piscidin 7
Moronidae	*D. labrax*	Dicentracin	97.3%	50.4%	69.9%	45.1%	35.4%	46.9%
Moronidae	*M. chrysops*	Moronecidin	97.3%	50.4%	67.3%	44.2%	34.5%	46.0%
	M. chrysops	Piscidin 3	69.9%	50.4%	92.9%	45.1%	34.5%	46.9%
	M. chrysops	Piscidin 4	46.9%	82.3%	47.8%	69.0%	45.1%	55.8%
	M. chrysops	Piscidin 5	45.1%	70.8%	51.3%	86.7%	44.2%	51.3%
	M. chrysops	Piscidin 6	44.2%	46.9%	45.1%	53.1%	77.9%	81.4%
Moronidae	*M. saxatilis*	Moronecidin	97.3%	50.4%	69.0%	44.2%	34.5%	46.0%
	M. saxatilis	Piscidin 3	69.9%	50.4%	92.9%	45.1%	34.5%	46.9%
	M. saxatilis	Piscidin 4	47.8%	78.8%	52.2%	69.0%	45.1%	54.9%
	M. saxatilis	Piscidin 6	44.2%	46.0%	45.1%	52.2%	78.8%	81.4%
	M. saxatilis	Piscidin 7	44.2%	51.3%	45.1%	53.1%	73.5%	89.4%
Moronidae	*M. chrysops_x_M_saxatilis*	Piscidin 4	47.8%	78.8%	52.2%	69.0%	45.1%	54.9%
	M. chrysops_x_M_saxatilis	Piscidin 5	45.1%	70.8%	51.3%	86.7%	44.2%	51.3%
Sinipercidae	*S. chuatsi*	Moronecidin	69.0%	49.6%	75.2%	46.0%	38.9%	49.6%
Cichlidae	*O. niloticus*	Piscidin 1	41.6%	50.4%	50.4%	43.4%	30.1%	44.2%
	O. niloticus	Piscidin 2	50.4%	43.4%	50.4%	39.8%	28.3%	38.9%
	O. niloticus	Piscidin 3	69.0%	47.8%	64.6%	45.1%	34.5%	48.7%
	O. niloticus	Piscidin 4	55.8%	41.6%	49.6%	36.3%	23.9%	38.9%
	O. niloticus	Piscidin 5	41.6%	45.1%	49.6%	42.5%	32.7%	45.1%
Gadidae	*G. morhua*	Gaduscidin 1	42.5%	43.4%	48.7%	36.3%	23.9%	37.2%

Supplementary Table 1. *Cont.*

Family	Species	Piscidin	Sea bass piscidins					
			Piscidin 1	Piscidin 2	Piscidin 4	Piscidin 5	Piscidin 6	Piscidin 7
	G. morhua	Gaduscidin 2	41.6%	46.0%	51.3%	37.2%	26.5%	39.8%
Serranidae	*E. coioides*	Epinecidin 1	57.5%	53.1%	69.0%	49.6%	34.5%	46.9%
	E. coioides	Piscidin 2	56.6%	54.9%	61.9%	50.4%	34.5%	46.0%
	E. coioides	Piscidin 3	57.5%	52.2%	57.5%	46.9%	35.4%	46.9%
	E. coioides	Piscidin 4	45.1%	64.6%	48.7%	58.4%	39.8%	50.4%
Pleuronectidae	*P. americanus*	Pleurocidin	45.1%	43.4%	53.1%	45.1%	33.6%	44.2%
Sciaenidae	*L. crocea*	Piscidin-like	56.6%	45.1%	60.2%	46.0%	38.1%	51.3%
	L. crocea	Piscidin5-like	43.4%	43.4%	50.4%	46.0%	38.9%	51.3%
Sciaenidae	*A. regius*	Piscidin	54.9%	46.0%	60.2%	46.9%	36.3%	49.6%
Oplegnathidae	*O. fasciatus*	Piscidin 1	58.4%	53.1%	62.8%	46.9%	37.2%	50.4%
	O. fasciatus	Piscidin 6	44.2%	49.6%	48.7%	45.1%	53.1%	61.1%
	O. fasciatus	Piscidin 7	49.6%	51.3%	46.9%	54.0%	57.5%	67.3%
Pleuronectidae	*H. platessoides*	Pleurocidin-like AP1	48.7%	41.6%	55.8%	44.2%	33.6%	43.4%

Identity scores were determined using the SIAS software, with BLOSUM62 scoring matrix and considering the length of multiple sequence alignment.

Supplementary Table 2. Primers used for gene expression analysis.

		FOR (5'→3')	REV (5'→3')
Actin, beta	*actb*	CAGAAGGACAGCTACGT	GTCATCTTCTCCCTGTTGGC
Piscidin 1	*pisc1*	TCGTCCTCATGGCTGAACC	GCGGTTAAAGCGCTGATATTG
Piscidin 2	*pisc2*	TCGTCCTCATGGCTGAACC	GGTTGCTCTTGATTGTTGTCCG
Piscidin 4	*pisc4*	TCGTCCTCATGGCTGAACC	GGAGCTGTGTTGAAGTTGAGT
Piscidin 5	*pisc5*	TCGTCCTCATGGCTGAACC	CACAGGTCTTCGAACGTATCC
Piscidin 6	*pisc6*	TCGTCCTCATGGCTGAACC	TCAGCGACGGTAGTTCTCTG
Piscidin 7	*pisc7*	TGGGACGTGTGAAATCCATGT	TCAGTTCTCTGGAGGTTGATC

Supplementary Table 3. Synthetic piscidin mature peptides.

Piscidin1	FFHHIFRGMHVGKTIHRLVTG-NH_2
Piscidin2	FLGRFFRRTQAILRGARQGWRAHKAVSRYRDRYIPETDNNQEQP-NH_2
Piscidin4	FIHHIFRGIINAGKSIGRFITG-NH_2
Piscidin5	LIGSLFRGAKAIFRGARQGWRAHKAVSRYRAGYVRRPVVYYHRVYP-NH_2
Piscidin6	LFGSLKAWFKGGKQASRDYKYQKDMAKMNKRYGPNWQQGGGQQPPANAQANDQPPADAQANDQPS-NH_2
Piscidin7	FLGRVKSMWSGVRNGYKAYKYQRNMAKMNKGYGPNWQQGGGQEPPADAQANDQPP-NH_2

Piscidins were commercially synthetized with an additional C-terminal amidation.

Chapter V

Beyond antimicrobial activity: the roles of piscidins during infection and iron modulation

Abstract

Fish present several antimicrobial peptides (AMPs), involved in many roles, including antimicrobial activity, immunomodulation and iron metabolism regulation. While the direct pathogen killing and the immunomodulatory functions are performed by different AMPs, the regulation of iron metabolism has been only attributed to hepcidin. In most mammals, hepcidin is found as a single gene, but in many fish species, including the European sea bass (*Dicentrarchus labrax*), two different hepcidin types are found: the single type 1 hepcidin is involved in iron modulation through the regulation of the iron exporter ferroportin; and the various type 2 hepcidins present antimicrobial activity against a number of pathogens. In the present study, we evaluated if other fish AMPs, particularly piscidins, may also have a role in the regulation of iron, using our experimental models of iron overload and peptide administration. Furthermore, the potential beneficial effect of piscidins was also assessed, by treating fish infected with *Photobacterium damselae* spp. *piscicida* with piscidin peptides. The results suggest that piscidins 2 and 7 might be involved in the regulation of iron, leading to decreases in the hematological parameters and an alternative distribution of iron in fish subjected to experimental iron overload. Moreover, the administration of piscidins 1 and 5 promoted significant survival rates, showing potential as novel therapeutic or prophylactic compounds, to replace the conventional treatments currently applied in aquaculture. Further studies are needed to understand the exact mechanisms by which piscidins interfere with iron metabolism, and how piscidins stimulated fish inflammatory responses, leading to an increased survival upon infection.

Introduction

A plethora of antimicrobial peptides (AMPs) can be found widespread throughout different fish species, showing different sequences, structures and functions, that include direct antimicrobial activity, immunomodulation or iron metabolism regulation [1]. In the European sea bass (*Dicentrarchus labrax*), a commercially important species for the Mediterranean aquaculture, several members of major AMP families have been described, namely hepcidins, piscidins and beta-defensins [2–5]. This fish species shows a high diversity of hepcidins and piscidins, that can be attributed to genome duplications and positive selection, influenced by host-pathogens interactions [3,4]. Consequences of genome duplications can be the loss of the duplicated gene or, through processes of

subfunctionalization and neofunctionalization, many duplicated genes are retained and even acquire novel functions [6].

Hepcidin (HAMP) is a small cysteine rich AMP highly produced in the liver, that shows antimicrobial activity *in vitro* [7,8]. However, the major function of hepcidin is the inhibition of the iron exporter ferroportin, through its internalization and degradation [9], leading to a decrease of iron release from hepatocytes, enterocytes and reticuloendothelial macrophages [10]. Hepcidin expression is regulated depending on the physiological condition, being increased during iron overload and inflammation/infection, and decreased during anemia and hypoxia [10,11]. During inflammation, hepcidin is induced by inflammatory cytokines, with IL-6 assuming a preponderant role, leading to a decreased mobilization of iron and limiting its availability for pathogens. However, as a long term effect, this decrease in circulating iron will also impair the erythropoietic processes of the host, leading to a condition known as anemia of inflammation [10]. Most mammals present a single hepcidin, but in sea bass, several hepcidins are found, whose functions are subfunctionalized. The type 1 hepcidin, usually present as a single gene, is involved in the regulation of iron through ferroportin inhibition, and the several type 2 hepcidins show diverse antimicrobial activities [3,12].

While most studies addressing sea bass AMPs are focused on hepcidins [3,12–15], other peptides, namely piscidins, are poorly understood. Piscidins comprise a large family of small, usually linear and amphipathic peptides present in several fish species with proved antimicrobial and immunomodulatory roles, in fish and mammalian models [16–23]. While Cyprinid and Salmonid species seem to lack these peptides, species from the Moronidae family present at least six piscidins, that are divided into different classes, according to the size of mature peptides and activity against different microorganisms [4,17,24,25]. Previous studies have focused on the structure and mode of action of the 22 amino acids piscidins 1 and 3, the smallest piscidins found in these species [26–29]. These two small piscidins seem to exert their antimicrobial functions using different mechanisms, with piscidin 1 being more membranolytic, while piscidin 3 shows stronger interactions with DNA [27, 28]. However, the roles of other piscidins, with sizes that range from 44 to 65 amino acids, are still unclear. The biggest sea bass piscidins (piscidins 6 and 7, with 65 and 55 amino acids, respectively) show poor antimicrobial and anti-parasitic activities [4], raising the question if other functions might be attributed to these atypical piscidins.

Different families of fish AMPs show similar functions, particularly the modulation of certain immune pathways, although the exact mechanisms triggered by these peptides are still poorly understood [1,30]. Furthermore, the subfunctionalization documented for the sea bass hepcidin family, with a single iron metabolism–related type 1 molecule and multiple type 2 antimicrobial hepcidins, raises the possibility for a more diversified role for

the antimicrobial peptides in sea bass [3]. As such, it led us to the hypothesis of a possible involvement of other AMPs in the regulation of iron, particularly piscidins. In the present study, we address that hypothesis, by analyzing the administration of piscidins in healthy fish, in order to evaluate the effects in iron related parameters, but also in models of iron overload, to understand how excess iron is regulated by piscine AMPs. Furthermore, as some piscidins exert, in fact, antimicrobial roles, we also tested these peptides as possible prophylactic or therapeutic compounds for aquaculture. The results presented in this study seem to indicate a possible role of some piscidins in iron metabolism regulation, as substantial variations were observed with the administration of these peptides. Also, the low mortalities observed in infected fish treated with piscidin 1 and 5 shows the potential of piscidins as alternatives to the conventional treatments currently applied in aquaculture. Further studies will be necessary to understand the role of piscidins in iron metabolism, and what is the connection between piscidins and hepcidins.

Results

Different piscidins show different hemolytic activities

Before peptide administration experiments, the hemolytic activity of sea bass piscidins was tested against sea bass erythrocytes (Table 1). Lysis of sea bass erythrocytes was observed with high concentrations of piscidins 1, 2 and 4. Piscidin 1 is highly hemolytic at 100 and 50 µM, inducing 97.5 and 54.9 % of hemolysis, respectively. Piscidin 2 and 4 are hemolytic only at the highest concentration level, resulting in 30.0 and 62.1 % of hemolysis, respectively. Piscidin 5 is the most hemolytic peptide, inducing 100.0, 94.3, 80.9, 47.0 and 13.8% of lysis at 100, 50, 25, 12.5 and 6.25 µM, respectively. Piscidins 6 and 7 had no significant impact on sea bass red blood cells, even at the highest concentrations.

Table 1. *In vitro* hemolytic activity of sea bass piscidins peptides.

	Hemolytic activity (%)							
μM	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.5625**	**0.78125**
Pisc1	97.5±3.6	54.9±4.6	12.5±10.6	0.6±0.9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pisc2	30.0±0.8	6.5±1.6	0.3±0.3	0.8±1.1	0.0±0.0	0.3±0.4	0.0±0.0	0.6±0.8
Pisc4	62.1±7.8	2.1±1.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.2
Pisc5	100.0±0.0	94.3±0.0	80.9±7.9	47.0±2.8	13.8±0.6	2.0±0.2	0.3±0.5	0.0±0.0
Pisc6	1.9±2.7	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.5	0.2±0.3	0.1±0.1	0.5±0.5
Pisc7	0.6±0.2	0.4±0.2	0.2±0.2	0.1±0.2	0.1±0.2	2.3±3.2	0.0±0.0	0.0±0.0

Erythrocytes were incubated with serial dilutions (μM) of the different synthetic piscidin peptides. Results are represented as means ± standard deviation and are representative of two independent experiments.

Piscidins have a marked effect in healthy sea bass hematological parameters

To study the effects of piscidins in fish, we first established a simple model of peptide administration in healthy animals (Supplementary Figure 6). Based on the results obtained in the previous test, we established a maximum concentration of 6.25 μM for each peptide to be administered, which is the highest concentration of piscidin 5 that did not cause significant adverse effects on erythrocytes *in vitro* (Table 1).

Blood and different tissues were collected at 1, 4, 7, 10 and 14 days post peptide administration, to determine the hematocrit and tissue iron content, and to evaluate the expression of several genes involved in iron metabolism and erythropoiesis.

Piscidin 2 administration led to a drastic reduction in the hematocrit, at 14 days (Figure 1A), while fish treated with piscidin 7 showed decreases starting from day 7 and peaking lowest values at day 14 (Figure 1C), leading to a condition of anemia in fish. Piscidin 5 also resulted in a significant reduction in the hematocrit, starting from day 10, until the end of the experiment (Supplementary Figure 1D). However, in this particular case, we cannot exclude a possible hemolytic effect of piscidin 5 *in vivo*, although neither mortalities or visible alterations in blood samples collected from these fish were observed. Administration of piscidins 1, 4 and 6 had no significant effects in the hematocrit of sea bass (Supplementary Figures 1A, C, E). Liver iron content of sea bass administered with each piscidin did not change significantly during the course of the experiment, although slight increases can be observed, when compared to the control group (Figures 1B, D; Supplementary Figure 2).

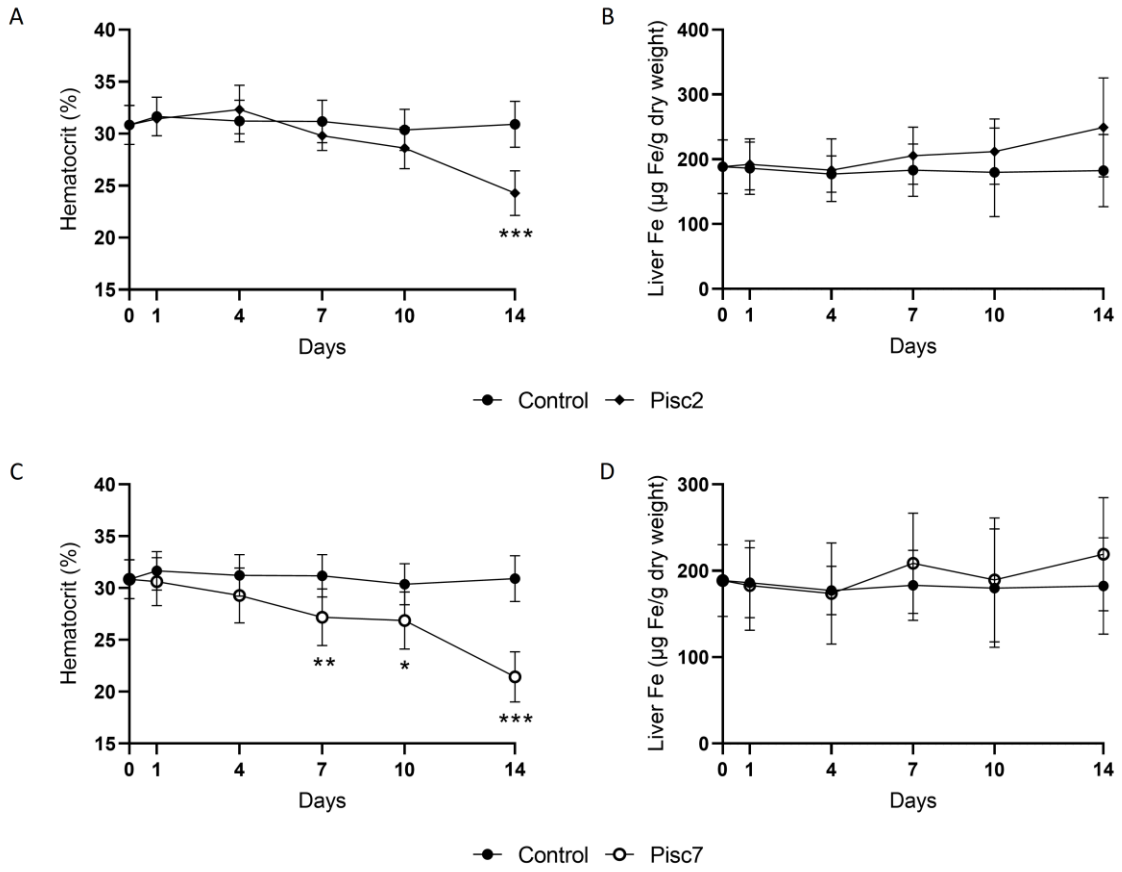

Figure 1. Hematocrit and liver iron content of fish treated with 6.25 µM of piscidin 2 or piscidin 7 peptides. (A) hematocrit and (B) liver iron content of piscidin 2 treated animals; (C) hematocrit and (D) liver iron content of piscidin 7 treated animals. Values are expressed as means ± standard deviation (n=10). Samples were collected at 1, 4, 7, 10 and 14 days post peptide administration. Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

Gene expression analysis was conducted in all experimental groups, with all showing slight variations in gene expression, but only fish treated with piscidins 2 and 7 showed significant variations in the various genes analyzed in the liver, posterior intestine, spleen and head kidney, tissues known to be involved in iron metabolism and erythropoiesis in fish.

In the liver of piscidin 2 treated animals, variations in the expression of *hamp1* were observed, with increases at days 4 and 10 post administration (Figure 2A), while in sea bass treated with piscidin 7, *hamp1* was up-regulated day 7, then returning to normal values (Figure 2D). The expression of *hamp2* was similar in both groups, being significantly up-regulated at day 4 of the experiment (Figures 2B, E). Either piscidin administration had no significant impact in the expression of *fpn1* (Figures 2C, F).

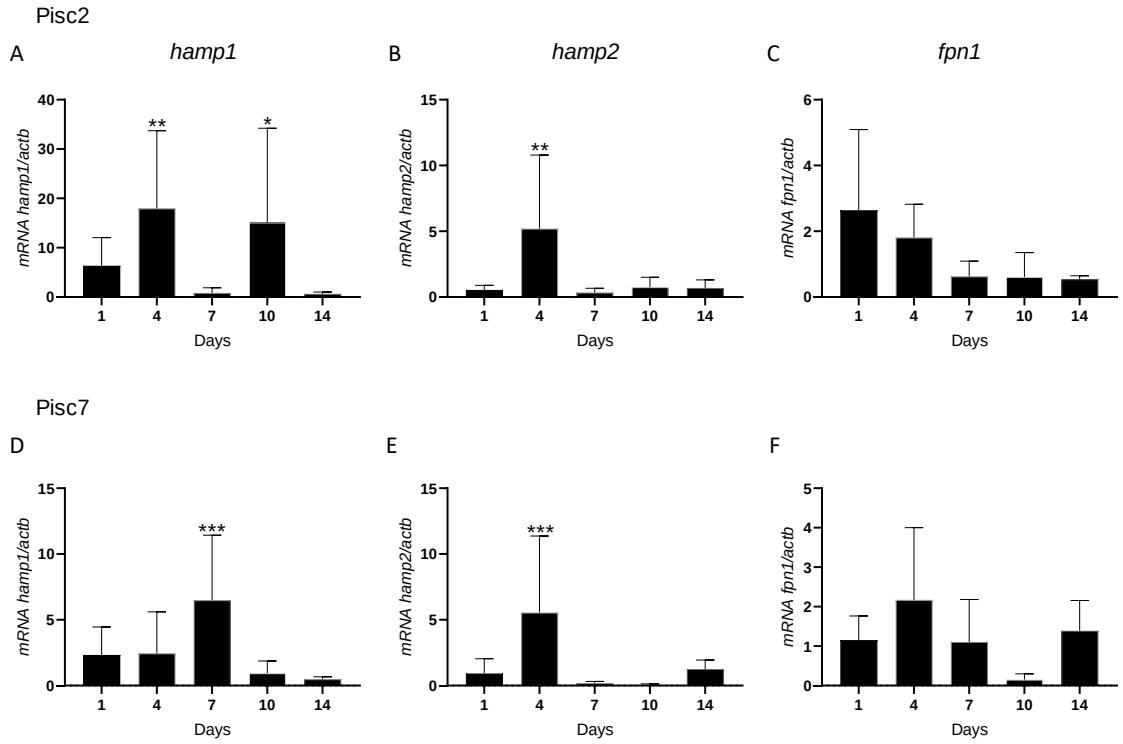

Figure 2. Gene expression in the liver at 1, 4, 7, 10 and 14 days after piscidin 2 or piscidin 7 peptide administration. (A) *hamp1*, (B) *hamp2*, (C) *fpn1* expression in the liver of sea bass treated with piscidin 2; (D) *hamp1*, (E) *hamp2*, (F) *fpn1* expression in the liver of sea bass treated with piscidin 7. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

In the posterior intestine, the pattern of gene expression was different between fish treated with each piscidin. In the piscidin 2 treated group, increases in the expression of *fth* and *fpn1* were observed at days 7 and 4, respectively (Figures 3B, C), while the expression of *slc11a2alpha* was not significantly altered by piscidin 2 treatment (Figure 3A). In piscidin 7 administered sea bass, an up-regulation in the expression of *slc11a2alpha* and *fth* were observed at days 4 and 14, respectively (Figures 3D, E), while no significant alterations were observed in *fpn1* (Figure 3F).

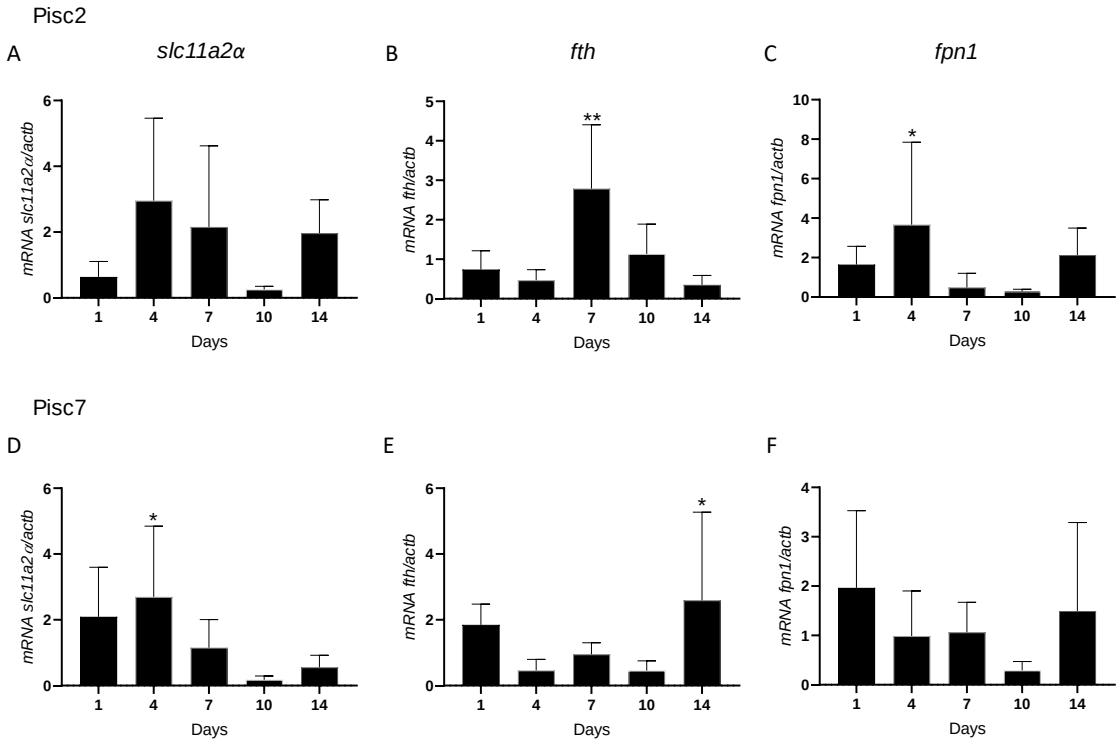

Figure 3. Gene expression in the posterior intestine at 1, 4, 7, 10 and 14 days after piscidin 2 or piscidin 7 peptide administration. (A) *slc11a2alpha*, (B) *fth*, (C) *fpn1* expression in the intestine of sea bass treated with piscidin 2; (D) *slc11a2alpha*, (E) *fth*, (F) *fpn1* expression in the intestine of sea bass treated with piscidin 7. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

In the head kidney, both groups showed similar profiles of expression, with *epo* and *epor* being down-regulated, while the expression of *fth* was up-regulated (Figure 4). The administration of piscidin 2 led to a down-regulation of *epo* at day 10 (Figure 4A) and of *epor* at days 7 and 10, returning then to normal values at the end of the experiment (Figure 4B). *Fth* expression increased significantly at day 7 post peptide administration, followed by a recovery towards day 14 (Figure 4C). In sea bass treated with piscidin 7, a significant down-regulation was observed in *epo* and *epor*, peaking lowest values at day 7 and gradually recovering to normal values, at the end of the experiment (Figures 4D, E). The expression of *fth* was increased at 4 days, and was kept up-regulated until the end of the experiment (Figure 4F).

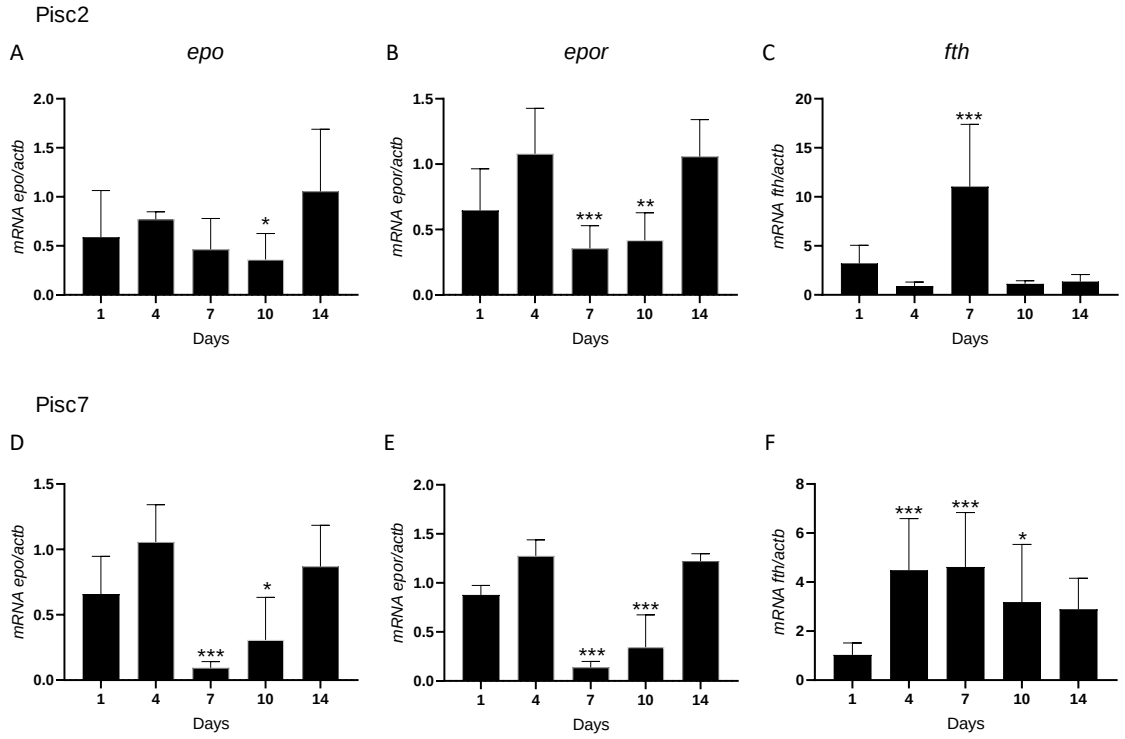

Figure 4. Gene expression in the head kidney at 1, 4, 7, 10 and 14 days after piscidin 2 or piscidin 7 peptide administration. (A) *epo*, (B) *epor*, (C) *fth* expression in the head kidney of sea bass treated with piscidin 2; (D) *epo*, (E) *epor*, (F) *fth* expression in the head kidney of sea bass treated with piscidin 7. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

The expression of these genes was also analyzed in the spleen, but no significant differences were observed (Supplementary Figure 3).

The status of iron overloaded fish treated with piscidins

To evaluate a possible effect of piscidins in iron overloaded fish, we chose piscidins 2 and 7, two peptides that caused the most significant alterations in the hematocrit of fish. We established three experimental groups, one with iron overload (as a baseline for comparison) and the other with iron overload followed by the treatment with a non-hemolytic concentration of 50 µM of either piscidin 2 or 7, similar to our previous experiment of iron overload and hepcidin administration [15]. We excluded piscidin 5, due to the high hemolytic activity that this peptide presented *in vitro* at 50 µM. After blood and serum sampling of this experiment, we tested the serum hemolytic activity of fish treated with both piscidins and no significant differences in terms of hemolytic activity were observed against sea bass erythrocytes, after treatment with either piscidin 2 or 7 (Table 2).

Table 2. Serum hemolytic activity of healthy and iron overloaded sea bass treated with piscidins 2 or 7.

Serum Hemolytic activity (%)							
Groups	**Days**						
	1	**4**	**7**	**10**	**14**	**21**	**28**
Fe+Pisc2	1.2±0.6	2.0±1.9	0.9±1.0	1.0±1.4	0.1±0.2	0.5±0.4	1.2±1.4
Pisc2	0.0±0.0	1.4±1.7	0.0±0.0	0.2±0.5	0.3±0.5	0.9±0.8	1.3±0.1
Fe+Pisc7	2.0±3.3	0.7±0.7	0.5±0.7	0.4±0.7	0.3±0.4	0.5±0.7	3.7±1.5
Pisc7	2.0±3.4	0.8±0.5	0.0±0.0	0.1±0.2	1.5±3.4	0.3±0.4	1.6±2.1

Fe+Pisc: iron overloaded fish treated with 50 µM of piscidins 2 or 7, 24 hours after overload; Pisc: healthy sea bass treated with 50 µM of piscidins 2 or 7. Serum samples were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration. Values are represented as means ± standard deviation (n=5).

We determined the hematocrit and iron content of the liver and spleen of fish only treated with 50 µM of piscidin 2 or 7. Results from this experiment reinforce the previous findings, that both peptides are not hemolytic and, most importantly, have a significant impact in the hematocrit levels, leading to a condition of anemia in fish. The observed response to both peptides presented some differences. Piscidin 2 administered fish showed a significant reduction in the hematocrit, peaking lowest values at day 7, followed by a slight recovery towards day 28 (Figure 5A). The decrease in this hematological parameter was accompanied by a significant increase in the liver iron content, starting from day 7 and until day 14, followed by a recovery to values similar to controls, at the end of the experiment (Figure 5B). When compared to the previous experiment, variations in the hematocrit and liver iron content occurred earlier, followed by a recovery (but lower than controls), with the accumulation of iron in the liver being much more pronounced when fish are treated with a higher dose of piscidin 2. In piscidin 7 treated animals, hematocrit was significantly reduced, showing the lowest values at days 7 and 10, followed by a recovery to normal values at the end of the trial (Figure 5C). In this experiment, we observed a recovery in the hematocrit at day 14, which was not observed when fish were treated with 6.25 µM of piscidin 7 (Figure 1C). As for the liver iron content, the administration of either 6.25 or 50 µM of piscidin 7 resulted in slight increases, although

not significant for both doses (Figures 1D and 5D). Spleen iron content showed no significant differences, although a tendency for increased iron deposition could be observed in fish treated with either piscidin 2 or piscidin 7 (Supplementary Figure 4).

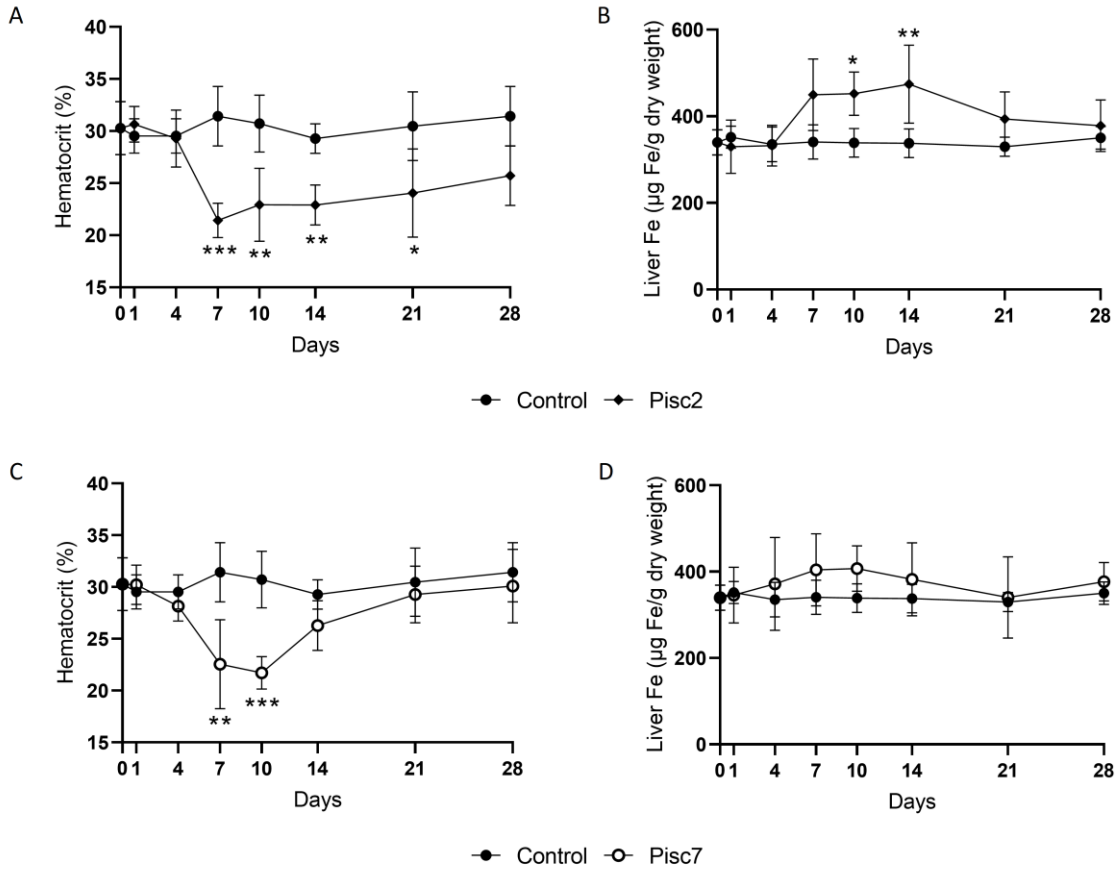

Figure 5. Hematocrit and liver iron content of fish treated with 50 µM of piscidin 2 or piscidin 7 peptides. (A) hematocrit and (B) liver iron content of piscidin 2 treated animals; (C) hematocrit and (D) liver iron content of piscidin 7 treated animals. Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration. Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

We then determined the hematocrit and iron content of the liver and spleen of iron overloaded fish treated with both piscidins. In the iron overload group, hematocrit increased from day 4 and showed the highest values at day 10, followed by a recovery towards day 28 (Figures 6A, C). Iron started accumulating in the liver as early as the first experimental day, reaching the highest values at day 7, followed by a recovery from day 10 up to day 28, but still kept elevated, when compared to controls (Figures 6B, D). In the spleen, there were no significant differences in iron content (Supplementary Figure 5).

Still, increases could be observed at days 1 and 4 of experiment. In iron overload followed by the administration of piscidin 2, there were also increases in the hematocrit, but occurring sooner and to a lesser extent that in iron overload alone, peaking highest values at day 4, and recovering towards the end of the trial (Figure 6A). Piscidin 2 administration led to a lower accumulation of iron in the liver, when compared to iron overload alone, although still higher than controls (Figure 6B). In iron overloaded animals treated with piscidin 7, we could see increases in the hematocrit and liver iron, but significantly lower than with iron overload alone. Still, hematocrit reached the highest values at day 10, followed by a recovery to percentages similar to controls (Figure 6C). Accumulation of iron was considerably reduced with piscidin 7 administration, but showed higher values when compared to controls, at day 4, followed by a recovery towards the end of the experiment (Figure 6D). Piscidin administration had no significant impact in spleen iron content of iron overloaded fish (Supplementary Figure 5). Still, differences could be observed between these groups. Piscidin 2 treated animals showed variations during the several experimental time points, but no accumulation of iron was observed, with fish presenting lower values, when compared to controls. In piscidin 7 administered animals, there were no increases of iron until day 10, followed by a decrease to values lower than controls.

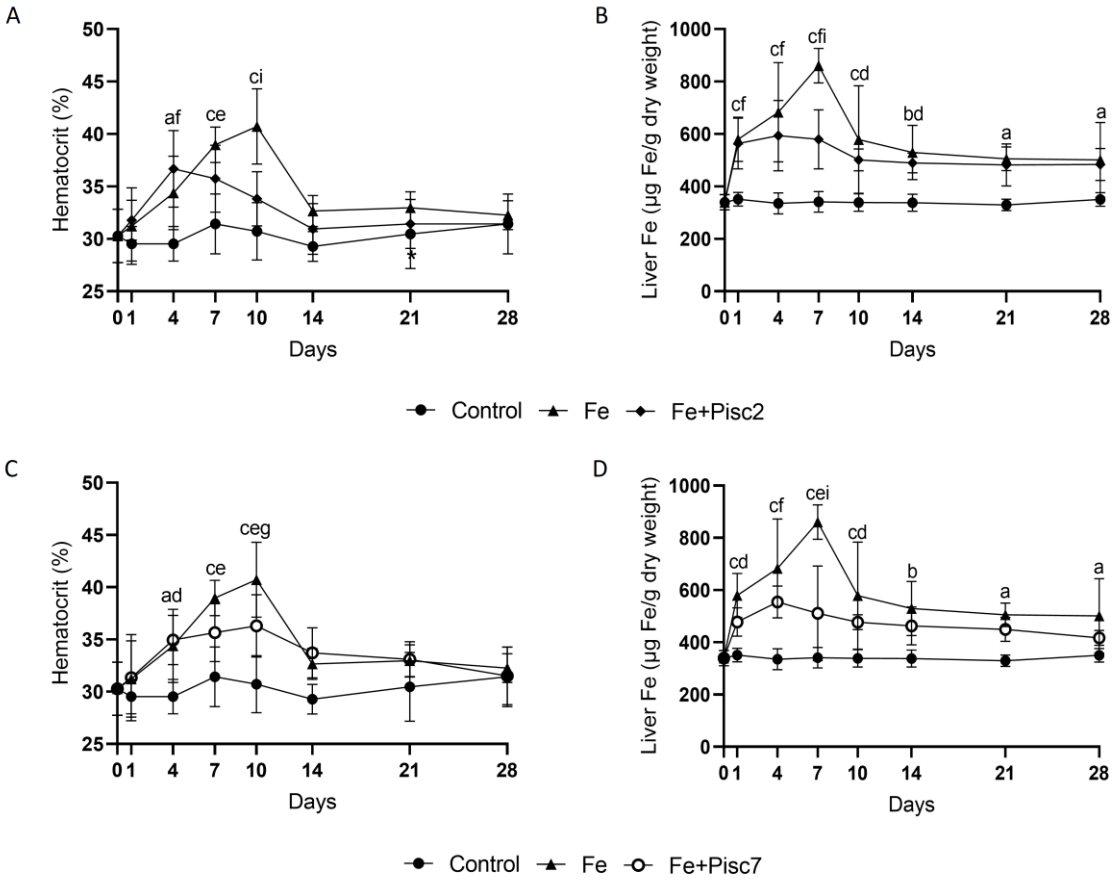

Figure 6. Hematocrit and liver iron content of sea bass after experimental iron overload (Fe) or iron overload and peptide administration (Fe+Pisc2/7). (A) hematocrit and (B) liver iron of piscidin 2 treated fish; (C) and (D) liver iron of piscidin 7 treated fish. Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration. Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters a, b, c between control and Fe animals, d, e, f between control and Fe+Pisc2/7 and g, h, i between Fe and Fe+Pisc2/7.

Piscidins protect fish against infection with *P. damselae*

To evaluate the potential of synthetic piscidins to prevent or treat bacterial diseases, we established experimental infections with the Gram-negative bacteria *Photobacterium damselae* spp. *piscicida* strain K1 (DSM 22834). Two major models were performed, one of peptide administration followed by infection (Pisc1/5 + inf - prophylactic potential) and of infection followed by peptide administration (inf + Pisc1/5 - therapeutic potential), as well as a simple model of infection without peptide administration (as a baseline for comparison). We chose piscidins 1 and 5 for being the two peptides that presented the highest antimicrobial activities [4], and established a non-hemolytic concentration of 6.25 µM, based on the previous results (Table 1).

The serum hemolytic potential of fish treated with piscidin 1 or 5 was also determined, with no significant alterations being observed in sea bass erythrocytes (Table 3).

Table 3. Serum hemolytic activity of infected sea bass treated with piscidins 1 or 5.

Serum Hemolytic activity (%)					
Groups	**Days**				
	1	**2**	**3**	**4**	**7**
Pisc1+Inf	0.0±0.0	2.5±3.7	0.0±0.0	0.7±1.2	0.2±0.5
Pisc5+Inf	0.2±0.4	0.0±0.0	0.5±1.1	0.0±0.0	1.6±2.8
Inf+Pisc1	1.6±1.8	0.1±0.1	0.0±0.0	0.2±0.3	0.0±0.0
Inf+Pisc5	1.0±2.1	0.0±0.0	0.3±0.5	2.8±4.8	0.0±0.0

Pisc+Inf: pre-treatment with 6.25 µM of piscidins 1 or 5, followed by infection with *P. damselae,* 24 hours later; Inf+Pisc: infection with *P. damselae,* followed by the post-treatment with 6.25 µM of piscidins 1 or 5, 24 hours later. Serum samples were collected

at 1, 2, 3, 4 and 7 days post peptide administration. Values are represented as means ± standard deviation (n=5).

In the model of peptide administration followed by infection, we observed a clear reduction in fish mortalities derived from the administration of both piscidins (Figure 7A), reducing from around 50% in the control infection to 6% in piscidin 1 and 2% in piscidin 5 administered animals. CFU counts in the spleen are similar in both piscidin treated groups, with reduced bacterial loads when compared to the infected animals, particularly at 4 and 7 days post infection (Figure 7B).

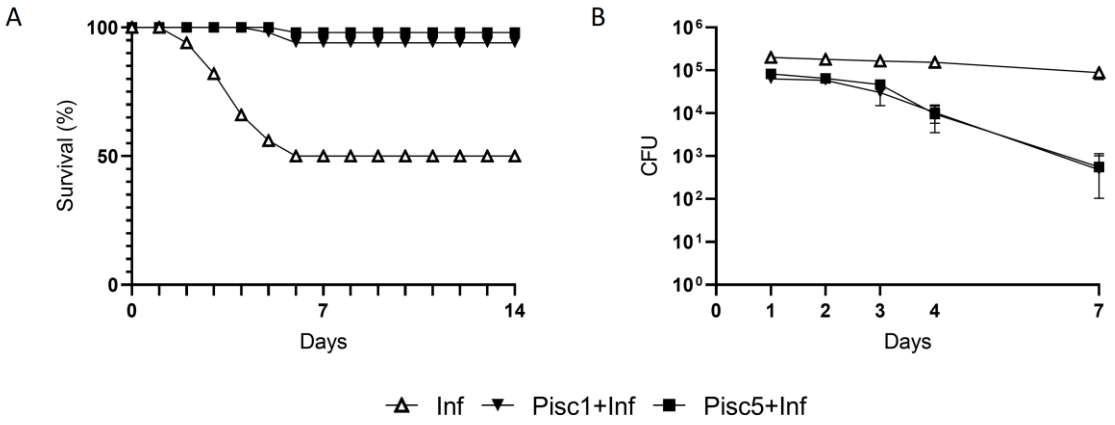

Figure 7. Survival curves (A) and CFU counts in the spleen (B) of sea bass treated with piscidin 1 or piscidin 5 peptides (pre-infection), followed by infection with *P. damselae,* 24 hours later. Mortality was assessed during 14 days of infection and colony forming unit (CFU) values are expressed as means ± standard deviation (n=5).

Infection alone led to significant decreases in hematocrit, up to day 4 post infection, with a recovery at the end of the experiment, but still lower than the controls (Figure 8). These decreases were not observed with piscidin administration, with the hematocrit being similar to controls in fish pre-treated with both piscidins (Figure 8).

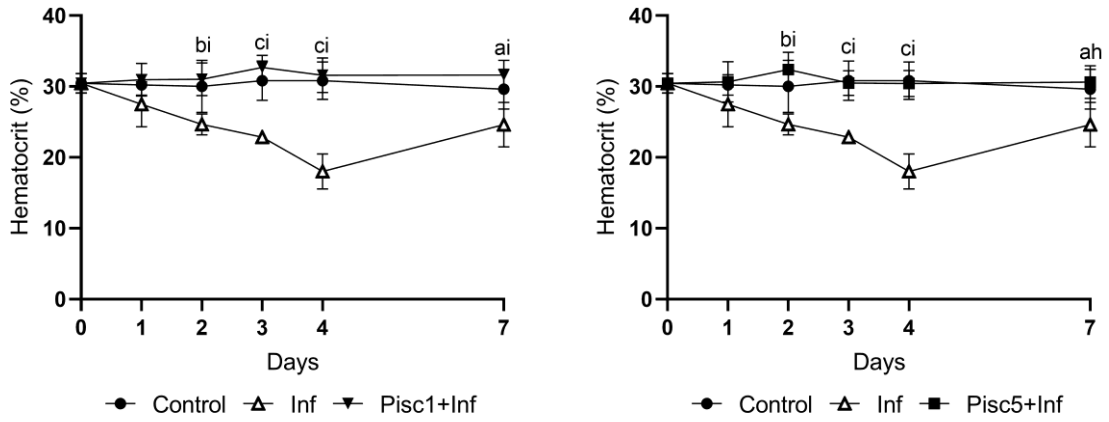

Figure 8. Hematocrit of sea bass pre-treated with piscidins 1 or 5, followed by infection with *P. damselae,* 24 hours later. Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 2, 3, 4 and 7 days post infection. Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters a, b, c between control and Inf animals, d, e, f between control and Pisc1/5+Inf and g, h, i between Inf and Pisc1/5+Inf.

In the model of infection followed by peptide administration, mortalities were slightly higher in fish post-treated with piscidins, when compared to the pre-infection experiment. Still, when compared to infection alone, there is a clear reduction in fish mortalities, from 50% in the control infection to 12% in piscidin 1 and 14% in the piscidin 5 treated animals (Figure 9A). CFU counts in the spleen were also considerably reduced in animals treated with both piscidins, when compared to infected animals (Figure 9B).

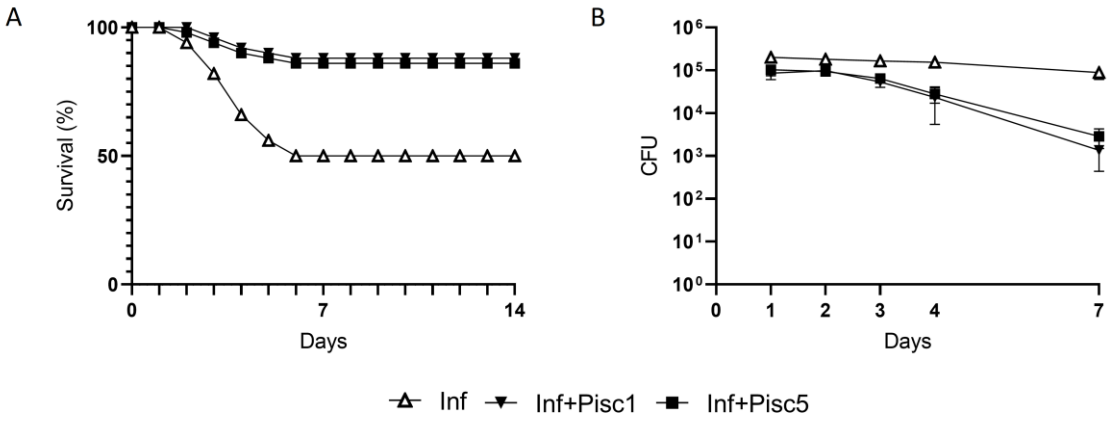

Figure 9. Survival curves (A) and CFU counts in the spleen (B) of sea bass infected with *P. damselae,* followed by the administration of piscidins 1 or 5, 24 hours later (post-

infection). Mortality was assessed during 14 days of infection and colony forming unit (CFU) values are expressed as means ± standard deviation (n=5).

Infected fish that were post-treated with both piscidins showed a slight reduction in the hematocrit at the beginning of the experiment, which was not observed in the pre-infection experiment. Still, these fish rapidly recovered to values comparable to controls, at day 3 post infection (Figure 10).

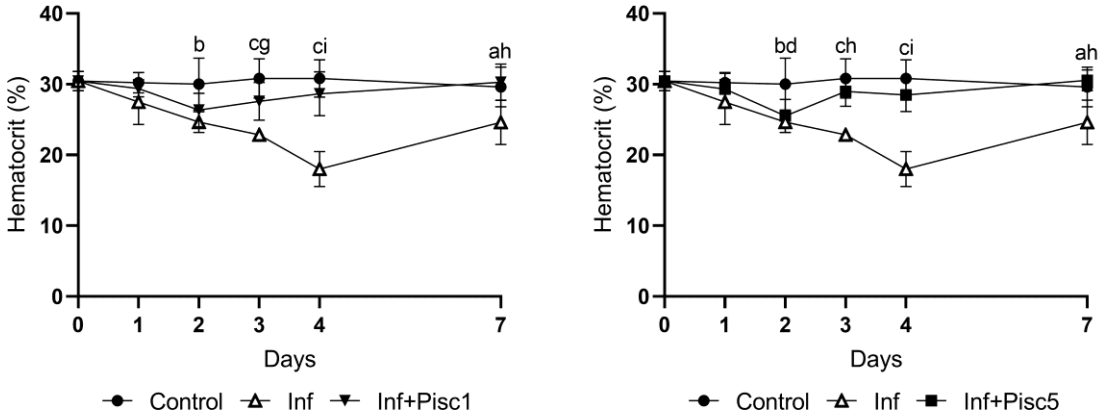

Figure 10. Hematocrit of sea bass infected with *P. damselae,* followed by the administration of piscidins 1 or 5, 24 hours later. Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 2, 3, 4 and 7 days post infection. Differences among groups were considered significant at $p<0.05$, $p<0.01$, and $p<0.001$, represented respectively by the letters a, b, c between control and Inf animals, d, e, f between control and Inf+Pisc1/5 and g, h, i between Inf and Inf+Pisc1/5.

Discussion

Fish antimicrobial peptides (AMPs) are involved in several functions, including antimicrobial activity, modulation of immune responses or iron metabolism regulation [1]. While the direct activity against pathogens and the immunomodulatory roles are attributed to different AMPs, although likely through different mechanisms, the regulation of iron is attributed to hepcidin, a small cysteine rich antimicrobial peptide. In the European sea bass (*Dichentrarchus labrax*), contrary to mammals, where a single hepcidin exerts both a role in antimicrobial response (albeit limited) and iron metabolism regulation, two types of hepcidin can be found, with the type 1 hepcidin (Hamp1) being involved in the regulation

of iron, and the several type 2 hepcidins (Hamp2) presenting antimicrobial activity against a variety of pathogens [3,12]. Furthermore, we recently described the protective effects of Hamp2, but not Hamp1, during infection with *Photobacterium damselae* spp. *piscicida*, reinforcing the roles of Hamp2 as an antimicrobial molecule. On the other hand, Hamp1 attenuated some of the effects of iron overload, which was not observed in Hamp2 treated animals [15]. As such, we raised the question if other AMPs, particularly piscidins, may also be involved in the regulation of iron.

To study the possible role of piscidins in iron metabolism, we first tested the administration of a low and non-hemolytic dose of each synthetic peptide and evaluated hematological parameters and liver iron content, as well as the expression of several genes involved in iron regulation and erythropoiesis. While piscidins 1, 4 and 6 had no impact in the parameters analyzed, administration of piscidins 2 and 7 resulted in significant decreases in the hematocrit. This resembles what is observed for hepcidin: while Hamp1 also promotes significant declines in hematological parameters, Hamp2 shows no such effect on healthy fish [14,15]. As such, different piscidin peptides may also be involved in different roles. Piscidin 5 also led to decreases in the hematocrit, but we first could not exclude a possible hemolytic effect of this peptide, based on *in vitro* evidences. Still, the dose used was theoretically low enough to not promote a significant hemolytic effect and, in fact, no signs of hemolysis were observed in samples collected from these animals. Thus, piscidin 5 likely induced some mechanism that led to the anemia observed during the last days of experiment, but further studies are needed to validate this hypothesis. Taking into consideration these observations, we focused on piscidins 2 and 7 and tested the administration of a higher dose of each piscidin in fish. This second experiment reinforced what was observed in the first one: piscidins lead in fact to a condition of anemia in fish, although the hematocrit and liver iron content show some differences, most likely due to the high dose administered.

The results obtained from these two experiments raised an important question: what are the mechanisms triggered by piscidins, resulting in a significant lower hematocrit? If we compare the administration of piscidins and Hamp1, both led to anemia in fish, but the dynamics seem quite different. Administration of Hamp1 leads to anemia as early as the first day of experiment and variations in the expression of several iron related genes – such as *sl11a2alpha, fth* and *fpn1* – reinforce the active role of type 1 hepcidin in the regulation of iron metabolism: this peptide binds to ferroportin, leading to it internalization and degradation in the hepatocytes, reticuloendothelial macrophages and enterocytes, suppressing the release of iron from these cells [9–11,31], which in turn leads to an increased iron storage in the form of ferritin and a further suppression of iron uptake in the intestine, mediated by *sl11a2alpha* [15]. In the head kidney and spleen, the hematopoietic

tissues of fish, decreases are observed in the expression of *epo,* coinciding with the development of anemia [32]. Likewise, during infection, hepcidin limits iron availability for pathogens as a mechanism of defense. However, as a long term effect, this will impair erythropoiesis, leading to a condition known as anemia of inflammation [10,31]. Fish treated with piscidins presented lower hematocrit values, but only during the last days of the experiment, and genes analyzed showed different patterns of expression. Furthermore, while Hamp1 led to a down-regulation of *epo* in both hematopoietic tissues, in piscidin treated fish, this effect was only observed in the head kidney, with no impact in the spleen. The results obtained points towards a different mechanism triggered by piscidins, not directly interfering with the uptake and mobilization of iron and acting specifically in the head kidney. The hormone erythropoietin (EPO) is essential to maintain the regular levels of erythrocyte cells. In mammals, erythropoietin deficiency is associated with anemia in chronic kidney disease and anemia of inflammation [33]. If piscidin is suppressing the expression of *epo,* it is probably leading to an impaired erythropoiesis and thus leads to the lower hematocrit levels during the last days of experiment. However, further studies will be required to better elucidate this matter.

Nevertheless, we must take into consideration the dose administered, as in our previous studies of Hamp1 administration, the dose used was higher than our first piscidin experiment [15,32]. The influence of different doses of hepcidin was previously documented, with the most prominent decreases in ferroportin expression being in fish treated with higher doses of hepcidin [12]. In fact, a high dose of piscidin resulted in more pronounced effects, with piscidin 2 leading to a significant iron accumulation in the liver, which was not observed for piscidin 7. Since we observed these effects with a higher dose, we can also consider another possibility: as the pathway by which each piscidin interferes with iron metabolism or erythropoiesis is still unknown, and the administration of a 6.25 µM solution of piscidin resulted in a significant up-regulation of *hamp1* expression, we cannot exclude a possible dose-dependent effect of piscidin on hepcidin. It is known that AMPs exert several immunomodulatory functions, one of them being the stimulation of other AMPs or inflammatory molecules, which can also explain the increased expression of *hamp2* [34]. During inflammation/infection, hepcidin is induced by inflammatory cytokines, one of them being IL-6, through the IL-6/JAK/STAT pathway [13], often leading to the anemia of inflammation. However, the data present in this study is insufficient to validate this hypothesis and expression analysis of genes involved in iron metabolism and hepcidin regulation will be needed.

To further study the involvement of piscidins 2 and 7 in iron regulation, we tested the administration of these two peptides in fish subjected to experimental iron overload. In fact, when iron overloaded fish are treated with both piscidin 2 or 7, the effects of iron are

attenuated, with the hematocrit and the liver iron content being lower, when compared to iron overload alone. As vertebrates do not present an active pathway of iron excretion, this element must be tightly regulated in order to avoid the toxic effects caused by its excess. While anemia or iron deficiency usually leads to decreased hematological parameters, the opposite does not necessarily happen, as in many cases iron overload can also have no impact on hematological parameters or can even lead to anemia [35,36]. However, in our models of iron overload in sea bass, hematocrit and red blood cells usually increase with iron overload, despite the increase in the expression of *hamp1* [13,15,32,37]. This is probably an unknown mechanism present in fish to cope with the excess of iron, using it in the production of more hemoglobin and red blood cells, keeping iron in a non-toxic form [15]. In the case of Hamp1 administration after iron overload, both RBC counts and hematocrit show no changes, and serum iron is reduced, but the accumulation of iron in the liver is even higher, and it is accompanied by an increased suppression of iron absorption in the intestine. As such, hepcidin signals the body to store iron in the liver and to suppress iron mobilization and absorption, due to the high amount of iron in circulation [15]. In the present study, piscidin administration led to variations in the parameters analyzed: while still higher than controls, the accumulation of iron was less pronounced with piscidins than in iron overload alone, which was not observed with the treatment with Hamp1. This is the first report that show a possible involvement of piscidins in iron metabolism. Thus, a detailed study is needed, as we did not determine serological parameters, such as serum iron or transferrin saturation, or evaluated the alterations in gene expression. Still, we could speculate that piscidins are leading to an alternative distribution of iron in excess in fish. To evaluate that, we determined the spleen iron content of fish treated with piscidins. In iron overload alone, there is a retention of iron during the first days of experiment. Assuming the erythropoietic function of the spleen, the excess iron is most likely used to produce more erythrocytes, coinciding with the usual increases in RBC counts and hematocrit observed in iron overloaded fish [13,15,32,37]. Thus, this leads to a fast decrease of iron content in this tissue, while in the liver, the major reservoir, this element is kept for a longer period of time. However, when iron overloaded fish are treated with piscidins, no accumulation of iron is observed. This is noteworthy, as in healthy fish treated with piscidins, it seems that peptides lead to a slight retention of iron in the spleen, but the same outcome is not observed in iron overloaded fish. As such, we cannot exclude the involvement of other tissues with functions on iron metabolism and erythropoiesis. The intestine is a good candidate, as this tissue shows the highest basal expression of piscidins (with the exception of piscidin 2, that is highly expressed in the spleen) [4], and may assume a yet unknown role. Considering the results obtained, there are some questions that must be addressed in the future: how do piscidins

lead to decreases in hematological parameters at such a low dose, accompanied by decreases in *epo* and *epor?*; does a high dose of piscidin will eventually lead to an up-regulation of *hamp1* and, consequently, an impairment in iron homeostasis?; what is the exact role of piscidin under a condition of iron overload and what is it relationship with hepcidin? Finally, the pathways triggered, tissues, cells and receptors involved are yet unknown, but it seems likely that they respond to more than one piscidin type, despite the differences in the amino acid sequences. Still, it seems that piscidins 2 and 7 (and perhaps piscidin 5) are more involved in this role, while the other may assume antimicrobial roles. Nevertheless, piscidin 2 seems to promote more significant effects than piscidin 7. Assuming the unique pattern of basal expression of piscidin 2, this peptide might be involved in other functions.

After studying the possible involvement of piscidins in iron metabolism, we tested the potential beneficial effects of piscidins during infection. Studies addressing peptide administration in sea bass are limited to hepcidin, with fish presenting high survival rates during infection with *V. anguillarum* or *P. damselae* [15,38]. We previously determined the antimicrobial activity of piscidins, with piscidins 1 and 5 being the most active peptides against bacteria and parasites [4]. Considering the low minimal inhibitory concentrations obtained with these two piscidins, including *P. damselae*, we chose them as the most promising ones to be used in infection experiments. Then, we took two different approaches: peptide administration before infection, to evaluate a possible prophylactic potential of piscidins; or peptide administration after infection, to test the potential therapeutic effect of these small proteins.

In both cases, data clearly show that piscidins were able to protect fish from infection. The results are even more pronounced in the pre-infection experiment, so piscidins seem to be promising prophylactic compounds. Still, infected fish treated with piscidins 1 or 5 recovered very well from infection, showing that piscidins can be also used as effective therapeutic agents during the first stages of infection, being highly effective in controlling the progression of infection and hamper the anemia of inflammation. However, we cannot ignore that piscidin 5, at 6.25 µM, led to decreases in the hematocrit of healthy fish. Still, during infection, fish did not show any sign of increased susceptibility with the administration of piscidin 5, but instead, they became resistant to infection. The effects of piscidin 5 in the hematocrit were only observed at days 10 and 14 of the experiment. Thus, this peptide may have a beneficial effect during the early stages of infection that supplants the late deleterious effects, either by direct effect on the pathogen, or triggering some immune mechanisms, limiting the progression of infection.

In our previous experiment of infection and peptide administration, the use of a 50 µM solution of Hamp1 actually made fish more susceptible to infection, showing slightly higher

mortalities, when compared to infected animals, while 50 µM of Hamp2 led to an increased fish resistance. The fact that Hamp1 led to a condition of anemia, as early as day 1 post administration, introducing a debilitative state in fish, is most likely the reason why these mortalities were observed [15]. Nevertheless, we must take into consideration the doses used, as in the case of Hamp1, lower doses would have a more limited impact [15]. The dose of piscidins administered was much lower, but we demonstrate that both piscidins 1 and 5 are effective at a low concentration. Considering the potential application in aquaculture at a large scale, the use of these peptides can become more cost-effective (particularly piscidin 1, with 22 amino acids), since the costs of mass producing synthetic peptides are still currently considerably high (when compared with antibiotics and other antimicrobial substances), but can be minimized by the fact that a low dose is effective in protecting fish. It is even possible that lower doses could produce similar effects, although that remains to be tested.

In summary, in this study we explored the potential roles of piscidins in sea bass, with piscidin 1 and 5 conferring a significant protective effect against bacterial infection, while piscidin 2 and 7 seem to be involved in the regulation of iron. Although these are preliminary studies, the results point to a function of piscidins in iron metabolism, as a possible alternative or complementary mechanism to hepcidin, the major iron regulator. Still, further studies are necessary to understand the exact pathways by which piscidins might regulate this element. The low mortalities observed during infection demonstrate that piscidins 1 and 5 can be considered as promising substitutes to the conventional treatments applied in aquaculture. The fact that these peptides are active against a wide range of pathogens including virus, bring the potential use of AMPs as effective anti-microbial compounds for aquaculture, something sorely lacking due to the limited activity of antibiotics. Finally, in a more practical point of view, testing other routes of administration is of the utmost importance in order to optimize the wide use of AMPs in aquaculture, as intraperitoneal administration, despite its proved effectiveness, is very costly and time consuming.

Material and methods

Animals

Healthy European sea bass (*Dicentrarchus labrax*), with an average weight of 50 g, were provided by a commercial fish farm in the north of Spain (Sonríonansa S.L., Pesués, Cantabria, Spain). Prior to the experiments, fish were acclimated for 30 days to the fish holding facilities of the Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Porto. Fish

were kept in 110 liters recirculating sea water (28±1 ‰ salinity) tanks at 22±1 ºC, with a 13/11-hour light/dark cycle and fed daily *ad libitum* with commercial fish feed with an iron content of approximately 200 mg iron/kg feed. Before each treatment, fish were anaesthetized with ethylene glycol monophenyl ether (2-phenoxyethanol, 3 mL/10 L, Merck, Algés, Portugal). All animal experiments were carried out in strict compliance with national and international animal use ethics guidelines (including ARRIVE guidelines), approved by the animal welfare and ethic committees of ICBAS (permit P293/2019/ORBEA, 05/04/2019) and conducted by experienced and trained FELASA Function A+B+D investigators.

***In vivo* experimental models**

The synthetic piscidin mature peptides were commercially produced (NZYtech, Lisbon, Portugal; Supplementary Table 1). To evaluate the effects of piscidin administration in different conditions, several experimental models were established (Supplementary Figures 6-8). Five fish from each experimental group were collected at the various time points after treatments and euthanized with an overdose of anesthetic. Blood and serum were collected for hematological and serological parameters determination. Fish were then dissected, tissues excised, snap frozen in liquid nitrogen and stored at -80 °C, for further use in tissue iron content determination and gene expression. Mortality was assessed during the experimental infections. Colony forming units (CFU) counts in the spleen of infected fish were performed. Briefly, the spleens were aseptically collected, homogenized in tryptic soy broth (TSB) 1% NaCl, serially diluted, plated on tryptic soy agar (TSA) 1% NaCl and incubated at 25ºC for 24-48 hours. No animals were excluded in any of the experiments.

Peptide administration. Fish were intraperitoneally injected with 100 µl of a 6.25 µM or 50 µM solutions of commercially synthesized sea bass piscidin peptides, diluted in sterile PBS. Samples were collected after 1, 4, 7, 10, 14, 21 and 28 days post-peptide administration (Supplementary Figures 6 and 7).

Iron overload. To induce iron overload, fish were intrap eritoneally injected with 100 µl of iron dextran (2 mg) (Sigma-Aldrich, St. Louis MO, USA) diluted in sterile PBS to a final concentration of 20 mg/ml. Samples were collected after 1, 4, 7, 10, 14, 21 and 28 days post-iron administration (Supplementary Figure 7).

Iron overload and peptide administration. Fish were first injected with iron dextran, followed by administration of 100 µl of a 50 µM solution of either piscidin2 or piscidin7

peptides 24 hours later, as previously described. Samples were collected after 1, 4, 7, 10, 14, 21 and 28 days post-iron administration (Supplementary Figure 7).

Infection. Photobacterium damselae spp. *piscicida* strain K1 (DSM 22834) was cultured to midlogarithmic growth in tryptic soy broth (TSB) growth medium, supplemented with 1% NaCl. After measuring absorbance at 600 nm, bacteria were washed and resuspended in sterile PBS to a final concentration of 10^6 CFU/ml. Fish were then intraperitoneally injected with 100 µl (10^5 CFU/fish) of bacterial suspension. Samples were collected after 1, 2, 3, 4 and 7 days post-infection (Supplementary Figure 8).

Peptide administration and infection. Fish were first injected with a 6.25 µM solution of either piscidin1 or piscidin5 peptides, followed by infection with *P. damselae* 24 hours later, as previously described. Samples were collected after 1, 2, 3, 4 and 7 days post-infection (Supplementary Figure 8).

Infection and peptide administration. Fish were first infected with *P. damselae*, followed by administration of a 6.25 µM solution of either piscidin1 or piscidin5 peptides 24 hours later, as previously described. Samples were collected after 1, 2, 3, 4 and 7 days post-infection (Supplementary Figure 8).

Controls. Fish were intraperitoneally injected with 100 µl of sterile PBS (Supplementary Figure 6, 7 and 8).

Hemolytic activity

The hemolytic activity of synthetic mature piscidin peptides and serum (obtained from the various experimental models) was determined. Briefly, blood was collected from healthy sea bass to a tube containing 100 uL of a 1:10 solution of EDTA diluted in PBS, centrifuged and supernatant discarded. The erythrocytes were then washed three times with PBS. Cells were then resuspended in PBS (1:20 dilution of washed erythrocytes) and incubated with serial dilutions of piscidin peptides or with 20 µL of each serum sample, for 1 h at 37ºC, in round-bottom 96-well plates, in a final volume of 200 µL. Plates were then centrifuged during 5 min at 4000 rpm, the supernatants (150 µL) were placed in flat-bottom 96-well plates and OD read at 540 nm in a plate reader. Erythrocytes in PBS and 0.1% Triton X-100 were used as negative and positive controls, respectively. The percentage of hemolysis was calculated using an equation described elsewhere [39]. *In vitro* hemolytic activity was obtained from two independent experiments.

Hematocrit and tissue iron content

Hematocrit was determined with microcapilaries, using 100 µl of blood in a 1:1 dilution with EDTA (1:10 diluted in sterile PBS) (BD Biosciences, San Jose CA, USA). Non-heme iron was measured in livers and spleens by the bathophenanthroline method [40]. Briefly, liver and spleen samples with an average weight of 100 mg were placed in unlidded 24-well plates and dried in and oven for 48h at 65°C. Subsequently, dry tissue weights were determined and samples digested with 37% hydrochloric acid, for 20h at 65°C. After digestion, supernatants were collected and a sample of each was added to a chromogen reagent (5 volumes of deionized water, 5 volumes of saturated sodium acetate and 1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycollic acid), in order to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The extinction coefficient for bathophenanthroline is 22.14 $mM^{-1}cm^{-1}$.

RNA isolation and cDNA synthesis

Total RNA was isolated from tissues with the NZY Total RNA Isolation kit protocol for tissue samples (NZYtech, Lisboa, Portugal) with the optional on-column DNase treatment, according to the manufacturer's instructions. Total RNA quantification was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and quality was assessed by running the samples in an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). For all samples, 2.5 µg of each were converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech) according to the manufacturer's protocol.

Gene expression analysis

Genes analyzed include genes involved in iron homeostasis and regulation (*hamp1* and *hamp2*), erythropoiesis (*epo* and *epor*), iron uptake (*slc11a2alpha*), iron storage (*fth*) and iron export (*fpn1*). The relative levels of these genes were quantified by real-time PCR analysis using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). A total of 2 µL of each cDNA sample was added to a reaction mix containing 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad), 2 µL double distilled H_2O, and 250 nM of each primer (Supplementary Table 2), making a total volume of 10 µL per reaction. A non-template control was included for each set of primers. The cycling profile was the following: 95 °C for 3.5 min, 40 cycles of 95 °C for 20 s and 59 °C for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reactions. Beta-actin (*actb*) was used as the housekeeping gene (M-value 0.177) (selected as the most stable gene among a suite of 5 candidates using the Delta CT method, Normfinder

and Genorm, through RefFinder, http://blooge.cn/RefFinder/) [41]. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold values was used to analyze gene expression levels.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 9 (GraphPad Software Inc, San Jose CA, USA). Multiple comparisons were performed with One-way ANOVA and *post hoc* Student Newman-Keuls test. A p value <0.05 was considered statistically significant.

Author Contributions

C.B. designed and conducted experiments, analyzed data and wrote the manuscript; J.V.N. designed, conducted and supervised the experiments, analyzed data and wrote the manuscript; P.N.S.R. designed and supervised the experiments; P.C., M.N. and J.F.M.G. conducted experiments.

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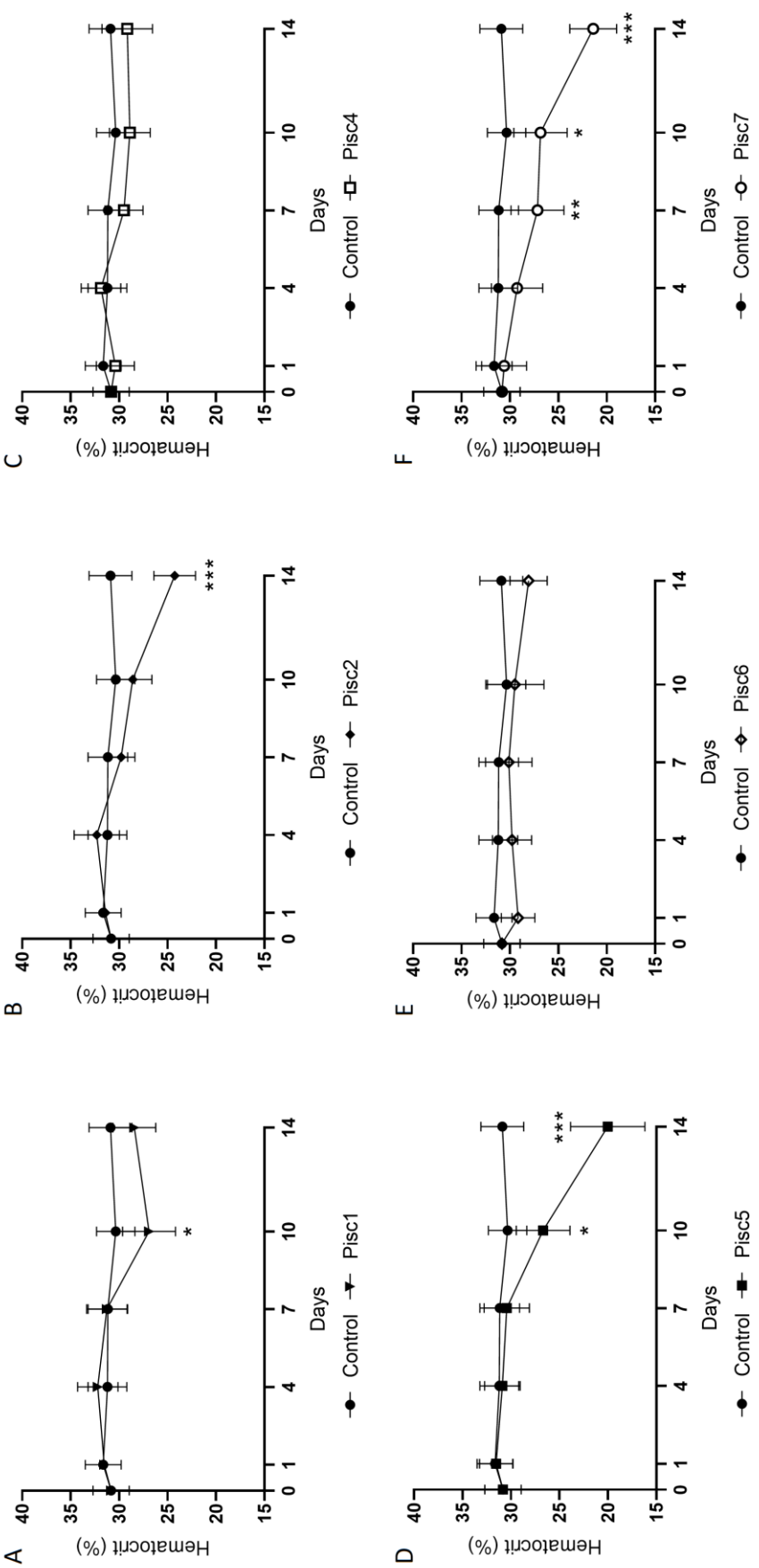
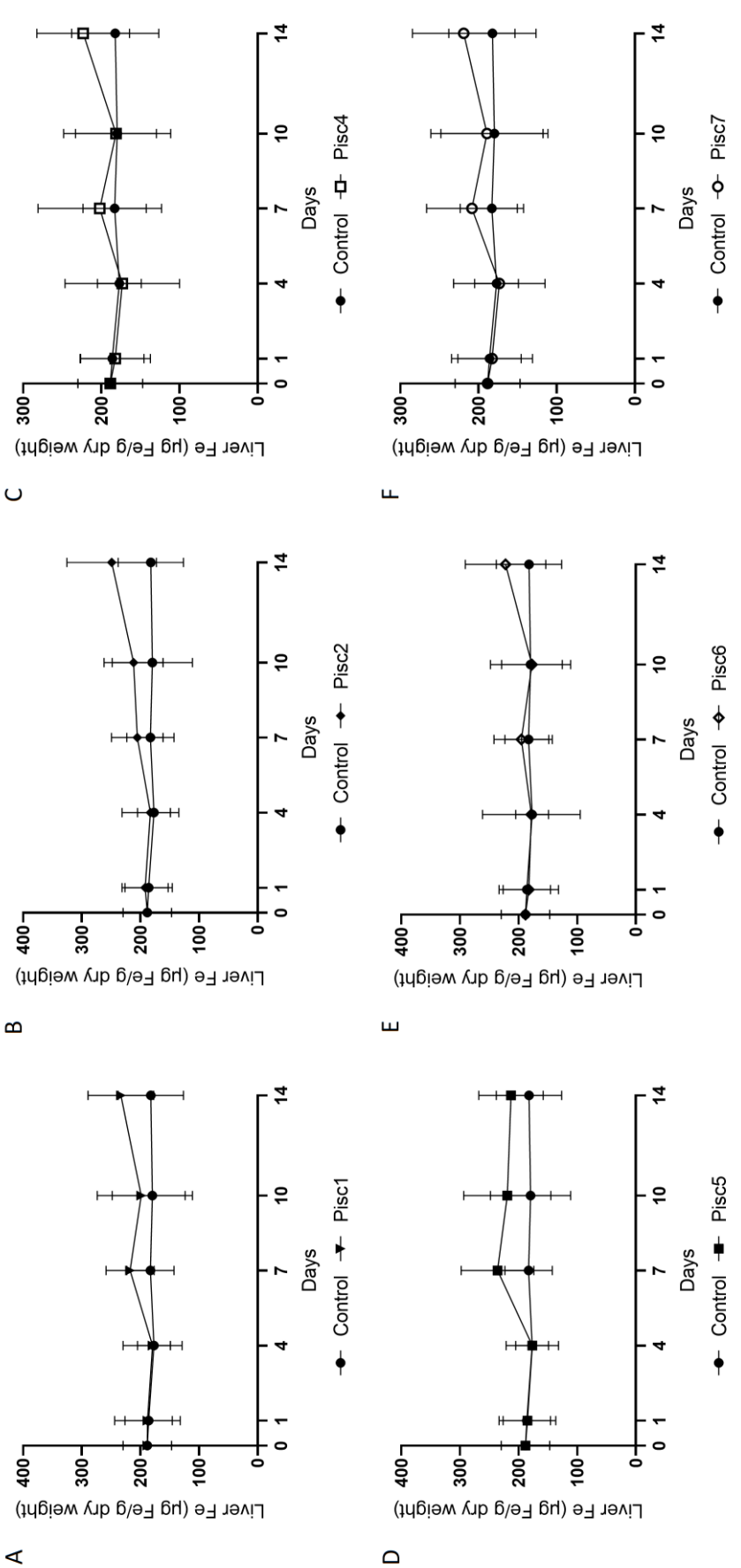
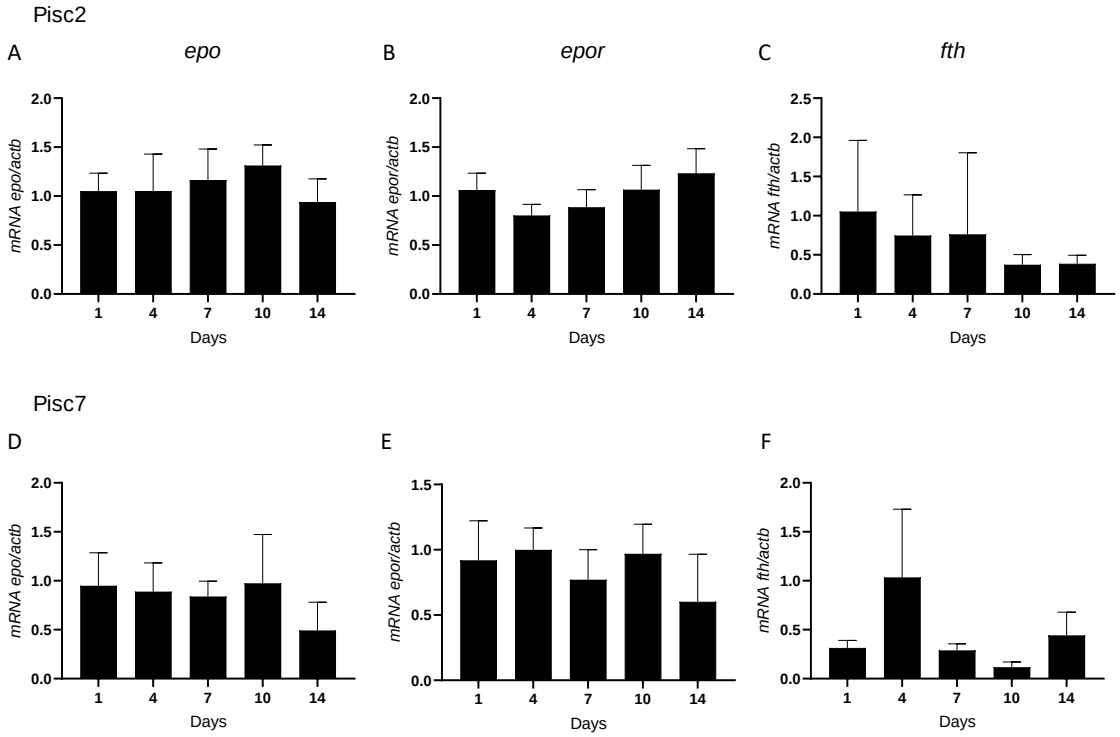
Supplementary Material



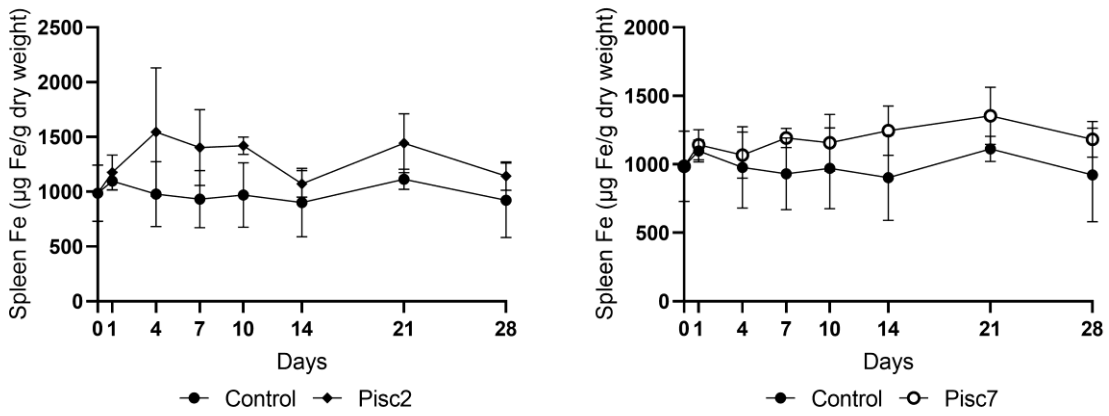
Supplementary Figure 1. Hematocrit of fish treated with each peptide. (A) Piscidin 1; (B) Piscidin 2; (C) Piscidin 4; (D) Piscidin 5; (E) Piscidin 6; (F) Piscidin 7. Values are expressed as means ± standard deviation (n=10). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.



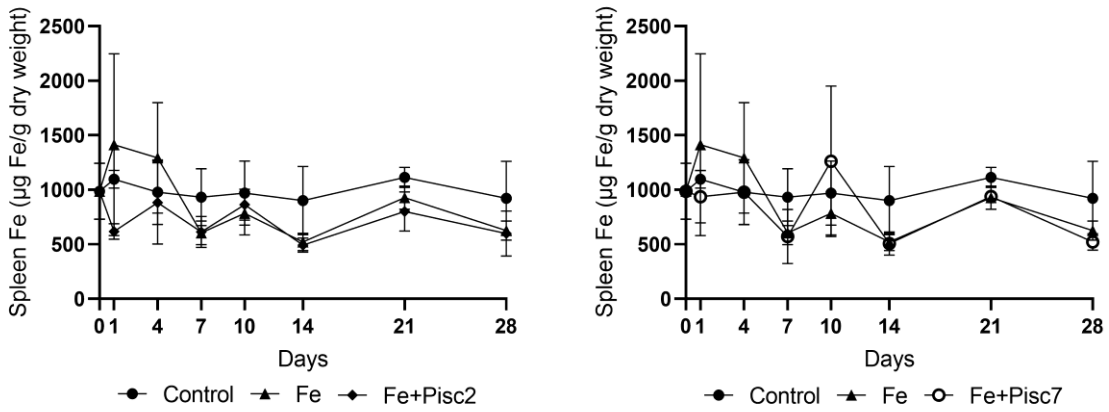
Supplementary Figure 2. Liver iron content of fish treated with each peptide. (A) Piscidin 1; (B) Piscidin 2; (C) Piscidin 4; (D) Piscidin 5; (E) Piscidin 6; (F) Piscidin 7. Values are expressed as means ± standard deviation (n=10). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.



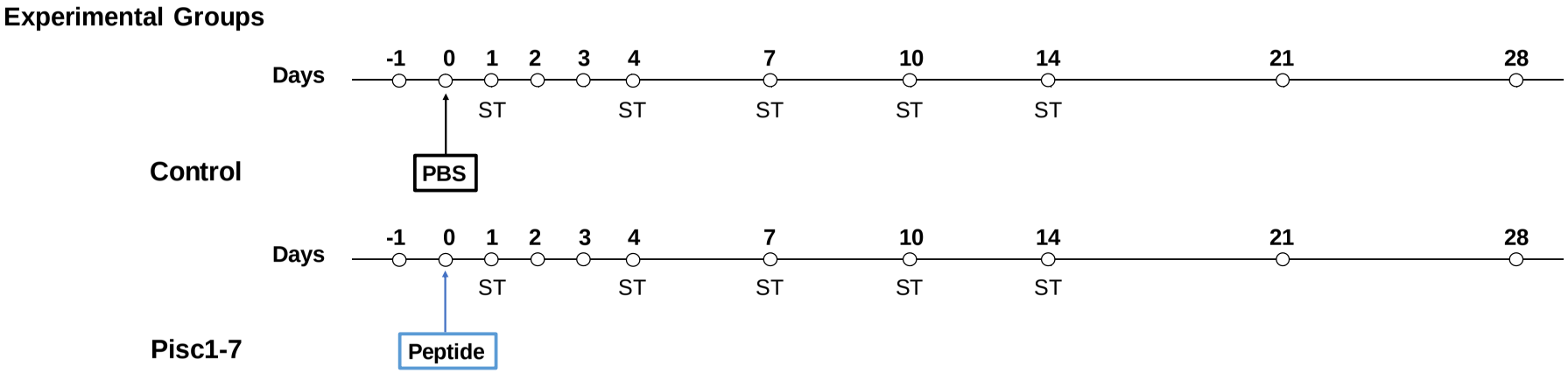
Supplementary Figure 3. Gene expression in the spleen at 1, 4, 7, 10 and 14 days after piscidin 2 or piscidin 7 peptide administration. (A) *epo*, (B) *epor*, (C) *fth* expression in the spleen of sea bass treated with piscidin 2; (D) *epo*, (E) *epor*, (F) *fth* expression in the spleen of sea bass treated with piscidin 7. *Actb* was used as the housekeeping gene. Values are expressed as means ± standard deviation (n=5). Differences among groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.



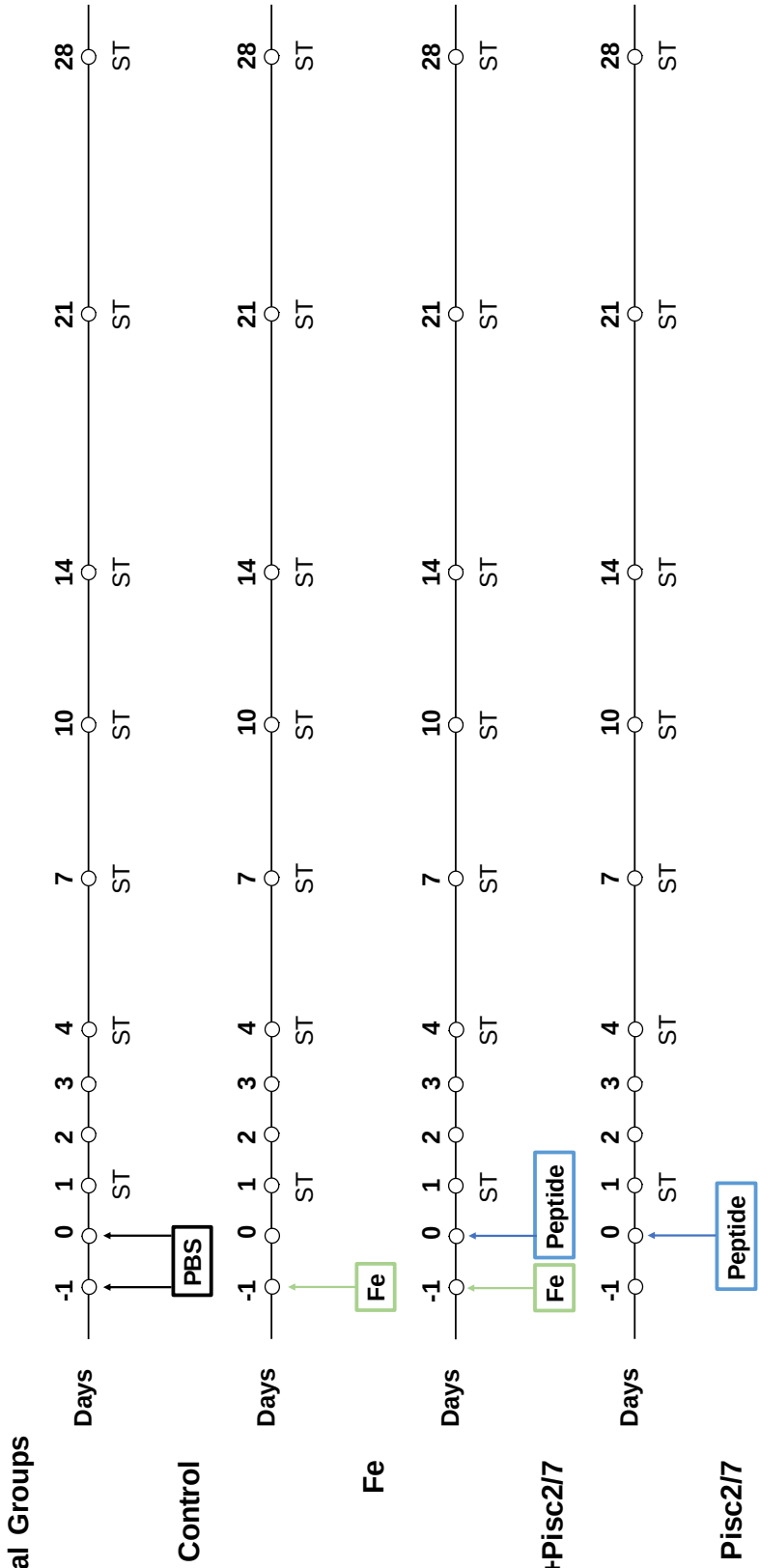
Supplementary Figure 4. Spleen iron content of fish treated with 50 µM of piscidin 2 or piscidin 7 peptides. Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration.



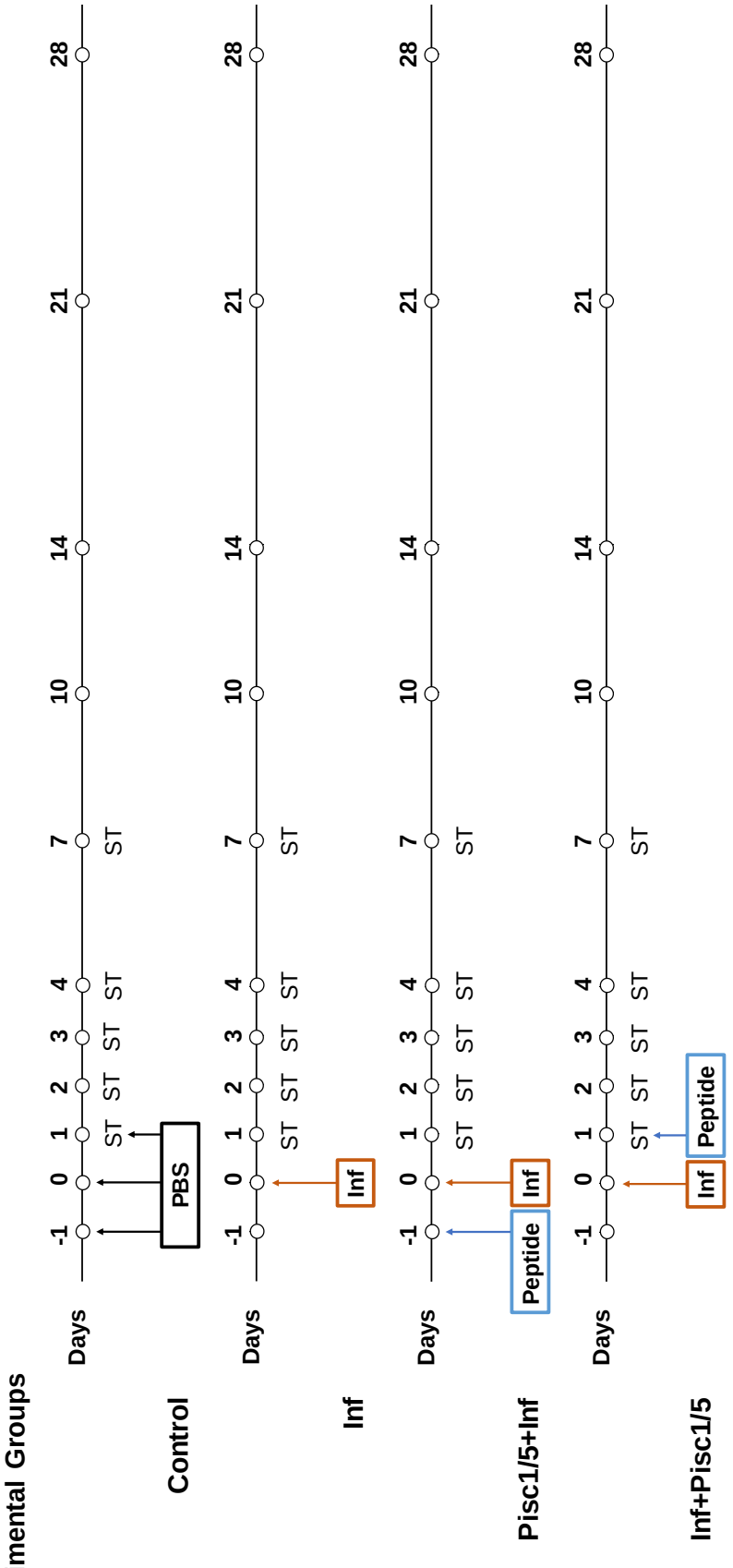
Supplementary Figure 5. Spleen iron content of sea bass after experimental iron overload (Fe) or iron overload and peptide administration (Fe+Pisc2/7). Values are expressed as means ± standard deviation (n=5). Samples were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration.



Supplementary Figure 6. Piscidin administration in healthy animals (Experiment 1). In our first experiment, healthy sea bass were treated with 6.25 µM of each piscidin (Pisc1 to 7). Blood and tissues were collected at 1, 4, 7, 10 and 14 days post peptide administration (ST – sampling time). The control group received PBS instead of the peptides.



Supplementary Figure 7. Iron overload and piscidin administration (Experiment 2). In our second experiment, different groups were established: Iron overload – fish were treated with 2 mg of iron dextran; Iron overload followed by peptide administration – fish received 2 mg of iron dextran, followed by the treatment with 50 µM of piscidins 2 or 7 (Fe+Pisc2/7), 24 hours later; Peptide administration – fish were treated with 50 µM of piscidins 2 or 7 (Pisc2/7). Blood and tissues were collected at 1, 4, 7, 10, 14, 21 and 28 days post peptide administration (ST – sampling time). The control group was equally manipulated, but received PBS instead of iron and peptides.



Supplementary Figure 8. Infection and piscidin administration (Experiment 3). In the third experiment, different groups were established: Infection – Infection (10^5 CFU of *P. damselae* strain K1/fish); Pre-infection - Pre-treatment with 6.25 µM of piscidin 1 or 5 (Pisc1/5), followed by infection with *P. damselae*, 24 hours later; Post-infection - Post-treatment with piscidin 1 or 5, 24 hours after infection with *P. damselae*. Samples from fish were collected after 1, 2, 3, 4 and 7 days of experiment (ST – sampling time). The control group was equally manipulated, but received PBS instead of bacteria and peptides.

Supplementary Table 1. Synthetic piscidin mature peptides.

Piscidin1	FFHHIFRGIVHVGKTIHRLVTG-NH_2
Piscidin2	FLGRFFRRTQAILRGARQGWRAHKAVSRYRDRYIPETDNNQEQP-NH_2
Piscidin4	FIHHIFRGIINAGKSIGRFITG-NH_2
Piscidin5	LIGSLFRGAKAIFRGARQGWRAHKAVSRYRAGYVRRPVVYYHRVYP-NH_2
Piscidin6	LFGSLKAWFKGGKQASRDYKYQKDMAKMNKRYGPNWQQGGGQQPPANAQANDQPPADAQANDQPS-NH_2
Piscidin7	FLGRVKSMWSGVRNGYKAYKYQRNMAKMNKGYGPNWQQGGGQEPPADAQANDQPP-NH_2

Piscidins were commercially synthetized (NZYTech, Lisbon, Portugal) with an additional C-terminal amidation.

Supplementary Table 2. Primers used for gene expression analysis.

		FOR (5' → 3')	REV (5' → 3')
actb	Actin, beta	CAGAAGGACAGCTACGT	GTCATCTTCTCCCTGTTGGC
hamp1	Hepcidin 1	CATTGCAGTTGCAGTGACACT	CAGCCCTTGTTGCCTCTG
hamp2	Hepcidin 2	CTGCTGTCCCAGTCACTGA	ACCACATCCGCTCATATTAGG
fpn1	Ferroportin	GGCCTACTACAACCAGAACAT	AGGCCGCACTTCTTGCGAA
fth	Ferritin H	AACCATGAGTTCTCAGGTGAG	TTAGCTGCTCTCTTTGCCCAG
slc11a2alpha	Solute Carrier Family 11 Member 2, alpha	CGCGTTCAACCTCCTCTCCTCT	AGCCCTCGCAGTACGGCACA
tfr1	Transferrin receptor 1	CTCCTTCAACCACACCCAGT	GACCAGTACCGAGGTTCCAA
epo	Erythropoietin	AGGCCAATCTGTGACCTGAG	GCAGTGCTGTGTTGGTGACT
epor	Erythropoietin receptor	GCCTATGTCACCCTCAATGC	GAGTCTGCCACTGCCATGTA

Chapter VI

General Discussion, Conclusions and Future Perspectives

The multiple antimicrobial peptides of the European sea bass (*Dicentrarchus labrax*)

During evolution, vertebrates have faced duplication of certain genes, through segmental, tandem, or even entire whole genome duplications (WGD). Jawed vertebrates likely arose after two events of WGD (2R), and a third WGD occurred in the lineage of ray-finned fishes (3R or fish-specific WGD), giving rise to the teleost genome organization, with an additional duplication occurring in the salmonid genome (4R or salmonid-specific WGD) [1–5]. The increased number of certain genes in fish species led to the hypothesis that these events of duplication separated fish from other vertebrates and, in particular, the teleost group from the non-teleostean lineages. The infraclass Teleostei is formed by nearly 30000 species, divided into 70 orders and 500 families, many more than the basal Actinopterygian lineages, that consist in approximately 40 species in 4 orders. The occurrence of a specific event of WGD likely provided the foundations for species radiation and diversification found among the teleostean [1,6,7]. An event of WGD can be followed by an extensive gene loss, as well as genomic rearrangements and evolutionary events on the retained duplicated genes, including subfunctionalization, in which the functions of the ancestral gene are divided among the duplicated genes, or neofunctionalization, through the acquisition of a new function by one of the duplicates [8,9]**.**

Results obtained in the present thesis and in previous work conducted in our lab [10] point towards a higher diversification of the hepcidin and piscidin families in the European sea bass (*Dicentrarchus labrax*, Moronidae family), while others, particularly beta-defensins, are more limited. We were able to isolate four type 2 hepcidins, asides the single type 1 [10], as well as six different piscidins (Chapter IV), but we have evidences of the presence of more piscidins and hepcidin type 2 isoforms. This is strikingly different from what is observed in other groups of fishes, particularly Cyprinids and Salmonids. These species seem to lack the presence of piscidin and hepcidin type 2 orthologues, but the beta-defensin family is much more diversified [11–15]**.** Due to the wide nature of beta-defensins, these peptides can be found in different fish species belonging to different superorders. However, piscidins and type 2 hepcidins are more restricted to certain orders or superorders, but are found in different groups of fish. While type 2 hepcidins seem to be limited to the Acanthopterygii Superorder, piscidins were also found in other species, particularly the Atlantic cod (*Gadus morhua,* Order Gadiformes, Paracanthopterygii Superorder). A significant diversity has been observed in piscidin and hepcidin sequences

among the different teleost species, likely a result of gene duplications and positive selection in the regions of AMP genes that encode for the mature peptides [16–19].

Piscidins have been well characterized in a variety of teleost fishes, being found in a different number for each species studied. Although the piscidin mature peptides present for each fish species are often dissimilar at the aminoacidic level, the main characteristics seem to be retained: they are usually small, comprised by a high number of hydrophobic and positive amino acids, fold into a linear and amphipathic alpha-helical peptide, and exert broad activity against several fish and non-fish pathogens [20–26]. In some species, such as the Nile tilapia (*Oreochromis niloticus*), striped bass (*Morone saxatilis*), white bass (*M. chrysops*) and their hybrid (*M. chrysops* × *M. saxatilis*), up to five piscidin peptides were documented, with profound differences in their sequences and antimicrobial activities [22,24,27,28]. Salger et al. [24] proposed to classify the six bass piscidins characterized into different classes, according to the size of the mature peptide and the antimicrobial activity: Class I, that includes the smaller piscidins (piscidins 1 and 3, with 22-aa), show broad antimicrobial activity against bacteria and parasites; Class II piscidins (piscidins 4 and 5, with 44 and 46-aa, respectively), also with antimicrobial activity against several pathogens; and Class III piscidins (piscidin 6 and 7, with 55-aa), mostly showing antiprotozoal activity. We can also divide sea bass piscidins into different classes or sub-groups, depending on the size of the mature peptide and amino acid composition, but the antimicrobial activities of the several piscidins are highly diverse, even within each sub-group. Piscidins 1 and 5 are highly active against most of the pathogens tested, while piscidins 6 and 7 show the weakest antimicrobial activities (Chapter IV). Still, we could speculate that these piscidins might have some antimicrobial activity against pathogens not analyzed in our study, such as marine parasites, as previously observed [24]. Furthermore, due to the atypical nature of piscidins 6 and 7 (65 and 55 amino acids, respectively, and low hydrophobicity and amphipathicity), we suggest that these peptides might be involved in other immune and non-immune functions, and do not act directly on pathogens. In fact, evidences of the present thesis indicate that piscidin 7 (as well as piscidin 2, that shows a moderate activity), might be involved in iron metabolism, while other piscidins mostly perform antimicrobial roles (Chapters IV and V). The systemic regulation of iron is performed by hepcidin, a cysteine rich AMP mostly produced in the liver. The mammalian hepcidin performs both functions, although the antimicrobial activity is limited. However, in many fish species, two different hepcidin types can be found, showing a significant degree of subfunctionalization [10]. In the case of piscidins, events of neofunctionalization might have led to these peptides gaining new functions, asides the direct bacterial killing and immunomodulatory roles.

The structures and interactions with membranes of bass piscidins 1 and 3 are very well demonstrated [29–34]. However, information regarding the structural features of other piscidins is limited and only one study aimed to determine the folding and mode of action of bass piscidin 4 (similar to our piscidin 2, with 44-aa). Piscidin 4 presents a disordered structure in aqueous solution, but folds into a weak alpha-helical structure in membrane-mimetic environments. Still, it has been suggested that this peptide can fold into additional conformations, that may be responsible for its selective activity [35]. We predict that all our piscidins are likely alpha-helical and show different degrees of hydrophobicity and amphipathicity. The amino acid sequences, secondary structures, hydrophobicity and amphipathicity are crucial for peptide activity on the different pathogens (and different pathogens show different degrees of resistance to cationic peptides) [36]. If we compare the activities of sea bass piscidins 1 and 4, that are more similar to bass piscidins 1 and 3, the first is generally more active than the second [24,37]. In a previous study, the lower amphipathicity was associated with the lower hemolytic activity of piscidin 3 (and also showed lower antimicrobial activity) [37]. In fact, there are differences between sea bass piscidins 1 and 4 in terms of amino acid sequence, hydrophobicity and amphipathicity, that can explain the differences in the *in vitro* antimicrobial and hemolytic tests. However, it was recently demonstrated that bass piscidin 1 and 3 actually act through different, but complementary, mechanisms, with piscidin 1 being more membrane permeabilizing and piscidin 3 showing stronger DNA interactions, such as DNA condensation or damage [31,38]. Still, whether sea bass piscidin 4 promotes its activity through similar mechanisms needs further examination. In the case of piscidins 6 and 7, the lower hydrophobicity and amphipathicity might explain the reduced activity on the pathogens analyzed. Nevertheless, the differences in these parameters do not fully explain the minimal inhibitory concentrations (MICs) obtained in the present study. In fact, piscidin 5, together with piscidin 1, showed the lowest MIC values and the highest hemolytic activities, but this peptide presents a much lower hydrophobicity and similar amphipathicity, when compared to piscidin 1. As such, the high activity of piscidin 5 is most likely not totally dependent on these features, but the exact mode of action remains to be elucidated.

Beta-defensins constitute a group of AMPs found in many different vertebrate species, and include six cysteines at conserved positions, that bind into a particular pattern of Cys1–Cys5, Cys2–Cys4 and Cys3–Cys6 [13,39–44]. On the contrary, alpha-defensins and the cyclic theta-defensins are typical of mammals, with theta-defensins being restricted to Old World monkeys [45–47]**.** The presence of beta-defensins in more primitive vertebrates, such as fish or reptiles, may indicate that these peptides constitute a more ancient type of defensin, and the others seem to arose only in the mammalian lineage [47]. Beta-defensins present some similarities with the invertebrate big defensins,

including the genomic organization, identical cysteine bridges and secondary structures [48–51]. Thus, it is generally accepted that beta-defensins arose from these big defensins, through processes of intronization of exonic sequences or exon shuffling, leading to the loss of the amino-terminal domain and the appearance of an ancestral vertebrate beta-defensin, with a two exon/one intron organization [15,52].Then, independent events of intron insertions have led to the appearance of the three exon/two intron structure observed in birds and fish, but mammals retained the original two exon/one intron organization [14,15]**.**

Within fish species, beta-defensins 1 and 2 show a high degree of conservation among the different species, with the exception of the olive flounder (*Paralichthys olivaceus*). However, other species, such as Salmonids, present a diverse group of beta-defensins [14,15,53]. In this particular case, the several beta-defensins found in this group of fishes is likely a result of the additional WGD that occurred in the Salmonid lineage [15]. Comparing with Perciform species, Salmonid beta-defensins 1 and 5 are much more similar with beta-defensins 1 and 2. The phylogenetic and syntenic analysis performed in the present thesis (Chapter II) reinforce these findings, as these peptides are positioned in the same cluster of the phylogenetic tree and the genetic loci show similarities. Nevertheless, Salmonids show species-specific differences, being not only the presence of additional defensins, but these two particular peptides (common to most of fishes) were duplicated in Salmonids, leading to the appearance of two similar beta-defensins 1 (1a and 1b) and beta-defensins 5 (5a and 5b) [15]. The succeeding evolutionary events that occurred in these different fish species led to the retention of the several duplicated genes in Salmonids, but in sea bass, beta-defensins became much more limited.

Sea bass beta-defensins show the main characteristics observed in the ones isolated in other species, including the six cysteines at conserved positions, that fold into an antiparallel triple-stranded beta-sheet [13,54–57]. Beta-defensin 2 also presents the motif CPRRYK, between Cys4 and Cys5, typical from the type 2 beta-defensins (although beta-defensin 3 also presents this motif, but this type 3 peptide was not yet isolated in Perciform species) [13,58]. Furthermore, beta-defensin 1 also folds into an extra alpha-helix at the N-terminal. Many fish defensins present this secondary structure, asides the three beta-sheets, but the alpha-helix can be predicted either in type 1 [13,54,57] or type 2 beta-defensins [56,59]. In our study, using the models available in SWISS-MODEL, we predicted the existence of an alpha-helix at the N-terminal of beta-defensin 1, but not in beta-defensin 2. Only one report showed a similar result, after isolation and structure prediction of zebrafish (*Danio rerio*) beta-defensins [13]. This fish species presents three different peptides, but it seems that only beta-defensin 1 folds into this particular tertiary structure. Still, there are two different factors that we must consider: these are theoretical

models, determined using structures available for other species; and many studies isolated a single defensin [54–57,59], but those species might express at least a second peptide. For instance, a single beta-defensin (belonging to the beta-defensin 2 subfamily) was recently characterized in the turbot (*Scophthalmus maximus*) and folds into a tertiary structure comprised by the three beta-sheets and an alpha-helix at the N-terminal [59]. However, analyzing the genome of turbot available in Ensembl database, we were able to identify the turbot beta-defensin 1 (Chapter II). As such, the alpha-helix can be found either in type 1 or type 2 beta-defensins. Nevertheless, in sea bass, using the templates available in SWISS-MODEL server, we were not able to predict an alpha-helix in the N-terminal of beta-defensin 2.

Fish beta-defensins are active against several bacteria and viruses (See Appendix 1), but the antimicrobial roles of these peptides against fungi and parasites were not yet addressed. These peptides are also chemotactic for fish leucocytes. The chemotactic activity of human and mice beta-defensins towards different cells is also documented [60,61]. Although the amino acid sequence of fish and human beta-defensins shows many differences, the overall tertiary structure of these peptides is similar. Still, in a previous study, the authors observed that head kidney leukocytes were attracted to the recombinant sea bream defensin 1, but the same was not observed using the recombinant human peptide [62]. The pathway by which fish defensins exert their chemotactic roles is still poorly understood, but it seems that there is a degree of specificity by fish receptors towards fish peptides, and not to the human ones [62]. Chemokines also present similarities in the tertiary structures and mammalian beta-defensins bind to certain chemokine receptors [60,61]. However, it is yet unknown if the same pathways are present in fish. Due to the high degree of conservation between sea bass and other fish peptides, we speculate that sea bass beta-defensins also exert similar functions, but further studies will be needed to confirm the antimicrobial and chemotactic activities of these peptides.

Antimicrobial peptides as novel sustainable compounds for aquaculture

During the last decades, the Mediterranean aquaculture has grown rapidly, with the European sea bass (*Dicentrarchus labrax*) and gilthead sea bream *(Sparus aurata*) being the dominant produced species. However, this large growth brought a number of major

problems, one of them being the higher occurrence of disease outbreaks and the use of large quantities of antibiotics to fight them [63,64].

Antibiotics are routinely used in aquaculture as prophylactic agents, mostly through medicated feed and occasionally by bath, before the appearance of the clinical signs of the disease, to avoid the potential emergence and spread of the infectious agent. Thus, the doses administered in aquaculture can be much higher than those established for terrestrial animals [65–67]. Due to the uncontrolled use of these antimicrobials, bacteria that cause major diseases in aquaculture facilities currently show mechanisms of resistance to many different antibiotics [64]. Furthermore, it has severe implications for human health and the environment, as high amounts of drug residues can enter in the human food chain and in the surrounding environment. These residues usually remain stable and at sub-therapeutic levels for long periods in water systems and sediments, exerting selective pressure in these areas and providing the optimal conditions for the emergence of resistant bacterial strains, including some important human-infecting bacteria [66].

While in Norway effective vaccination procedures led to a profound reduction of the use of antibiotics [68], in the Mediterranean area, the methodologies established have been proven less effective. Nevertheless, there are already some commercial vaccines for the prevention of diseases caused by *Betanodavirus*, *Vibrio anguillarum* and *Photobacterium damselae*, with others being currently under development for other pathogens, such as *Tenacibaculum maritimum* [69]. Due to the reduced number of vaccines available, or the limited protection that those vaccines may provide, researchers are now focusing their attention on alternatives to antibiotics, with AMPs being promising candidates.

Most of the studies available used tilapia piscidins 3 and 4 [70–74] and grouper (*Epinephelus coioides*) epinecidin [75–81], peptides that were tested in fish or mammalian models. Some authors also addressed the applications of bass (*Morone* spp.) piscidin 1 in rodents [82–85], but the studies testing the administration of piscidins belonging to Moronidae species in fish are extremely limited. The use of AMPs as novel therapeutic or prophylactic compounds in the production of sea bass is almost inexistent, with only one study addressing the effects of a synthetic hepcidin in sea bass infected with *V. anguillarum,* with fish showing high survival rates [86]. One of the issues that hinder a broader study on the use of AMPs in sea bass is the lack of knowledge on the families of antimicrobial molecules in this fish species. In the present thesis, we were able to isolate several peptides belonging to two different AMPs families and provide some information regarding their sequences, structures and antimicrobial activities (Chapters II and IV). Furthermore, we also tested synthetic peptides derived from sea bass AMPs in experimental models of infection, in order to understand the protective effects of sea bass

antimicrobial molecules (Chapters III and VI). We chose different peptides that show a high antimicrobial activity: Hamp2, that was previously described as a molecule highly active against *P. damselae* spp. *piscicida* [10], and piscidins 1 and 5, that show the lowest MIC values against this particular bacterium (Chapter IV). The approach adopted was similar, with fish being treated with AMPs either 24 hours before or 24 hours after the experimental infection, but the doses administered differ - 50 μM of Hamp2 and 6.25 μM of piscidins 1 or 5. Still, despite the differences between peptides and the doses administered, the overall results were comparable: either Hamp2 or piscidins 1 and 5 led to significantly higher survival rates and lower CFU counts. These three synthetic peptides proved to be effective not only before, but also during the first stages of infection, controlling the progression of the disease and hampering the anemia of inflammation.

A question that remains to be addressed is how peptides led to this resistance in fish. The action of piscidins or Hamp2 is surely not only limited to having a direct antimicrobial activity, but they are likely also involved in other immunomodulatory processes which are described for a variety of antimicrobial peptides. These include higher leukocyte proliferation, recruitment and activity, or stimulation of the overall inflammatory response [54,57,62,87–93,94]. *In vivo,* increased resistance of fish was observed after treatment with AMPs, accompanied by lower pathogen loads and modulation of immune related genes, with these changes being dependent on the peptide, route of administration and infectious agent [73,77,79,80,86,88,95,96]. For instance, the intramuscular administration of a plasmid containing a zebrafish beta-defensin conferred protection to fish against Spring Viraemia of Carp Virus (SVCV), associated with the activation of the type I interferon (IFN)-system, recruitment of T-helper (Th) cells, and up-regulation of *ifnγ*, in a Th1 immune cell response [88]. Atlantic cod (*Gadus morhua*) beta-defensin is likely involved in the stimulation of phagocytic activity of head kidney leucocytes in response to *V. anguillarum* [54]. In another report, the expression of immune related genes either increased or decreased, after electroporation of plasmid expressing tilapia piscidin 3 or 4, during infection by two different bacteria, *V. vulnificus* or *Streptococcus agalactiae* [72]**.** Due to the extracellular nature of *P. damselae* spp. *piscicida,* both hepcidin type 2 and piscidins may act: i) directly on the pathogen, inhibiting the proliferation of bacteria; ii) indirectly, by triggering the overall inflammatory response, with the activation and recruitment of phagocytic cells and increased expression of pro-inflammatory cytokines and other soluble factors, such as AMPs. Nevertheless, the exact immunomodulatory roles of hamp2 or piscidins 1 or 5 remain to be elucidated.

Although there is a wealth of information regarding the antimicrobial and immunomodulatory activities of different AMPs, the establishment of these peptides as therapeutic or prophylactic compounds in aquaculture faces several critical challenges.

The main constrains may include i) the capacity to achieve the desirable activity or effect under host's physiological conditions, particularly using certain routes of administration, such as the oral route; ii) potential cytotoxicity; iii) susceptibility to proteases and iv) high production costs [97,98]. The results of the present thesis clearly open the door to the use of piscidins and hepcidin type 2 derived peptides as novel drugs for sea bass production, but there is still work to be done. The ideal compound for aquaculture is the one that elicits a robust protection and is administered with safety and without causing stress to fish. In our experiments, we used single doses of peptide, delivered through the intraperitoneal route and using the model of infection by *P. damselae* spp. *piscicida* (Chapters III and V).

Considering that a 6.25 μM solution of both piscidins led to percentages of survival that range from 86 to 98%, although the costs of production of synthetic peptides are still considerably higher, when compared to other antimicrobials, the efficacy of the low dose in protecting fish opens the door to the use of piscidins in sea bass aquaculture. The results described in the present thesis place piscidin 1 as an ideal candidate to replace the current treatments in aquaculture, due to the high effectiveness at a low dose and the short amino acid sequence (only 22-aa). Nevertheless, there are three different approaches that we can consider in order to optimize the use of these (and other fish and non-fish) AMPs. The first is to reduce the dose administered. This is particularly important for Hamp2, that was administered to fish at 50 μM and lower doses could produce similar effects. The second is to establish a proper route of administration. Some previous studies addressed the effects of the oral administration of AMPs [78,99,100]. In one of those studies, the use of a transgenic Artemia expressing a recombinant epinecidin led to increased survival rates of zebrafish infected with *V. vulnificus* [99]. The increased resistance of zebrafish was associated with an increased expression of AMPs, particularly beta-defensins and hepcidin. However, in another study, the use of a cathelicidin-coated feed resulted in higher mortalities of rainbow trout (*Oncorhynchus mykiss*) fry infected with *Yersinia ruckeri*, when compared to the injection route. The authors attributed this poor effectiveness to the activity of proteases on the peptide, leading to a proteolytic degradation of cathelicidin in the gastrointestinal tract [100]. The fact that these peptides are usually susceptible to proteases leads to the third approach: to modify the amino acid sequence. Different modifications on the template sequence can be implemented: amino acid substitutions, including the use of D-amino acids; post-translational modifications, such as C-terminal amidation; or cyclization of synthetic peptides [97]. Alterations in the native sequence may not only increase the resistance of AMPs to a possible degradation, but can also reduce the cytotoxicity to host's cells, while maintaining (or even increasing) their antimicrobial activities [85,101–105]. It is to note that our piscidins were synthetized

with an additional C-terminal amidation. In fact, piscidins can be naturally amidated, as described by Lauth et al. [106]. Still, the effect of amidation in the antimicrobial activity of bass piscidins 1 and 3 was later tested and authors did not observe differences in MICs between amidated and non-amidated forms [32]. The treatment of fish with piscidin 5 led to the highest percentages of survival observed (98% in the pre-infection experiment). The fact that this peptide is constituted by 46-aa and shows a high hemolytic activity may limit its use. However, we demonstrated the high effectiveness of this peptide at low doses and thus, the high production costs can be minimized. Furthermore, the modifications above mentioned may be implemented in piscidin 5, in order to reduce its toxicity, while retaining its effectiveness.

Novel functions for piscidins: a missing link for iron metabolism in fish

Iron is an essential element for most organisms. It is involved in many processes, but the deficiency or excess of iron causes serious problems to the organism [107]. Since there is no active pathway of iron excretion, this element is tightly regulated, in order to promote its absorption, transport, storage and utilization in physiological processes, through different pathways coordinated by different cells and molecules [108,109]. These mechanisms are very well conserved among vertebrates, including fish [110–114], but some differences can be observed, such as the apparent lack of HFE (although the TfR mediated pathway still responds to changes in the iron status and inflammatory stimuli [115]) and the presence of multiple hepcidins [10].

The functions of the mammalian hepcidin are well described, the main being the regulation of iron, through the inhibition of the iron exporter ferroportin, and antimicrobial activity (although limited) [116–118]. However, in sea bass and some other fish species, two different hepcidin types are found, whose functions were subfunctionalized: the single type 1 hepcidin, homologous of the mammalian counterpart, presents an inhibitory role on ferroportin, but is not active on pathogens; on the contrary, the several type 2 hepcidins show activity against different microorganisms, but no effect on ferroportin [10,114]. The presence of multiple hamp2 indicates a more significant role of hepcidin as an antimicrobial molecule in fish [10]. The direct antimicrobial activity and immunomodulatory functions are attributed to different AMPs, but the regulation of iron is only attributed to hepcidin. As such, we raised the question if other AMPs, particularly piscidins, might also be involved in the regulation of iron in fish.

While the function of hepcidin as an iron regulator is well demonstrated, the pathway by which piscidins interfere with the absorption, distribution or usage of iron is unclear. The fact remains that piscidins 2 and 7 led to significant decreases in the hematocrit, either when administered at 6.25 or 50 μM concentrations, during the last days of experiment. On the contrary, Hamp1 led to lower hematocrit values as early as day 1 post peptide administration. This peptide effectively inhibits the function of ferroportin, limiting iron export, as well as the absorption of iron in the posterior intestine (Chapter III). Instead, piscidins seem to be more active in the head kidney, by decreasing the expression of *epo* and *epor*, that may translate in an impairment of erythropoiesis and, consequently, in lower hematocrit values. Together with this down-regulation of erythropoietin and its receptor, we observed increases in the expression of *fth* in the head kidney, and a slight accumulation of iron in the liver. This means that piscidins might be limiting erythropoiesis in the head kidney and, consequently, the amount of iron that is not used in this process is likely stored in the head kidney and the liver (although in this tissue the increment of iron was not significant). When fish are treated with 50 μM of piscidin 2 or 7, the mentioned differences in the hematocrit and liver iron content were even more pronounced. The expression of iron related genes in these animals remains to be analyzed, but the effects of piscidins in healthy animals seem to be dose-dependent (as previously observed for hepcidin [114]), so we expect to observe more differences in the expression of iron related genes, when fish are treated with 50 μM. Considering these results, we can consider two different possibilities: i) piscidins do not directly interfere with iron, but instead, they are actually interfering with the normal erythropoietic processes, leading to the lower hematocrit values observed; ii) if there is an effect of the dose administered, and the administration of 6.25 μM led to increases of *hamp1* expression, we anticipate that 50 μM of piscidins increased significantly the expression of *hamp1*, leading to the alterations observed in the hematological parameters and liver iron. Thus, piscidin may interfere indirectly with iron metabolism, by promoting a significant up-regulation of *hamp1*, but in a dose dependent manner. This is not so surprising, since AMPs present several immunomodulatory functions including the stimulation of other AMPs and other inflammatory molecules [73,99,119], and thus may also explain the increases in *hamp2*, the antimicrobial hepcidin.

When iron overloaded fish are treated with Hamp1, the peptide attenuates some of the effects caused by the excess of iron. Hepcidin leads to an even more pronounced iron withholding in the liver, and effectively limits iron export through ferroportin and uptake in the posterior intestine through Slc11a2alpha. The hematological parameters analyzed did not change significantly, when compared to controls, and there is also a reduction in the serum iron, when compared to iron overload (Chapter III). Thus, Hamp1 has the potential

to be used to treat iron disorders. The administration of piscidins also attenuated some of the effects of iron overload, but led to an alternative distribution of iron in the organism, since iron content in the liver is also reduced, contrary to what was observed with the administration of hepcidin. If iron is not being stored in the liver, the major iron reservoir, is most likely being accumulated in other tissues. To evaluate that, the spleen iron content was also determined. In the experimental model of iron overload, there was an increase (albeit non-significant) of iron in the spleen. This tissue performs erythropoietic functions and the iron in excess is probably used for the production of erythrocytes, coinciding with the increases in RBC counts and hematocrit observed in our previous models of iron overload [112,115,120; Chapter III]. As such, while iron is stored in the liver and remains for a longer period of time, in the spleen, iron is rapidly used during erythropoiesis. However, when iron overloaded fish were treated with 50 μM of piscidins, there were no increases in the spleen iron content. Therefore, while in healthy animals, piscidins lead to a slight retention of iron in the spleen, the same is not observed in iron overloaded fish. It seems that, in the presence of iron in excess, piscidins signal the body to distribute iron across different organs, asides the liver, but not in the spleen. Thus, it is possible that there are other tissues involved in this process, particularly the intestine or the head kidney. The intestine shows the highest basal expression of piscidins, with the exception of piscidin 2, that is highly expressed in the spleen (Chapter IV), and may assume an important (yet unknown) role. The basal expression of piscidins in the head kidney is not so pronounced, but when fish are treated with piscidin peptides, there are some significant changes in gene expression, so an involvement of the head kidney is also a possibility.

Although the present study points towards a possible role of piscidins in iron metabolism regulation or erythropoiesis, there are many questions that remain to be answered: what is the connection between piscidins and hepcidin, the major iron regulator? How do piscidins lead to decreases in hematological parameters at such lower dose, accompanied by decreases in the expression of *epo* and *epor?* Does a high dose of piscidin will eventually lead to an up-regulation of *hamp1* expression and, consequently, an impairment in iron homeostasis? What are the mechanisms triggered by piscidins? The pathways activated, and the tissues, cells and receptors involved are yet unknown, but it seems that there is a response to more than one piscidin peptide, despite the differences in the amino acid sequences. Furthermore, piscidins constitute a group of peptides highly diverse in their number and sequences, and piscidins 2 and 7 seem to be more restricted to species belonging to the Moronidae family. Thus, are piscidins also involved in the regulation of iron/erythropoiesis in other fish? Finally, in fish that lack the presence of these peptides, what is the alternative? Do these fish not present this alternative

mechanism of iron regulation/erythropoiesis, or do they have other molecules that may perform this function?

The results of this study show that while some piscidins mostly perform antimicrobial roles (akin to type 2 hepcidins), others might be involved in other functions, particularly iron metabolism regulation, in a complementary or alternative mechanism to type 1 hepcidin. Still, these models need to be studied in detail, by analyzing the expression of iron and erythropoiesis related genes, evaluating other hematological or serological parameters, such as serum iron or transferrin saturation, or determining the iron content in other tissues involved in iron metabolism or erythropoiesis, as we observed some differences in the liver and spleen.

Concluding remarks and future perspectives

Fish present many AMPs, that can be divided into different families, and show several functions and potential applications (reviewed in [121–124]). However, in the European sea bass (*Dicentrarchus labrax*), only hepcidins have been extensively studied [10,114,115,125], but other AMPs, such as piscidins and beta-defensins, are poorly understood. In the present thesis, we were able to expand the number of novel AMPs isolated in sea bass, and provided some insights into their sequences, functions and potential applications (Figure 1). We showed that sea bass presents several members belonging to different AMP families, with piscidins and type 2 hepcidins being much more diversified in terms of number and amino acid sequences [10; Chapter IV], while the beta-defensin family shows a much less degree of diversification (Chapter II).

Sea bass piscidins exert a broad antimicrobial activity *in vitro* against several fish and non-fish pathogens, with piscidins 1 and 5 being the most active peptides, while piscidins 6 and 7 show a poor effect on microorganisms (Chapter IV). These two peptides might be involved in other functions, but further studies are needed to understand the possible roles of these atypical piscidins. Nevertheless, we have evidences of a role of piscidins 2 and 7 in the regulation of iron. The several sea bass piscidins may have acquired novel functions, particularly iron metabolism regulation, asides the antimicrobial or immunomodulatory activities.

In this thesis, we tested the protective effects of synthetic piscidins and hepcidins during infection with *Photobacterium damselae* spp. *piscicida.* We demonstrated that Hamp2 and piscidins 1 and 5 have potential to be used either as prophylactic or therapeutic compounds, resulting in low mortalities and CFU counts (Chapters III and V). The present study clearly opens the door to the use of sea bass AMPs as novel compounds to replace

the conventional antimicrobials currently used in aquaculture, with both type 2 hepcidins and several piscidins proving to be effective in protecting fish from infection, but further studies will be needed in order to understand how these peptides led to this resistance of fish. Beta-defensin derived peptides can also be tested, due to their antimicrobial activities and immunomodulatory roles. Still, for the wide use of sea bass AMPs in aquaculture, implementing appropriate doses and more effective ways of administration is crucial. Piscidin 1 seems to be the most promising candidate, due to the high effectiveness at a low dose and the short amino acid sequence (only 22-aa), but the use of other AMPs, such as Hamp2, can be optimized. The intraperitoneal route, despite being effective, is costly and time consuming, but oral supplementation also presents significant challenges. Peptide modification can also be performed, in order to develop novel peptides that maintain their activity, but with reduced cytotoxicity and higher resistance to potential proteolytic degradation (that may occur in the gastrointestinal tract, after oral administration). The effect of these peptides must also be tested using other pathogens, particularly virus, that also cause severe mortalities in aquaculture. However, there is no doubt that fish AMPs are excellent candidates to replace antibiotics, due to the low probability to induce microbial resistance and broad antimicrobial activity (while antibiotics are mainly active on bacteria).

We also addressed the involvement of piscidins in other functions, particularly iron metabolism regulation. Hepcidin type 1 is the key regulator of iron metabolism in fish, by inhibiting the function of ferroportin, the iron exporter [114]. Piscidins may also have a role in this matter, but the exact mechanisms are still unknown (Chapter V). Nevertheless, piscidins likely act through different pathways, as they did not change significantly the expression of ferroportin. In seems that piscidins may act directly on the head kidney, the tissue that presented some of the most significant changes in gene expression, after piscidin administration. However, a low dose of piscidins triggered the expression of *hamp1* and thus, we cannot exclude a possible indirect effect of piscidins in iron metabolism, through the up-regulation of type 1 hepcidin, in a dose dependent manner. We also demonstrate that Hamp1 can attenuate some of the effects of excess iron, presenting the potential to be used in the treatment of iron disorders (Chapter III). Piscidins also attenuated some of these effects, but led to an alternative distribution of iron. Iron is likely being accumulated in different tissues, but not in the spleen. We cannot exclude the involvement of other tissues asides the liver, such as the intestine and the head kidney, tissues known to be involved in iron metabolism and erythropoiesis. The intestine, the organ that shows the highest basal expression of piscidins (with the exception of piscidin 2), may perform a yet unknown role. Our various models of piscidin administration and iron overload must be studied in further detail, with the analysis of the

expression of iron and erythropoiesis related genes, as well as other hematological parameters and tissue iron content, to better assess the role of piscidins in iron metabolism and their connection with other AMPs, especially hepcidin.

In the present thesis, we focused on some piscidins, namely piscidins 1, 2, 5 and 7, but the functions of piscidins 4 and 6 must also be addressed, particularly piscidin 6. This peptide is constituted by 65-aa, shows low hydrophobicity, amphipathicity and reduced antimicrobial activity. As such, piscidin 6 might be involved in other functions, such as immunomodulation, but further studies will be needed to support this hypothesis. Nevertheless, peptides derived from sea bass present themselves as viable alternatives to the current treatments applied in aquaculture. They can act directly on pathogens, but can also act as host defense peptides, by modulating the immune responses. However, the wide use of these peptides in aquaculture is still limited, and further studies will be needed in order to establish more effective doses and routes of administration.

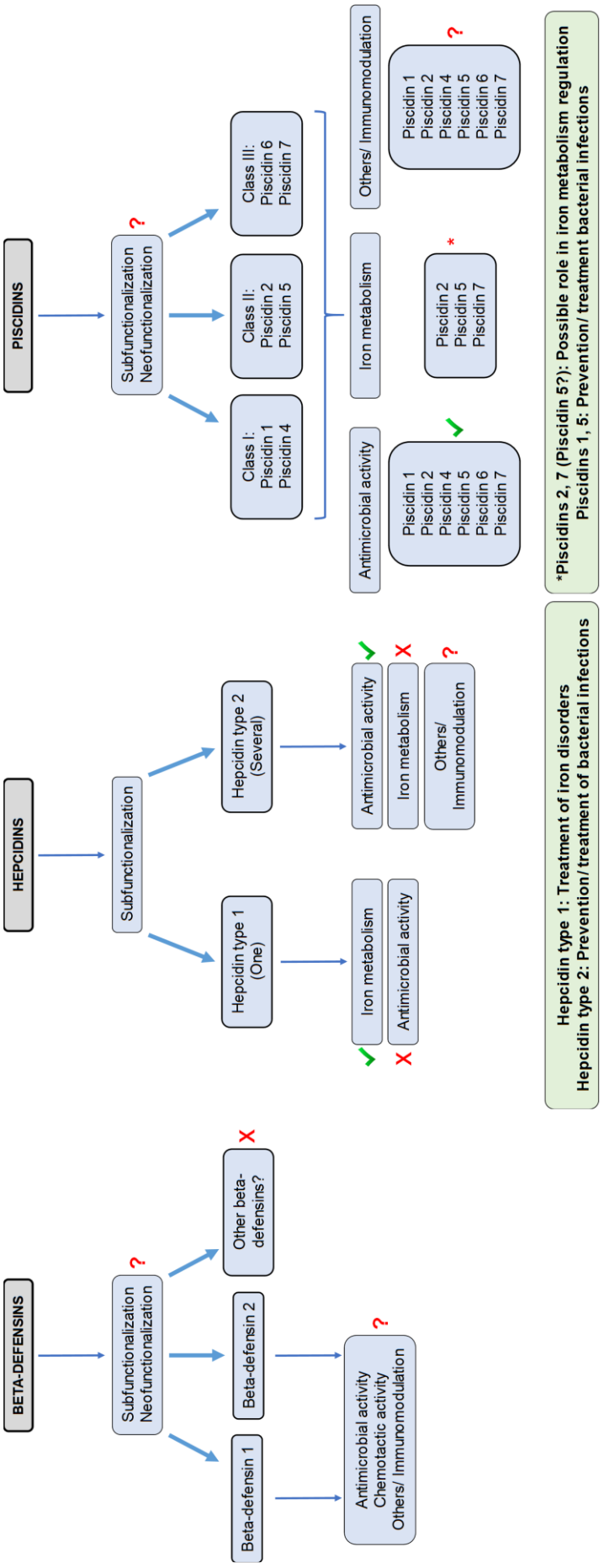


Figure 1. Overview of the antimicrobial peptides in the European sea bass (*Dicentrarchus labrax*).

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Chapter VII

Appendix

Appendix 1

Microorganisms showing susceptibility to beta-defensin derived peptides

Microorganisms	References
Gram-negative bacteria	
Aeromonas hydrophila	Zhao et al [1]; Wang et al [2]; Chen et al [3]; Zhu et al [4]; Yang et al [5]
Aeromonas sobria	Jin et al [6]
Aeromonas veronii	Zhu et al [4]
Edwardsiella tarda	Zhuang et al [7]
Edwardsiella ictaluri	Zhu et al [4]
Escherichia coli	Zhao et al [1]; Nam et al [8]; Jin et al [6]; Wang et al [2]; Dong et al [9]; Zhu et al [4]; Zhuang et al [7]
Pseudomonas aeruginosa	Jin et al [6]
Pseudomonas plecoglossicida	Li et al [10]
Vibrio alginolyticus	Li et al [10]
Vibrio anguillarum	Zhao et al [1]; Jin et al [6]; Cuesta et al [11]; Zhuang et al [7]
Vibrio campbellii	Li et al [10]
Vibrio harveyi	Zhou et al [12]; Li et al [10]
Vibrio fluvialis	Zhao et al [1]; Jin et al [6]
Vibrio parahaemolyticus	Li et al [10]
Yersinia ruckeri	Zhu et al [4]
Gram-positive bacteria	
Bacillus cereus	Jin et al [6]
Bacillus subtilis	Cuesta et al [11]; Chen et al [3]; Li et al [10]
Micrococcus luteus	Zhao et al [1]; Jin et al [6]; Ruangsri et al [13]
Planococcus citreus	Ruangsri et al [13]
Staphylococcus aureus	Zhao et al [1]; Jin et al [6]; Wang et al [2]; Zhu et al [4]
Streptococcus agalactiae	Dong et al [9]; Zhu et al [4]
Streptococcus iniae	Zhu et al [4]; Zhuang et al [7]
Virus	
Nervous necrosis virus (NNV)	Guo et al [14]
Singapore grouper iridovirus (SGIV)	Guo et al [14]
Spring viremia carp virus (SVCV)	García-Valtanen et al [15]
Viral haemorrhagic septicaemia virus (VHSV)	Falco et al [16]

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Appendix 2

Microorganisms and cancer cell lines showing susceptibility to hepcidin derived peptides

Microorganisms/ Cancer cell lines	References
Gram-negative bacteria	
Acinetobacter baumannii	Huang et al [1]
Aeromonas hydrophila	Wang et al [2]; Zhang et al [3]; Yang et al [4]; Cai et al [5]; Mu et al [6]; Go et al [7]; Huang et al [1]; Shirdel et al [8]; Hu et al [9]; Chen et al [10]
Aeromonas salmonicida	Huang et al [1]
Edwardsiella tarda	Wang et al [11]; Zhang et al [12]; Chen et al [13]; Go et al [7]; Hu et al [9]
Escherichia coli	Lauth et al [14]; Hirono et al [15]; Cuesta et al [16]; Wang et al [2]; Zhang et al [3]; Yang et al [4]; Cai et al [5]; Wang et al [11]; Lin et al [17]; Álvarez et al [18]; Tao et al [19]; Liu et al [20]; Mu et al [6]; Go et al [7]; Shirdel et al [8]; Hu et al [9]
Klebsiella pneumoniae	Lauth et al [14]; Liu et al [20]
Photobacterium damselae	Zheng et al [21]
Photobacterium damselae spp. *piscicida*	Hirono et al [15]; Neves et al [22]
Pseudomonas aeruginosa	Tao et al [19]; Liu et al [20]; Go et al [7]; Huang et al [1]
Pseudomonas fluorescens	Qu et al [23]
Salmonella enterica	Go et al [7]
Shigella flexneri	Lauth et al [14]; Go et al [7]
Shigella sonnei	Lauth et al [14]; Go et al [7]
Vibrio alginolyticus	Wang et al [2]; Zhang et al [3]; Chen et al [13]; Mu et al [6]
Vibrio anguillarum	Wang et al [11]; Lin et al [17]; Zhang et al [12]; Neves et al [22]; Liu et al [20]; Chen et al [13]; Go et al [7]
Vibrio fluvialis	Zhang et al [3]
Vibrio harveyi	Wang et al [2]; Zhang et al [3]; Yang et al [4]; Wang et al [11]; Chen et al [13]; Mu et al [6]
Vibrio parahaemolyticus	Wang et al [2]; Zhang et al [3]; Wang et al [11]; Chen et al [13]; Mu et al [6]; Hu et al [9]
Vibrio vulnificus	Zhou et al [24]; Chen et al [13]; Chen et al [10]
Yersinia enterocolitica	Lauth et al [14]
Gram-positive bacteria	
Bacillus cereus	Wang et al [2]; Yang et al [4]
Bacillus subtilis	Cuesta et al [16]; Wang et al [2]; Zhang et al [3]; Yang et al [4]; Lin et al [17]; Liu et al [20]; Mu et al [6]; Go et al [7]; Shirdel et al [8]
Corynebacterium glutamicum	Wang et al [2]; Yang et al [4]; Cai et al [5]; Qu et al [23]; Zheng et al [21]
Enterococcus faecium	Huang et al [1]

Microorganisms/ Cancer cell lines	References
Gram-positive bacteria	
Lactococcus garviae	Hirono et al [15]; Neves et al [22]; Go et al [7]
Listeria monocytogenes	Tao et al [19]; Chen et al [13]
Micrococcus lysodeikticus	Zhang et al [3]; Yang et al [4]; Qu et al [23]; Zheng et al [21]
Micrococcus luteus	Wang et al [2]; Zhang et al [12]; Go et al [7]; Shirdel et al [8]
Staphylococcus aureus	Hirono et al [15]; Wang et al [2]; Zhang et al [3]; Yang et al [4]; Zhou et al [24]; Cai et al [5]; Wang et al [11]; Qu et al [23]; Lin et al [17]; Tao et al [19]; Zhang et al [12]; Liu et al [20]; Chen et al [13]; Mu et al [6]; Go et al [7]; Huang et al [1]; Hu et al [9]
Staphylococcus epidermidis	Wang et al [2]; Yang et al [4]
Streptococcus agalactiae	Hu et al [9]
Streptococcus iniae	Hirono et al [15]; Chen et al [13]; Go et al [7]; Shirdel et al [8]
Streptococcus parauberis	Neves et al [22]
Virus	
Nervous necrosis virus (NNV)	Chia et al [25]; Wang et al [26]
Singapore grouper iridovirus (SGIV)	Zhou et al [24]; Mu et al [6]
Parasites	
Cryptocaryon irritans theronts	Zheng et al [21]
Trypanosoma carassii	Xie et al [27]
Fungi	
Aspergillus niger	Lauth et al [14]; Wang et al [2]; Yang et al [4]
Candida albicans	Go et al [7]
Candida glabrata	Huang et al [1]
Fusarium graminearum	Wang et al [2]; Yang et al [4]
Fusarium solani	Wang et al [2]; Yang et al [4]
Cancer cell lines	
Human cervical adenocarcinoma (HeLa)	Chang et al [28]
Human fibrosarcoma (HT1080)	Chen et al [29]; Chang et al [28]
Human hepatocellular carcinoma cell (HepG2)	Chang et al [28]

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activity but limits its antibacterial activity. *Dev. Comp. Immunol.* 2021, *114*, 103845.

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Appendix 3

Microorganisms and cancer cell lines showing susceptibility to piscidin derived peptides

Microorganisms/ Cancer cell lines	References
Gram-negative bacteria	
Acinetobacter baumannii	Pan et al [1]; Taheri et al [2]; Olivieri et al [3]; Buonocore et al [4]
Aeromonas hydrophila	Silphaduang and Noga [5]; Yin et al [6]; Peng et al [7]; Mao et al [8]; Yang et al [9]; Pan et al [10]; Prior et al [11]; Portelinha et al [12]
Aeromonas salmonicida	Cole et al [13]; Silphaduang and Noga [5]; Douglas et al [14]; Patrzykat et al [15]; Iijima et al [16]; Bae et al [17]; Milne et al [18]
Edwardsiella tarda	Acosta et al [19]; Mao et al [8]
Escherichia coli	Cole et al [13]; Silphaduang and Noga [5]; Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Iijima et al [16]; Yin et al [6]; Chekmenev et al [21]; Sun et al [22]; Lee et al [23]; Noga et al [24]; Buonocore et al [25]; Ruangsri et al [26]; Acosta et al [19]; Mao et al [8]; Niu et al [27]; Bae et al [28]; Lee et al [29]; Pan et al [1]; Bae et al [30]; Salger et al [31]; Li et al [32]; Umasuthan et al [33]; Zhuang et al [34]; Libardo et al [35]; Taheri et al [2]; Olivieri et al [3]; Buonocore et al [4]; Milne et al [18]; León et al [36]; Prior et al [11]; Qiao et al [37]; Portelinha et al [12]
Flavobacterium columnare	Prior et al [11]
Helicobacter pylori	Narayana et al [38]; [39]
Klebsiella pneumoniae	Cole et al [40]; Silphaduang and Noga [5]; Lauth et al [20]; Pan et al [1]; Olivieri et al [3]; Buonocore et al [4]
Photobacterium damsela subsp. d*amselae*	León et al [36]
Photobacterium damsela subsp. *piscicida*	Noga et al [24]; Milne et al [18]
Pseudomonas aeruginosa	Cole et al [13]; Cole et al [40]; Silphaduang and Noga [5]; Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Pan et al [41]; Lee et al [23]; Peng et al [7]; Acosta et al [19]; Mao et al [8]; Lee et al [29]; Libardo et al [35]; Taheri et al [2]; Olivieri et al [3]; Pan et al [10]; Buonocore et al [4]; Qiao et al [37]
Pseudomonas fluorescens	Sun et al [22]; Mao et al [8]; Niu et al [27]; Pan et al [10]; Qiao et al [37]
Salmonella typhimurium	Cole et al [13]; Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Lee et al [23]; Lee et al [29]
Serratia marcescens	Cole et al [13]
Shigella flexneri	Silphaduang and Noga [5]; Lauth et al [20]; Noga et al [24]; Salger et al [31]; Pan et al [10]; Qiao et al [37]
Vibrio alginolyticus	Silphaduang and Noga [5]; Yin et al [6]; Pan et al [41]; Peng et al [7]; Mao et al [8]; Bae et al [28]; Bae et al [30]; Yang et al [9]; Bae et al [17]; Qiao et al [37]
Vibrio anguillarum	Iijima et al [16]; Sun et al [22]; Noga et al [24]; Ruangsri et al [26]; Salger et al [31]; Yang et al [9]; Milne et al [18]; León et al [36]
Vibrio cholera	Lauth et al [20]

Microorganisms/ Cancer cell lines	References
Gram-negative bacteria	
Vibrio harveyi	Iijima et al [16]; Pan et al [41]; Mao et al [8]; Bae et al [28]; Bae et al [30]; Bae et al [17]; León et al [36]; Qiao et al [37]; Portelinha et al [12]
Vibrio ordalii	Bae et al [30]; Bae et al [17]
Vibrio parahaemolyticus	Yin et al [6]; Mao et al [8]; Li et al [32]; Yang et al [9]; Zhuang et al [34]; Qiao et al [37]
Vibrio vulnificus	Iijima et al [16]; Yin et al [6]; Pan et al [41]; Peng et al [7]; Bae et al [28]; Bae et al [30]
Yersinia enterocolitica	Lauth et al [20]; Pan et al [41]
Yersinia ruckeri	Sun et al [22]; Ruangsri et al [26]; Milne et al [18]
Gram-positive bacteria	
Bacillus cereus	Chekmenev et al [21]; Buonocore et al [25]; León et al [36]
Bacillus subtilis	Cole et al [13]; Iijima et al [16]; Lee et al [23]; Acosta et al [19]; Mao et al [8]; Niu et al [27]; Lee et al [29]; Pan et al [10]; Qiao et al [37]
Enterococcus faecalis	Lauth et al [20]; Peng et al [7]; Wang et al [42]; Salger et al [31]; Olivieri et al [3]
Lactococcus garviae	Silphaduang and Noga [5]; Iijima et al [16]; Noga et al [24]; Salger et al [31]; Milne et al [18]; León et al [36]
Listeria monocytogenes	Lauth et al [20]; Pan et al [41]; Yang et al [9]
Micrococcus lysodeikticus	Ruangsri et al [26]; Pan et al [10]; Qiao et al [37]
Micrococcus luteus	Lauth et al [20]; Pan et al [41]; León et al [36]; Qiao et al [37]
Staphylococcus aureus	Cole et al [13]; Cole et al [40]; Silphaduang and Noga [5]; Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Chekmenev et al [21]; Pan et al [41]; Lee et al [23]; Noga et al [24]; Ruangsri et al [26]; Acosta et al [19]; Mao et al [8]; Niu et al [27]; Huang et al [43]; Lee et al [29]; Salger et al [31]; Li et al [32]; Yang et al [9]; Zhuang et al [34]; Libardo et al [35]; Taheri et al [2]; Olivieri et al [3]; Pan et al [10]; Buonocore et al [4]; Qiao et al [37]
Staphylococcus epidermidis	Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Pan et al [41]; Lee et al [23]; Lee et al [29]; Taheri et al [2]; Olivieri et al [3]; Buonocore et al [4]
Streptococcus agalactiae	Lauth et al [20]; Pan et al [41]; Peng et al [7]
Streptococcus faecalis	Silphaduang and Noga [5]; Peng et al [7]; Salger et al [31]
Streptococcus iniae	Silphaduang and Noga [5]; Lauth et al [20]; Iijima et al [16]; Noga et al [24]; Bae et al [28]; Bae et al [30]; Salger et al [31]; Umasuthan et al [33]; Bae et al [17]
Streptococcus pneumoniae	Lauth et al [20]; Pan et al [41]

Microorganisms/ Cancer cell lines	References
Virus	
Channel catfish virus (CCV)	Chinchar et al [44]
Foot and mouth disease virus (FMDV)	Huang et al [45]
Human Immunodeficiency Virus (HIV)	Wang et al [46]
Infectious pancreatic necrosis virus (IPNV)	León et al [36]
Japanese encephalitis virus (JEV)	Huang et al [47]
Nervous necrosis virus (NNV)	Wang et al [48]; León et al [36]
Spring viremia carp virus (SVCV)	León et al [36]
Viral septicaemia haemorrhagic virus (VHSV)	León et al [36]
Parasites	
Amyloodinium ocellatum dinospore	Colorni et al [49]
Cryptocaryon irritans theronts	Colorni et al [49]; Zhuang et al [34]; Chen et al [50]
Cryptocaryon irritans trophonts	Mao et al [8]; Niu et al [27]; Chen et al [50]
Ichthyophthirius multifilii theronts	Colorni et al [49]
Miamiensis avidus	Umasuthan et al [33]
Tetrahymena pyriformes	Ruangsri et al [26]; Salger et al [31]
Trichodina	Colorni et al [49]
Fungi	
Aspergillus niger	Mao et al [8]; Niu et al [27]
Candida albicans	Cole et al [40]; Lauth et al [20]; Douglas et al [14]; Patrzykat et al [15]; Yin et al [6]; Jung et al [51]; Sung and Lee [52]; Sung et al [53]; Cho and Lee [54]; Acosta et al [19]; Mao et al [8]; Niu et al [27]; Umasuthan et al [33]; Taheri et al [2]
Microsporosis canis	Yin et al [6]
Neurospora crassa	Lauth et al [20]
Saccharomyces cerevisiae	Jung et al [51]; Sung and Lee [52]; Umasuthan et al [33]
Saprolegnia sp	Zahran and Noga [55]

Microorganisms/ Cancer cell lines	References
Cancer cell lines	
Human breast adenocarcinoma (MCF7)	Hilchie et al [56]; Hilchie et al [57]
Human breast adenocarcinoma (MDA-MB-231)	Hilchie et al [56]; Hilchie et al [57]
Human breast adenocarcinoma (MDA-MB-468)	Hilchie et al [56]; Hilchie et al [57]
Human breast adenocarcinoma (SKBR3)	Hilchie et al [56]; Hilchie et al [57]
Human breast carcinoma (T47-D)	Hilchie et al [56]; Hilchie et al [57]
Human fibrosarcoma (HT1080)	Lin et al [58]
Human glioblastoma (U251)	Su et al [59]
Human histiocytic lymphoma (U937)	Chen et al [60]
Human lung adenocarcinoma (A549)	Lin et al [58]; Ting et al [61]; Cheng et al [62]
Human osteosarcoma (MG63)	Cheng et al [62]; Yuan et al [63]
Human osteosarcoma (143B)	Cheng et al [62]
Human ovarian adenocarcinoma (SKOV-3)	Cheng et al [62]
Human synovial sarcoma (Aska-SS)	Su et al [64]
Human synovial sarcoma (SW982)	Su et al [64]
Mouse mammary carcinoma (4T1)	Hilchie et al [56]; Hilchie et al [57]

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