U.PORTO

MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS

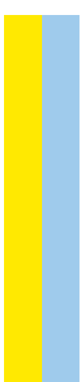
AQUACULTURA E PESCAS

# Immune responses of gilthead seabream (*Sparus aurata*) to *Photobacterium damselae* subs. *piscicida* infection: searching for health biomarkers

Paulo Joaquim da Silva Santos

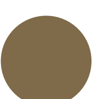
**M**

**2020**



U.PORTO

**Paulo Joaquim da Silva Santos.** Immune responses of gilthead seabream (*Sparus aurata*) to *Photobacterium damselae* subs. *piscicida* infection: searching for health biomarkers

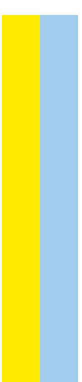


**M**.ICBAS **2020**

**Immune responses of gilthead seabream (*Sparus aurata*) to *Photobacterium damselae* subs. *piscicida* infection: searching for health biomarkers**

Paulo Joaquim da Silva Santos

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



Paulo Joaquim da Silva Santos

# Immune responses of gilthead seabream (*Sparus aurata*) to *Photobacterium damselae* subsp. *piscicida* infection: searching for health biomarkers

Tese de Candidatura ao grau de Mestrado em Ciências do Mar - Recursos Marinhos submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

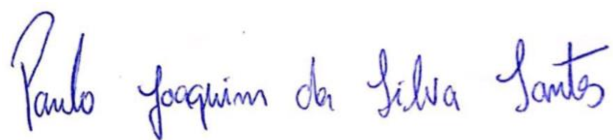
Orientador – Doutor Benjamín Costas Refojos, Investigador Auxiliar, Centro Interdisciplinar de Investigação Marinha e Ambiental

Coorientador – Teresa Maria Coelho Baptista, Professora adjunta (especialista), Instituto Politécnico de Leiria

Porto 2020

## Declaração de honra

Declaro que a presente dissertação é da minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plagio e auto-plágio constitui um ilícito académico.



Paulo Joaquim da Silva Santos

# Acknowledgments

This work was supported by project BE4AQUAHEALTH: RASTREIO NACIONAL DE PATOLOGIAS DE PEIXES DE AQUACULTURA: UMA APOSTA NA PREVENÇÃO (16-02-05-FMP-0013), funded by Mar2020 Operational Programme and the European Union through FEDER, and by national funds through FCT - Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020.

The elaboration of this dissertation was only possible with the collaboration of a great number of people which I would like to thank.

To my supervisor, Benjamin, thank you so much for supporting me on all the decisions, for guiding me on the most difficult moments and for being one of the major resposibles for my research interest. I fell I learned so much on the last three years, and there is no doubt that it was caused by the confidence and cooperation values you transmit to all people that work with you, creating an amazing lab group.
I am also very thankfull to my co-supervisor, Professor Teresa Baptista, for letting me be part of this great project that allies immunology and pathology and was exactly what I was wishing to study. Your experience, knowledge, enthusiasm, dedication and joy have contributed so much to complete this work.
I would also like to extend my deepest gratitude to professor Eduardo Rocha, whose constructive advises and counselling helped to face the many obstacles along master course.

To my lab friends: Diogo, Inês, Carla, Marina, Bruno, Rita, Lourenço, Sérgio, Ana Garcia, Ana Rocha, Mariana, João and André. Thanks for all the advices and help through this process, specially to the one's that went to sample with me at 300 km from home, and for the patience and support on all the "windy" days! I also want to thank to my "amigos de Peniche": Ricardo, Pedro, Damiana e Beatriz. Your help and simpathy have turned all 12 hours sampling much lighter and funny. All of you have contributed on a very significant way to this dissertation.

I can´t forget my master course mates. Bernardo, Frederico, Gina, Inês, Joana, Rúben and Rute. Even though we were a little class and these two years have passed with a blink of an eye, it was a pleasure to share with you all the moments and I hope we can continue to see each other and celebrate everyone's achievements.

And last but not least, I would like to thank my family for all the effort, support, for being a role model on my development and never letting me give up on my objectives. To you, Giulia, words will never be enough to show you my gratitude for all the comprehension, patience and cooperation, always pushing me to grow as researcher and human being.

To all of you THANK YOU SO MUCH!

## Abstract

Aquaculture is an emergent industry challenged by the occurrence of different pathogenic agents which may lead to productive and monetary losses. In order to better understand the disease and develop tools to protect fish from this threat, the present study was designed for studying haematological and innate immune responses of gilthead seabream (*Sparus aurata*) within the first days of infection with *Photobacterium damselae* subsp. *piscicida* (*Phdp*).

A time-course study was performed at CETEMARES (Politécnico de Leiria, Peniche, Portugal) facilities with 132 seabream juveniles (9.8 ± 2.2 g). Among them, 12 fish were selected and sampled before infection (time 0). Thereafter, the remaining animals were randomly selected and intraperitoneally (i.p.) injected with 100 µl PBS (control group) or 100 µl of exponentially growing *Phdp* ($10^6$ CFU/ mL; infected group) and distributed as a complete randomized design in 6 independent recirculating seawater systems (i.e. triplicates per experimental condition). Two animals per tank (n=6 per treatment) were sampled at 3, 6, 9, 24 and 48 h after i.p. injection. At each sampling point, fish were anaesthetized and samples of blood and head-kidney were collected for haematological procedures and immune-related gene expression analyses. The remaining blood was centrifuged and plasma was collected for innate humoral parameters determination (i.e. antiproteases, proteases and peroxidase activities).

Peripheral erythrocyte levels decreased in infected animals compared to sham injected groups regardless time, whereas haematocrit and haemoglobin levels were found diminished in infected animals at 24 and 48 h post infection. Even though total peripheral leucocytes did not change between both conditions, circulating neutrophil and monocyte populations showed augmented numbers in infected animals since these constitute the first cell defence line. These results go along with molecular findings that registered increased expression on immune genes related to phagocytic activity and inflammation.

Future studies should be performed with other bacterial pathogens for a better comprehension of the host response to infection and provide us more robust data for health biomarkers definition, contributing for a safer and more efficient aquaculture.

**Keywords**

Animal health; infection; immune response; red blood cells; neutrophils; IL-1β, IL-34.

# Resumo

A Aquacultura é uma indústria em expansão desafiada pela ocorrência de diversos agentes patogénicos que podem levar a perdas produtivas e monetárias. A fim de compreender melhor a doença e desenvolver ferramentas que protejam os peixes desta ameaça, o presente estudo foi desenhado para estudar as respostas hematológicas e imunes inatas da dourada (*Sparus aurata*) nos primeiros dias de infecção com *Photobacterium damselae* subsp. *piscicida* (Phdp).

Foi realizado um estudo ao longo do tempo nas instalações do CETEMARES (Politécnico de Leiria, Peniche, Portugal) com 132 juvenis de dourada (9,8 ± 2,2 g). Entre estes, 12 peixes foram selecionados e amostrados antes da infecção (tempo 0). Posteriormente, os animais restantes foram selecionados aleatoriamente e injetados intraperitonealmente (ip) com 100 µl de PBS (grupo controlo) ou 100 µl de Phdp em crescimento exponencial ($10^6$ UFC / mL; grupo infectado) e distribuídos de forma aleatória em 6 tanques de recirculação independente (ou seja, triplicados por condição experimental). Dois animais por tanque (n=6 por tratamento) foram amostrados às 3, 6, 9, 24 e 48 horas após injeção. Em cada ponto de amostragem, os peixes foram anestesiados e amostras de sangue e rim anterior foram recolhidas para procedimentos hematológicos e análises de expressão génica relacionada com o sistema imunológico. O sangue restante foi centrifugado e o plasma foi separado para determinação dos parâmetros humorais inatos (atividades de antiproteases, proteases e peroxidase).

Os níveis de eritrócitos periféricos diminuíram em animais infectados em comparação os grupos com infeção simulada, independentemente do tempo, enquanto que os níveis de hematócrito e hemoglobina diminuíram em animais infectados 24 e 48 horas após a infecção. Ainda que os leucócitos periféricos totais não tenham mudado entre as duas condições, as populações de neutrófilos e monócitos circulantes apresentaram números aumentados em animais infectados, uma vez que constituem a primeira linha de defesa celular. Esses resultados vão de acordo com os achados moleculares que registraram aumento da expressão em genes relacionados com a atividade fagocítica e inflamação.

Estudos futuros devem ser realizados com outros patógenos bacterianos para uma melhor compreensão da resposta do hospedeiro à infecção e poder fornecer dados mais robustos para definição de biomarcadores de saúde, contribuindo para uma aquacultura mais segura e eficiente.



**Palavras passe:**

Saúde animal; infeção; resposta immune; eritrócitos; neutrófilos; IL-1β, IL-34.

## INDEX

**1- Introduction** 1

1.1. Aquaculture Development and Importance 1

1.2. *Sparus aurata* 3

1.3. Pathogenic Features 4

1.4. Photobacteriosis 5

1.5. Fish Immune System and inflammation 7

1.6. Scope of the study 12

**2- Materials and Methods** 13

2.1. Experimental Design 13

2.2. Bacterial Challenge 13

2.3. Sampling 14

2.4. Heamatological Analysis 14

2.5. Innate Humoral Parameters 14

2.6. Gene Expression Analysis 16

2.7. Statistical Analysis 18

**3- Results** 19

3.1. Bacterial Challenge 19

3.2. Heamatological Analysis 19

3.3. Innate Humoral Parameters 20

3.4. Gene Expression Analysis 24

**4- Discussion** 27

**5- Conclusion** 31

**6- References** 32

# 1- Introduction

## 1.1. Aquaculture Development and Importance

Aquatic organisms are considered one of the most promising sources of protein to face human hunger since they present good nutrient and protein values accounting, in 2017, with 17 percent of total animal protein ingestion and near 10 percent of total protein ingestion **(FAO 2020)**. Alongside, fish availability also represents an advantage on this nutriment, once its production, on the last decades, has followed population growth and food ingestion, which had sharply rose.

Still regarding aquatic species importance, we can notice the galloping increase in fish supply, mainly due to aquaculture production, which resulted in more than 140 million tonnes of fish available for human consumption in 2014, a value that is more than three times higher when compared with 40 million tonnes registered in 1970 **(Figure 1)**. We can also verify that the increase rate in fish supply is considerably higher than population growth, leading to a higher amount of fish available for each person, also known as average per capita availability, reaching a value of 20kg/person in 2014 **(FAO 2018)**.

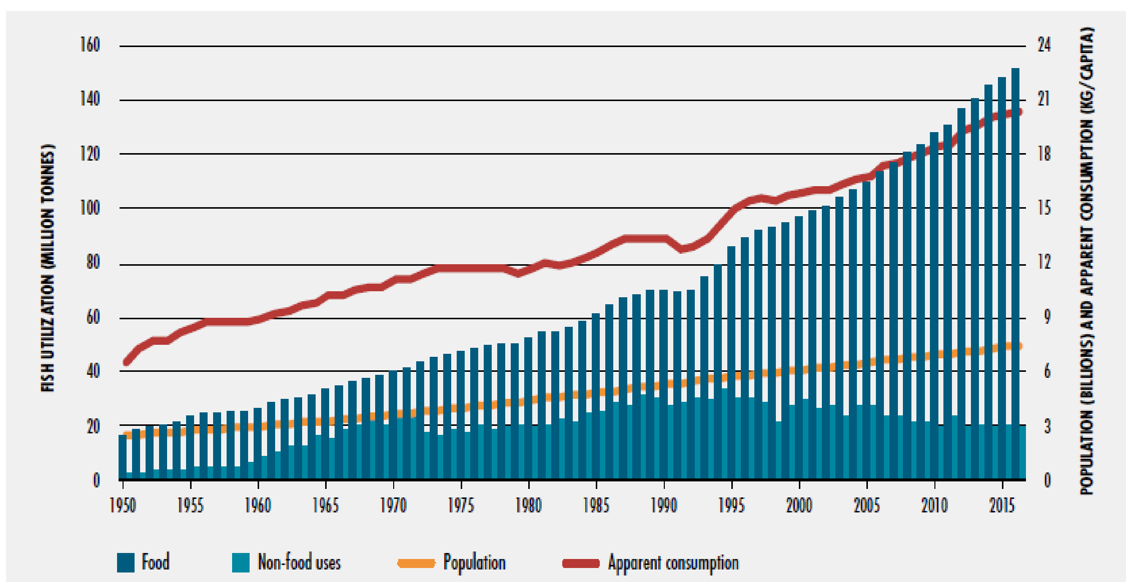



**Figure 1-** Evolution of population, fish and fish usages from 1950 to 2015. Source: FAO 2018.

The human controlled production of aquatic organisms is defined as aquaculture and is possible to culture finfish, molluscs, crustaceans, amphibians, reptiles and aquatic plants. This activity is crucial for sustainability of fish consumption without compromising

aquatic ecosystems since it permits us to breed animals in high quantities and healthier conditions with relative ease, contributing to the decrease of overfishing **(NOOA 2019)**. In addition, fishing captured quantities are starting to stagnate due to non-controllable factors such as global warming, water pollution, insufficient food supply and predation.

Although some consumers are still reluctant to eat farmed fish, many companies from all over the world are investing on innovative, ecological and profitable aquacultures. The culture of fish in Europe is still a novelty and the quantities produced by this industry are below the world's average (represents only about 17 percent of the total european supply) **(FAO 2020)**. On the other hand, Portugal is among the countries with more investment on imported farmed fish, a signal that shows the opportunity for fish farmers to raise their business a diminish foreign dependence. The production of fish from aquaculture in Europe reached, in 2014, 2,930 million tonnes, with the Southern Europe countries, where Portugal is included, contributing with 595 thousand tonnes **(FAO 2016)**.

The number of different marine cultured fish species in Europe is vast, being Atlantic salmon (*Salmo salar*) the most produced and commercialized finfish. Specifically in Portugal, and according to Instituto Nacional de Estatistica, about 13,992 tonnes of marine animals were produced in aquaculture in 2018 **(INE 2019)**. Still on Portuguese aquacultures, between marine organisms produced, finfish contributes with almost 5,000 tonnes, from which we can emphasize turbot (*Scophtalmus maximus*), gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) **(Table I)**.

**Table I –** Fish production on inland and oceanic waters by type of regimen and species **(INE 2019)**.

| | **Production (Tonnes)** | **Extensive (Tonnes)** | **Semi-intensive (Tonnes)** | **Intensive (Tonnes)** |
|---|---|---|---|---|
| **Freshwater fish** | **697** | **0** | **0** | **697** |
| **Rainbow trout (*Oncorhynchus mykiss*)** | 655 | 0 | 0 | 655 |
| **Brackish/Marine water fish** | **3,860** | **0** | **511** | **3,349** |
| **Gilthead seabream (*Sparus aurata*)** | 898 | 0 | 308 | 590 |
| **Turbot (*Scophtalmus maximus*)** | 2,582 | 0 | 0 | 2,582 |
| **European Sea Bass (*Dicentrarchus labrax*)** | 200 | 0 | 0 | 200 |

## 1.2. *Sparus aurata*

The gilthead seabream *Sparus aurata* (Linnaeus 1758) is an oval finfish of the Actinopterygii class, Perciformes order and Sparidae family. This species presents a greyish coloration with a golden strip between his eyes and a dark patch in the origin of the lateral line **(Martins & Carneiro 2018).** It is an euryhialine and eurythermic carnivore fish and its commonly distributed in the Mediterranean and Black Seas and along the Eastern of the Atlantic Ocean, swimming alone or in small groups **(Moretti *et al.* 1999).**

Gilthead seabream is a protandrous hermaphrodite (species that are born as male where population suffers a sexual change during its life cycle) and individuals become females after 2 years or 30 cm in length. This process can also be influenced by biological reproductive factors and sex reversion was also observed in captivity due to social and hormonal action **(Zohar 1989).** In Mediterranean, spawn occurs between October and December and females can lay 80,000 eggs a day. Eggs are pelagic, small in size and hatching starts approximately 48 hours after fertilization **(Mitcheson & Liu 2008).**

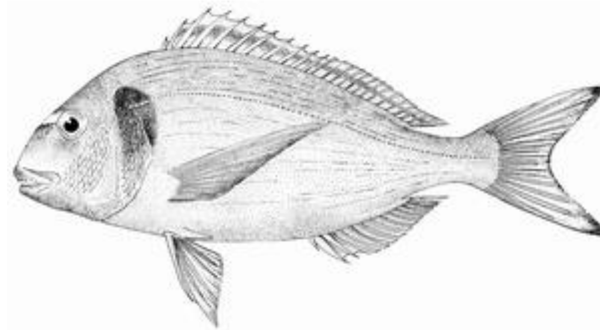


**Figure 2**- *Sparus aurata*. Source: FAO 2005

The natural breeding of seabream take place generally on coastal lagoons and saltwater ponds, being artificially intensively produced on the Mediterranean countries since 1980, with special contribution from Greece, Italy, Turkey and Spain **(FAO 2020b).**

The intensive production of this species represents a huge contribution for the Mediterranean aquaculture, resultant of research effort for bigger and more efficient production. Nevertheless, several mortalities and economical losses have been reported caused by pathogenic outbreaks, from which we can emphasize bacterial processes such as vibriosis and photobacteriosis **(Borrego *et al.* 2017).**

### 1.3. Pathogenic Features

The occurrence of animal infectious disease is a multifactorial process, that does not only involve the pathogen itself as the exclusive way for a pathology to occur, but an interaction between host, pathogen and environment, also known as the epidemiological triad **(Snieszko 1974)**. When balance is broken, defence mechanisms of the host are activated to try to solve it by an immunological cascade divided in 3 main stages:

I. The alarm reaction with release of signallers for general immunological answer;

II. The stage of resistance consisting in changes on the host's physiology and anatomy in order to recover to an equilibrium;

III. The stage of exhaustion when defence mechanisms are unable to maintain balance and disease develops.

Diseases can be classified according to their biological features. Regarding its capacity to propagate, a pathogen is defined as non-contagious if its action remains strict to one individual or contagious when pathogen can be transmitted between individuals. According to pathogens interaction on host, pathogens can act by their own, leading to isolated infections, or as two or more different infectious agents with cumulative effects on host, resulting on co-infections **(Roberts 2012)**.

Studying diseases according to its occurrence and spread, Kinne **(Kinne 1980)** has defined pathological processes as:

**Sporadic** – when a disease occurs in few individuals of a population, without a temporal or spatial pattern.

**Epizootic** – if many individuals are affected but for a restricted time and space.

**Panzootic** – when an outbreak of large scale takes in, affecting several geographical areas.

**Enzootic** – if a disease persistent or re-occurs on a determined area, with low level of intensity.

Ictic bacteriosis evolution can assume 4 different forms, depending on various factors related with host resistance (genetical constitution, immunity, stress and nutritional state), pathogen virulence and environmental conditions to pathogen resistance **(Menezes 2000)**. The acute, systemic or septicemic form is frequently caused by Gram-negative bacteria that are in blood circulation, causing fast and massive destruction of stock, sudden lack of appetite, skin darkening and exophthalmia. At necropsy is observable inflammatory exudates, kidney haemorrhages and spleen enlargement. An

attenuated form of acute evolution, caused by reduced pathogenic virulence and/or enhanced host resistance is designated as subacute or ulcerative form, resulting on a slower and local infection that affects mainly the muscle and skin tissues, forming ulcers with variable length and depth that can reach blood vessels and spread the infection to other tissue. Chronic, granulomatous or proliferative infections are defined as resultant of a long term and well isolated process, with formation of nodules containing the pathogen surrounded with epithelioid cells, with later accumulation of lymphocytes and fibrocytes on the outside layer **(Austin & Austin 2007)**.

The main causes of infectious disease on aquaculture are bacteria (54.9%), followed by virus (22.6%), parasites (19.4%) and fungi (3.1%) **(Kibenge *et al.* 2012)**. Although a wide group of bacteria can infect fish, a strict group of this pathogens are responsible for important monetary losses on aquaculture farms. In Portugal, information regarding the most important fish diseases is lacking, constituting a gap for studies that intend to improve fish health.

## 1.4. Photobacteriosis

Photobacteriosis or fish pasteurellosis is a septicemic disease caused by the Gram-negative, facultative intracellular halophilic bacteria *Photobacterium damselae* subsp. *piscicida (Phdp)*, being responsible for significant monetary costs in aquaculture production globally **(Andreoni & Magnani 2014)**. The disease was first reported in 1963 in a wild population of white perch in the USA **(Sniezko *et al.* 1964)**, and first isolation on aquacultures from the mediterranean countries occurred in 1990 on a Spanish gilthead seabream fish farm **(Toranzo *et al.* 1991)**. Infection can take part on a wide diversity of marine fish, including seabream, seabass, Atlantic salmon **(Romalde *et al.* 2002)**, sole species (*Solea senegalensis* and *Solea Solea*) **(Pellizzari *et al.* 2013)**, meagre (*Argyrosomus regius*) **(Costa *et al.* 2017)**, yellowtail (*Seriola quinqueradiata*) and cobia (*Rachycentron canadum*) **(Andreoni & Magnani 2014),** among others.

This bacterial septicemia occurs mainly during warm periods and pathology progress has been associated with temperatures above 18-20 ºC **(Magariños *et al.* 2001)**, low salinity, and poor water quality **(Romalde *et al.* 2002)**. Gilthead seabream susceptibility to this disease varies with fish development, being larvae and juveniles more disposed to the infection (with mortalities reaching 90-100%) whereas fish over 50 g present more resilience caused by phagocytosis efficiency **(Pellizzari *et al.* 2013)**.

Severity of disease can evolve on acute (generally related to younger fish) or chronic forms. Clinical external findings are usually non observable even on acute

outbreaks, with some fish presenting mild heamorrhagic regions in the head and gills **(Romalde *et al.* 2002, Baptista *et al.* 1996)**, anorexia and dark skin **(Magariños *et al.* 2001).** Internally, infected fish usually show multifocal necrosis in the liver, spleen, and kidney **(Andreoni & Magnani 2014)** and in some cases pale liver **(Baptista *et al.* 1996)**, splenomegaly and kidney enlargement **(Costa *et al.* 2017).** Chronic lesions can result on whitish granulomatous nodules about 0.5-3.5 mm in diameter in visceral organs such as kidney and spleen **(Magariños *et al.* 1996a).**

The infection process is complex and initially depends on bacterial invasion and adhesion of host cells. Although pathogenesis is still not fully understood, especially the invasion of non-phagocytic cells, several virulence factors are considered to increase *Phdp* resistance and proliferation. The capsule of the bacteria is one of them once its polysaccharide composition permits its resistance to bactericidal serum activity **(Magariños *et al.* 1996b)**. In addition, bacterial phagocytosis by neutrophils and monocytes allied to intracellular survival can act as a reservoir for the pathogen and decrease its elimination by host defences and exogenous antimicrobial agents such as antibiotics **(Andreoni & Magnani 2014).** Other strategies including host cell lesion by extracellular products with haemolytic and phospolipase activities were also described **(Magariños *et al.* 1992a)**. Also the acquisition of host's iron by high affinity iron-binding siderophores permits bacteria to obtain this metal from transferrin and heme compounds, contributing to the synthesis of proteolytic enzymes that enhance bacterial survival **(Magariños *et al.* 1994)**.

More recent studies have concluded that virulent *Phdp* strains can induce phagocytic cells apoptosis under natural or experimental infection on European seabass, by secretion of an exotoxin protein named AIP56 **(do Vale *et al.* 2007)**.

Rapid diagnosis of pasteurellosis is essential for accurate management and efficient control of outbreaks **(Carraro *et al.* 2017)**. Biochemical and serological tests, such as Analytica Profile Index-20 (API-20E) with result 2005004 **(Magariños *et al.* 1992b)**, slide agglutination **(Toranzo *et al.* 1987)** or ELISA are routinely used to identify the bacterium. Still, molecular approaches have been developed on the last 20 years since this method presents higher precision and is less time consuming. On the other hand, some constrains regarding the discrimination between *Photobacterium damselae* subsp*. damselae* and *Phdp* have been found since genetic sequences codifying for target genes are shared for both subspecies **(Romalde *et al.* 2002)**. Thus, current molecular approaches for the detection *of Phdp* involves more than one single step. Two of them include a multiplex PCR assay for 16S gene and ureC gene **(Osorio *et al.* 2000)** or Pbp-1A gene and UreC gene as internal amplification control **(Amagliani *et al.* 2009)**, while other one consisted on the amplification of the capsular polysaccharide gene (CPS)

with an additional culture step on TCBS (Thiosulfate-Citrate-Bile Salts-Sucrose) **(Rajan *et al.* 2003)**.

The treatment measures to face photobacteriosis are similar to those used for fish main bacterial infections and consists on chemotherapeutics administration such as antibiotics. However, the indiscriminate and unregulated use of broad spectrum antibiotics resulted on resistance to this drugs by *Phdp* strains, with studies confirming resistance to kanamycin, sulphonamide, tetracycline, ampicillin, chlorfenicol, florfenicol, erythromycin **(Andreoni & Magnani 2014)**, cloxaciclin and cefoperazone **(Parin *et al.* 2016).** In addition, recent concerns about environmental pollution as well as animal and human health are promoting the drastic decrease of the antibiotics use **(Cabello 2006)**, that are highly restrictive by european legislation, direting reseach efforts for novel alternative immune enhancers, namely prebiotics, probiotics and functional diets.

Prevention for this infection can be obtained through vaccination but differences like fish species, fish size and vaccine formulation affect their effectiveness **(Toranzo *et al.* 2005)**. As photobacteriosis affects frequently seabream juveniles from 10-30 g, conventional vaccines consisted of inactivated products resultant from heat or formalin killed bacteria, that were administered by dipping fish on early larval stages (1-2g) **(Magariños *et al.* 1999)**. In 2016, the veterinary pharmaceutical company HIPRA has been permited to comercialize a vaccine, ICTHIOVAC®PD, specifically formulated for juvenile seabream, confering 5 months of protection after dip administration of 2 inactivated *Phdp* strains for fish with 1 to 2 grams **(Miccoli *et al.* 2019)**. Depending on fish rearing facilities and prophylactic strategies, fish might be revaccinated through intraperitoneal administration, and boost usually occur when fish reach 15 to 20 grams.

## 1.5. Fish Immune System and Inflammation

In this section, a presentation of fish principal defence mechanisms will be presented. Teleost fish have an important role on the evolution of immunological answer since they are the first vertebrates to present both humoral and cellular acquired immunity **(Schluter *et al.* 1999)**. Although immunological steps for infected fish show many similarities to mammals, slight differences are present related to its physiological specificity. These differences are mainly caused by fish inability to control their temperature, affecting their defence mechanisms and time of actuation, leading to a major preponderance of innate immunity or primary response **(Tort 2003)**.

Immunological answer can be divided into three major components **(Figure 3)**: phagocytosis, innate/humoral immunity and adaptative/cellular immunity.

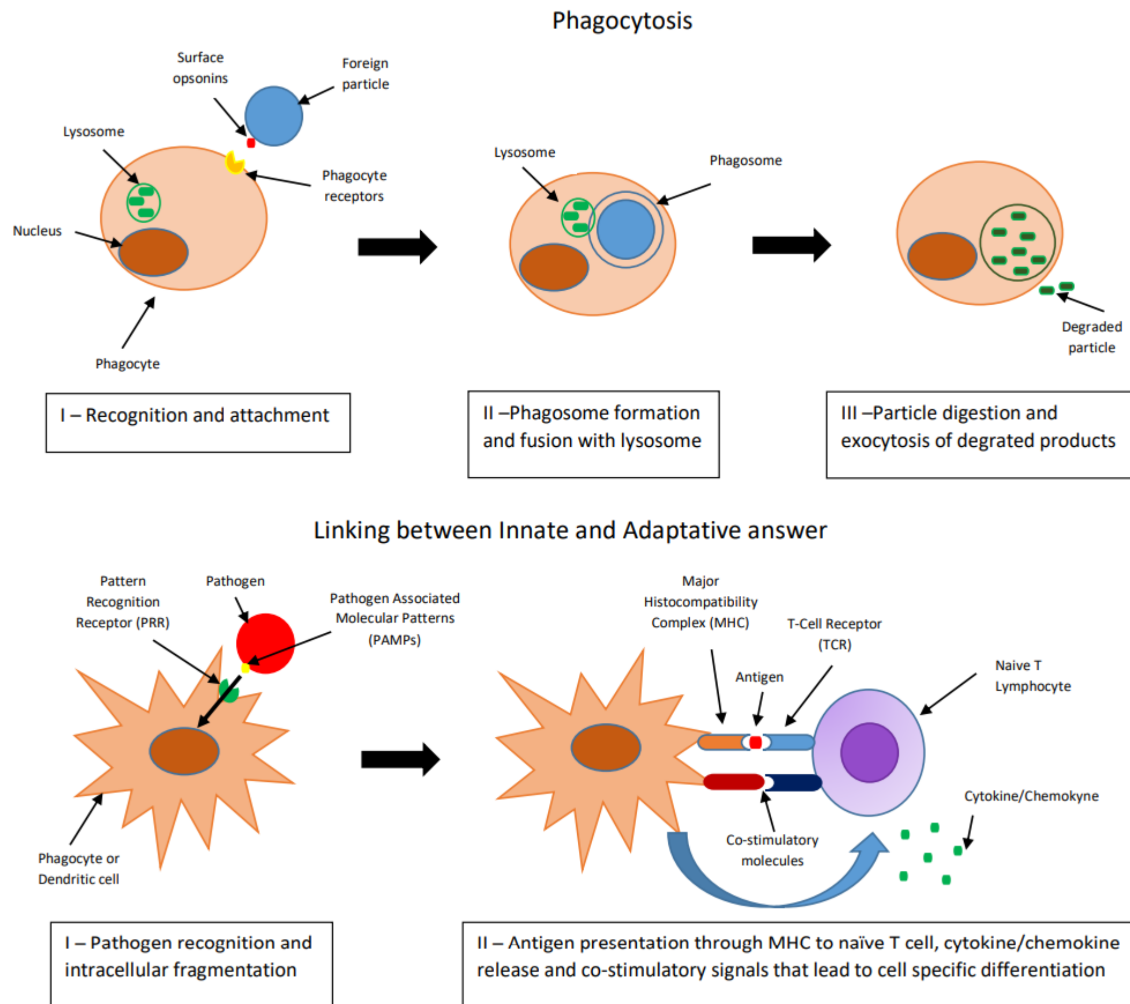



**Figure 3-** Phagocytosis process and innate/adaptative connection.

Phagocytosis is the most ancient immune mechanism reported (being present since the first unicellular organisms such as protozoans), that occurs due to a pathway that involves recognition and attachment of a foreign particle, with further internalization and vacuole formation (phagosome), digestion with help of lysosomes and ends up with exocytosis of the processed content **(Corbel 1975)**. The phagocytic process is modulated by cells called phagocytes (such as neutrophils and monocytes/macrophages) which are activated after surface receptor stimulation **(Uribe-Querol & Rosales 2017)**. Neutrophils are round-shaped cells with high capacity to migrate **(Griffin 1984)** and a strong non-specific cytotoxic activity (**Sasaki *et al.* 2002)**. These myeloid cells contain myeloperoxidase in their cytoplasmic granules **(Afonso *et al.* 1997)** that begins its action in the presence of halide ions and hydrogen peroxide by halogenation of the bacterial cell walls as well as production of bactericidal hypohalite

ions **(Klebanoff & Clark 1978)**. Similarly, monocytes/macrophages are phagocytic cells containing high amounts of mitochondria, vacuoles **(Dogget & Harris 1989)** and reactive oxygen species **(Hodgkinson *et al.* 2015)**. Although some authors use both cell nomenclature indiscriminately, they are easily distinguished regarding their location once monocytes act as circulating cells and macrophages exert their immune role on tissue and other places like the peritoneal cavity and natatory bladder **(Lamas *et al.* 1994)**. Another feature of this cell lineage is related to the expression of a specific receptor that was found to be distinctive on gilthead seabream cells called colony stimulating factor-1 receptor (CSF-1r) **(Roca *et al.* 2006)**.

Innate immunity is a fast and strong immunological answer provided by dendritic cells and phagocytes, with low specificity to the pathogen, that is able to discriminate self and non-self organisms by identifying molecules or proteins of pathogens (also known as pathogen associated molecular patterns), with the use of signallers of the host's recognizing cells called pattern recognition receptors (PRR) **(Magnadóttir 2006)**.There are several PRR families described in fish, being the most studied the Toll-like Receptors (TLRs), transmembranar proteins that after stimulation develop a signalling cascade ending on increased expression of Interferon Regulatory Factors (IRF) and Nuclear Factor-kB (NF-kB) **(Li *et al.* 2016)**. NF-kB is of special importance on immune response and inflammation since it leads to a large number of components release (such as cytokines and adhesion molecules) and can also influence host's cellular proliferation, differentiation and survival **(Liu *et al.*2017)**. Cytokines are small proteins produced by different kind of cells with specific functions and targets, contributing for cell to cell comunication. These mediators can be divided according to their production organ (being lymphokines produced by lymphocytes and monokines produced by monocytes) or their function (having chemokines a chemotactic activity and interleukins contributing to linkage between two leucocytes) **(Chang & An 2007)**. The wide interaction between different cytokines and defence cells from both humoral and adquired immunity provides a complete and coordinated answer that will be more detailed below. The inflammatory process is essential for physical and chemical homeostasis maintenance after infection occurrence or tissue lesion **(Kiron 2012)**, and can be sorted in three main stages: recognition, response and repair. Recognition phase involves the release of signals such as Heat Shok Proteins (HSPs), chaperones whose role is to facilitate cytokine production and macrophage diferentitation **(Breloer *et al.* 1998)**. Macrophages have been found to have two opposite effects and, since then, two different phenotypes were stablished, with M1 polarization being related to pathogen or damaged cell presence, and M2 being involved on repair processes **(Ley 2017)**. The intensity of inflammation is mediated through many different mechanisms but a simple way to explain it can be using this dual

macrophagic activity, with M1 cells acting as pro-inflammatory cytokine producters (e.g. IL-1β, Il-6, Il-12 and tumor necrosis factor-α) while M2-macrophages are responsible for anti-inflammatory cytokines (e.g. transforming growth factor-β and Interleukin 10) **(Zou & Secombs 2016)**.

The latest defence mechanism entering on action is adaptative immunity, an immunological response specific to the pathogen infection, that is developed by presentation of an antigenic compound from antigen presenting cells to lymphocyte cells through major histocompatibility complex (MHC) **(Chaplin 2010).** This MHC-T cell bound is important since it activates different T lymphocyte subsets, being MHC class I recognized to be linked with cytotoxic T lymphocytes (CD8+) that can induce by its own damage on the pathogen **(Fischer *et al.* 2006)**, while MHC class II binds to helper T cells (CD4+) with further cytokine release **(Yoon *et al.* 2016)**.These cytokines are IFNɣ and IL-2 when a non specific answer mediated by macrophages is required, while IL-2 and IL-4 are secreted in order to obtain a specific B lymphocyte stimulation **(Ashfaq *et al.* 2019).** B lymphocyte cells have high relevance on the adaptative process since they acquire the ability to differentiate and produce immunoglobulins, being immunoglobulin M the most common in fish **(Uribe *et al.* 2011).** After immune system synthesis of the immunoglobulin that is able to eliminate the antigen, T lymphocytes enter in action by producing interleukins, on a signalization process that ends with memory cell's production for that kind of infection, through a mechanism of action that is also influenced by temperature **(Ellis 1999)**.

Other fundamental host defence mechanism that is nor humoral nor cell-mediated immunity are called nonspecific mediators of immunity. Their function is to block pathogen invasion before immune cells and humoral factors take place. Examples of this immunological walls are mucus and skin. Fish epidermis is composed of nonkeratinized living cells **(Roberts & Bullock 1980)**, constituting an adaptive advantage once it allows fish to balance osmolarity **(Ellis 1981)**. Mucus is an external barrier that is present on skin, gills and gastrointestinal mucosa, preventing microorganism's colonization and proliferation through several proteins and enzymes (such as lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and IgMs) **(Magnadóttir 2006).** Antibacterial compounds are a recent study group of peptides that are divided into linear α-helix peptides (piscidin, gaduscidin, moronecidin, grammistins, pleurocidin, chrysophsin, pardaxin, epinecidin and chemokine derived peptides), dissulfide bond peptides (cathelicidins, defensins, hepcidins) and peptides with different structures. The study of this compounds is of major importance on the development of new strategies for pathogen degradation and fish immune enhancement **(Valero *et al.* 2020)**

An affected host can be recognized by five observable clinical signs (redness, heat, swelling, pain and loss of function), but since fish temperature is affected by water temperature, heat and redness can be masked and are not useful as markers for disease's diagnosis **(Roberts 2012)**. The developmental mechanism for the inflammatory process results on a vascular phenomenon with histamine release that initiates a fast and local increased blood flow, ending in hyperaemia. Alongside, a chemotaxis process takes place, through inflammatory mediator's production and blood stream release, guiding neutrophils to the damaged local **(Junger 2008)**. These phagocytic cells possess a set of different antimicrobial agents such as lytic enzymes, antiproteases, bactericidal reactive oxygen species **(Ellis 2001)** and complement factors **(Barton 2008),** which can directly activate mechanisms for immediate elimination, colonization, survival and proliferation of microorganisms.

Lysozyme is a phagocytic cell's enzyme which hydrolyses N-acetylmuramic acid and N-acetylglucosamine which are constituents of the peptidoglycan layer of bacterial cell walls (**Ingram 1980)**. The complement system can be activated by antigen-antibody reactions or by the so-called alternative route, via binding to microbial cell wall polysaccharides, which results in opsonization and/or lysis of foreign cells **(Bayne & Gerwick 2001)**. Antiproteases are substances that have yet been found in fish serum **(Ellis *et al.* 1981)** and their role is to maintain body fluid homeostasis, being involved on acute phase reactions as non-specific answer and also on bacterial proteases inhibition, decreasing pathogen possibility to use host proteins as substrate for their maintenance **(Magnadóttir 2006)**. The main protease inhibitors are α1-anti-protease, α2-anti-plasmin and α2-macroglobulin **(Ellis 2001)**.

## 1.6. Scope of Study

Although aquaculture's growth potential and sustainability are undeniable, the increase of fish density for a higher profitability presents an increased preponderance for the occurrence of infectious pathologies that cause relevant losses on fish farms. A specific study for each species involving all components of disease development is necessary to understand the infectious process in order to produce and implement control and treatment measures.

Therefore, the objective of this study was to understand farmed juvenile gilthead seabream immune modulation after *Phdp* infection. For this, fish were kept in farming conditions with further division of two groups (Control and Infected). After challenge, individuals were followed for a period of 48 hours and cellular, humoral and gene expression analysis were performed to provide a better insight of the defence mechanisms affected.

The remaining 30 animals from each treatment were maintained for 14 days for mortality rate data assessment.

# 2- Materials and Methods

## 2.1. Experimental Design

The current study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese Veterinary Authority following FELASA category C recommendations. This experiment was performed accordingly to the guidelines on the protection of animals used for scientific purposes (European Union directive 2010/63/EU).

Gilthead seabream juveniles were transferred from Estação Piloto de Piscicultura de Olhão (Olhão, Portugal) to Politécnico de Leiria facilities (CETEMARES, Portugal), and quarantined for a period of 90 days. After this period, 132 fish (9.8 ± 2.2 g) were individually weighted and randomly distributed into 6 recirculating tanks of 60 L of seawater (n = 22, animal initial density = 19.6 $Kg/m^3$ , photoperiod 12 hours light/12 hours dark). The physicochemical parameters such as oxygen saturation (6.62 ± 0.04 mg/L), salinity (30.95 ± 0.06) and pH (8.04 ± 0.05) were monitored on a daily base. Both temperature and ammonium/nitrite levels were kept constant throughout the trial (T = 25 ± 1 °C; $NH_4$ and $NO_2$ respectively under 0.33 and 1.61 mg/L).

## 2.2. Bacterial Challenge

*Photobacterium damselae subsp. piscicida* (AQP17.1), kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiologia y Parasitologia, Facultad de Biologia, Universidade de Santiago de Compostela, Spain) was cultured on Erlenmeyer flasks containing 50 mL of TSB (1.5% of NaCl) (Difco Laboratories) and grown under continuous agitation (25 °C) for 48 hours. After that, the content of the flasks was transferred to 50 mL falcon tubes and centrifuged for 30 minutes at 3,500 rpm. The supernatants of the centrifuged tubes were then discarded and the remaining pellet was dissolved in PBS (GIBCO). Bacterial concentration was read at 600 nm and adjusted to $1 \times 10^6$ CFU/mL. Half of individuals were infected through peritoneal injection with 100 µl of the above suspension ($1 \times 10^5$ CFU/fish), while the other half of individuals were kept as control group and injected with the same volume of phosphate buffered saline (PBS). Infection was followed for 14 days and animals that died during this period were registered in order to obtain mortality rates.

## 2.3. Sampling

Both infected and control groups were sampled immediately before infection (Time 0), and then 3, 6, 9, 24 and 48 h after challenge. Two fish per tank were randomly sampled for each time point (n = 6 for treatment) and euthanized using 2-phenoxyethanol (0.5 mL/L). Blood samples were collected from the caudal vein using 1 mL syringes (previously prepared with 3,000 units/mL of heparin). Blood samples were then placed in 1.5 mL heparinized tubes and gently homogenized for hematological analysis as described below. The remaining blood was centrifuged for 10 min at 10,000 x g at 4 $^{o}$C and afterwards plasma was collected and stored at -80 $^{o}$C. Head-kidney was also aseptically collected for gene expression analysis and stored in RNA later (with a proportion of 1/10 w/v), at 4 $^{o}$C for the first 24 h and then stored at -80 $^{o}$C.

## 2.4. Hematological Analysis

Before centrifugation of homogenized blood, a small aliquot was reaped for white blood cells (WBC) and red blood cells (RBC) counts, haematocrit (Ht) and haemoglobin determination (Hb, SPINREACT kit, ref. 1001230, Spain). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MHCH) were also calculated:

**MCV ($mm^3$)** = (Ht/RBC) x 10

**MCH (pg/cell)** = (Hb/RBC) x 10

**MHCH (g/100 mL)** = (Hb/Ht) x 100

The smears from heparinized blood were run through a single blood droplet and air dried. After drying, the slides were fixed with a solution of formaldehyde-ethanol (90 % absolute ethanol to 10 % of 37% formaldehyde) for one minute **(Kaplow 1965)**. Neutrophils were then marked for detection of peroxidase activity, following a protocol described by **Afonso *et al.* (1998).** Subsequently, slides were stained with Wright's stain (Haemacolor, Merck) and observed under oil immersion (1,000 X). Leucocytes were identified and a differential count of neutrophils, monocytes, lymphocytes and thrombocytes was made in a total of 200 cells/smear. Relative counts were further converted for absolute values ($\times 10^4$/mL) of each cell type using WBC results.

## 2.5. Innate Humoral Parameters

1) Peroxidase activity: Total peroxidase activity in plasma was measured following the procedure described by **Quade and Roth (1997).** To do so, 15 µl of plasma in duplicate were diluted in 135 µl of HBSS without $Ca^{2+}$ and $Mg^{2+}$ in flat bottomed 96-well plates. Then, 50 µl of 20 mM 3,3',5,5'-tetramethybenzidine hydrochloride (TMB; Sigma) and 50 µl of 5 mM hydrogen peroxide were added, resulting on a change of colour of the mixture that turned blue **(Figure 4).** The colour change reaction was stopped after 2 minutes by adding 50 µl of 2M sulphuric acid **(Figure 5)** and the optical density was read at 450 nm in a Synergy HT microplate reader, Biotek. Two wells with 150 µl of HBSS were used as blanks. The peroxidase activity (units/mL plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 Optical Density (OD).

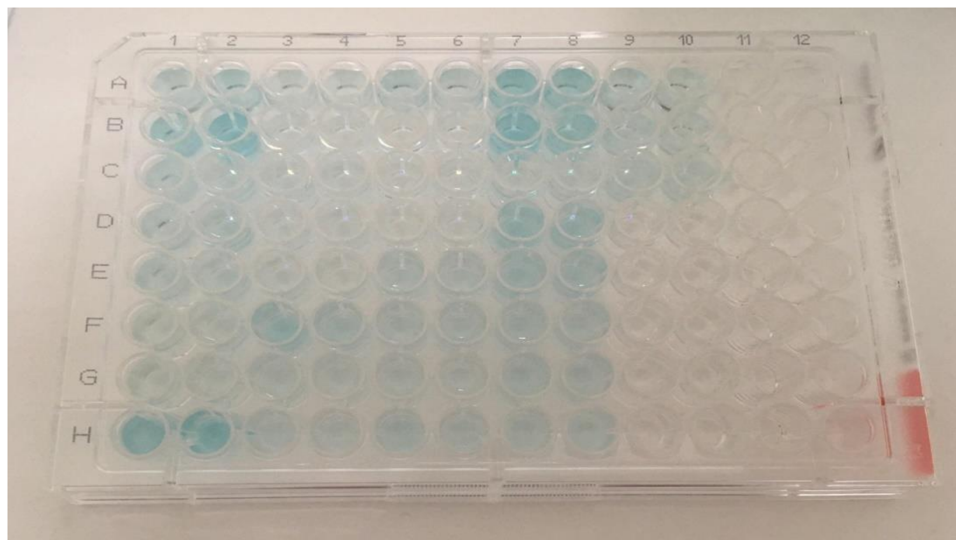



**Figure 4-** Microplate after adding hydrogen peroxide.

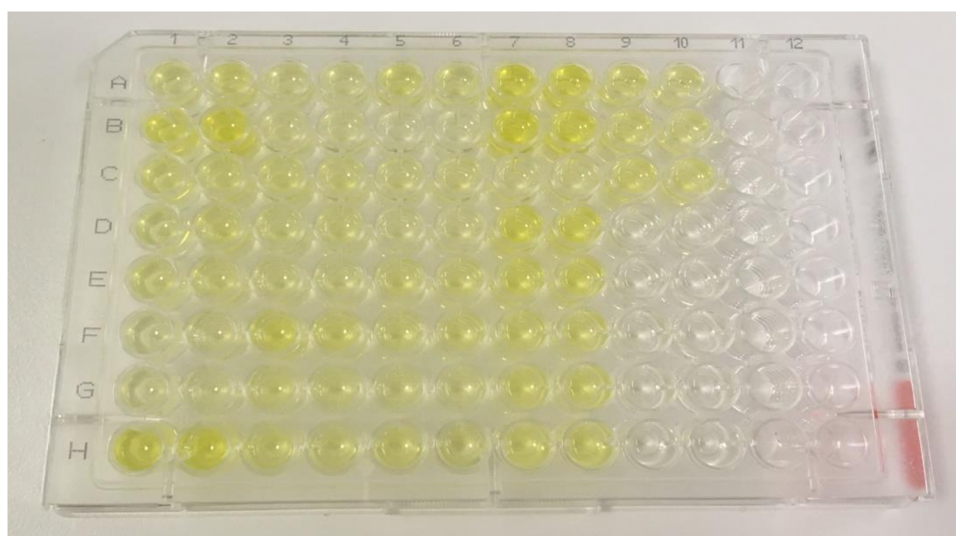



**Figure 5-** Microplate after adding sulphuric acid.

2) Antiprotease Activity: The method described by **Ellis (1990)** was modified and adapted for 96-well microplates (**Machado *et al.* 2015**). Firstly, 10 µl of plasma were incubated with the same volume of trypsin solution (5 mg/mL in $NaHCO_3$ 5 mg/ml, pH 8.3) for 10 minutes at 22 ºC in polystyrene microtubes. Afterwards, 100 µl of phosphate buffer ($NaH_2PO_4$,13.9 mg/ml, pH 7.0) and 125 µl of azocasein (20 mg/ml in $NaHCO_3$, 5 mg/ml, pH 8.3) were added and the mixture was incubated for 1 h at 22 ºC. 250 µl of trichloroacetic acid were then added to the microtubes and incubated for 30 min at 22 ºC. Finally, the mixture was centrifuged at 10,000 × g for 5 min at room temperature and 100 µl of supernatants were transferred to a 96-well plate in duplicate containing 100 µl of 1N NaOH. One blank of phosphate buffer saline only was used in the protocol, and the reference sample was obtained using phosphate buffered saline instead of plasma **(Figure 6)**. The percentage of trypsin activity was calculated as follows:

**% non-inhibited trypsin** = (Sample absorbance × 100) / Reference sample

**% inhibited trypsin** = 100 - % non-inhibited trypsin

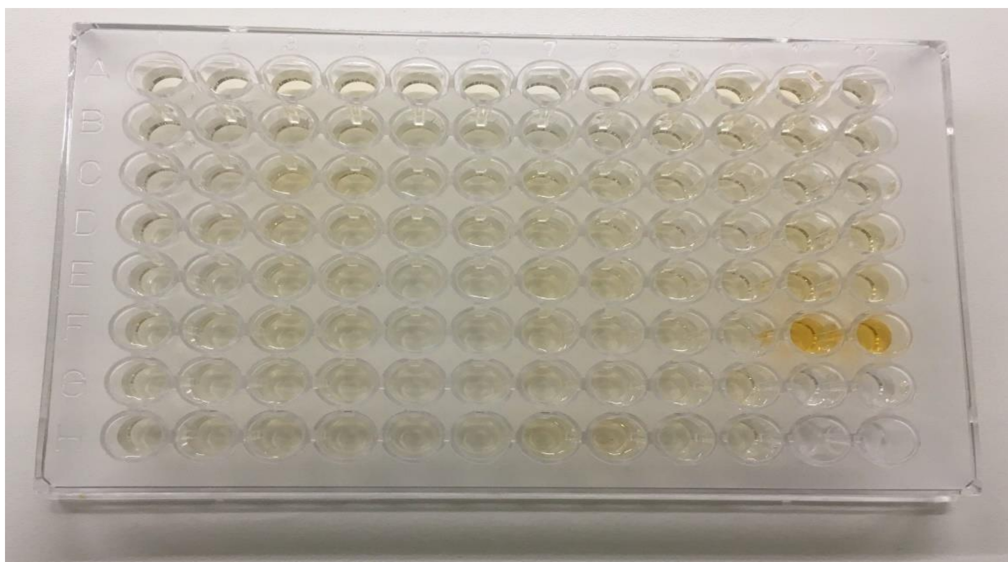


**Figure 6-** Antiprotease microplate before reading.

3) Protease activity: The method described by **Ellis (1990)** was modified and adapted for 96-well microplates **(Machado et al. 2015)**. All procedure followed the same order and quantities of antiprotease activity protocol except for the period of incubation with phosphate buffer and azocasein that was maintained in constant agitation for 24 h.

## 2.6. Gene Expression Analysis

The extraction of head kidney's RNA was performed with NZY total RNA isolation kit (NZYTech, Lisbon, Portugal), following manufacturer's instructions. After extraction,

RNA samples were quantified and purity was assessed by spectrophotometry using DeNovix DS-11 FX (Wilmington, DE, USA). Samples varied on RNA quantity from 197.61 ng/µl to 912.43 ng/µl and presented 260:280 ratios between 2.15 and 1.91.

NZY first-strand cDNA synthesis kit (NZYTech, Lisbon) was used for transcription of the obtained RNA to cDNA. This step also allowed us to standardize our samples (50 ng/µl of cDNA) on a final volume of 20 µl. Reverse transcriptase was then performed on Veriti DX 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

Real-time Quantitative PCR was carried out in duplicate for each reaction with the CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA), being 12 genes **(Table II)** selected and studied according to their influence on the immune answer. Primer efficiency was tested for each gene with results varying between 111 and 86 %. Cycling conditions were identical among different genes, varying only on the annealing temperature, consisting on:

A) 10 min at 95 ºC for initial denaturation,

B) 40 cycles of 2 steps of:

I) 95 ºC for denaturation for 15 sec

II) primer annealing temperature for each different gene for 1 minute

III) 72 ºC for extension.

C) 1 min at 95 ºC, followed by 30 sec at anneling temperature and ending with 95 ºC for 15 sec. For each target gene samples were normalized using EF-1α gene as housekeeping and subsequently Pfaffl method **(Pfaffl 2001)** was used for gene expression calculations.

**Table II**- Immune related genes analysed by Real-time PCR.

| Gene | Acronym | Efficiency (%) | Annealing temperature (ºC) | Amplicon length (bp) | Primer Sequence (5´-3´) |
|---|---|---|---|---|---|
| **Elongation Factor 1α** | EF-1α | 92 | 58 | 87 | F: CTGTCAAGGAAATCCGTCGT<br>R:TGACCTGAGCGTTGAAGTTG |
| **Heat-Shok Protein 70** | HSP70 | 104 | 55 | 124 | F:ACGGCATCTTTGAGGTGAAG<br>R:TGGCTGATGTCCTTCTTGTG |
| **Non-specific cytotoxic cell receptor protein 1** | NCCRP1 | 106 | 60 | 100 | F: ACTTCCTGCACCGACTCAAG<br>R:TAGGAGCTGGTTTTGGTTGG |
| **Interleukin 34** | IL-34 | 102 | 60 | 214 | F: CATCAGGGTTCATCACAACG<br>R: GACTCCCTCTGCATCCTTGA |
| **Hepcidin** | Hep | 111 | 60 | 382 | F:GCCATCGTGCTCACCTTTAT<br>R:CCTGCTGCCATACCCCATCTT |
| **Major histocompatibility complex I** | MHCI | 107 | 60 | 104 | F:CGATGGAACCTTCCAGATGA<br>R:CCTCGTTCACACCAGAGAGC |
| **Major histocompatibility complex II γ** | MHCII | 100 | 60 | 107 | F:ACAACATGAACGCTGAGCTG<br>R:CTCGTCCACAGAGTCATCCA |
| **Interleukin 1 β** | IL1β | 112 | 60 | 245 | F: TCTTCAAATTCCTGCCACCA<br>R:CAATGCCACCTTGTGGTGAT |
| **Colony stimulating factor-1 receptor** | Csf1r | 96 | 60 | 129 | F: ACGTCTGGTCCTATGGCATC<br>R:AGTCTGGTTGGGACATCTGG |
| **Transforming growth factor β1** | TGFβ1 | 96 | 58 | 132 | F:TCTGGGGTGGAAATGGATAC<br>R: CTCCTGGGTTGTGATGCTTA |
| **Caspase 1** | Casp-1 | 87 | 59 | 92 | F:ACGAGGTGGTGAAACACACA<br>R:GTCCGTCTCTTCGAGTTTCG |
| **β-Defensin** | β-Def | 107 | 60 | 101 | F:CCCCAGTCTGAGTGGAGTGT<br>R:AATGAGACACGCAGCACAAG |
| **Interleukin 10** | IL-10 | 91 | 57 | 65 | F:AACATCCTGGGCTTCTATCTG<br>R: GTGTCCTCCGTCTCATCTG |

## 2.7. Statistical Analysis´

The group of animals were fold increased by dividing each fish parameter with mean basal condition (Time 0) with further addition of one. Afterwards, mean and standard deviation were calculated for each treatment and time group. Data were analysed for normality and homogeneity of variance and Log transformed before statistical treatment when needed. Data were analysed by Two-way ANOVA (Tukey post hoc test) and, when interaction was observed, one-way ANOVA was performed. The performance of statistical analyses occurred under SPSS 26 program for WINDOWS. The level of significance used was $p \leq 0.05$ for all statistical tests.

# 3- Results

## 3.1. Bacterial Challenge

Evaluating the effect of bacterial infection on fish survival for 14 days within different treated groups (n=60), **(Figure 7)** presents a clear and marked difference ($X^2$= 0.0053) between fish that were inoculated with *Phdp* (cumulative mortality of 36.7 %) and fish injected with PBS (cumulative mortality of 6.7 %). It is also possible to observe that fish that died due to bacterial infection have only been found during the first 6 days of disease, being this the normal death timing of this bacterial disease.

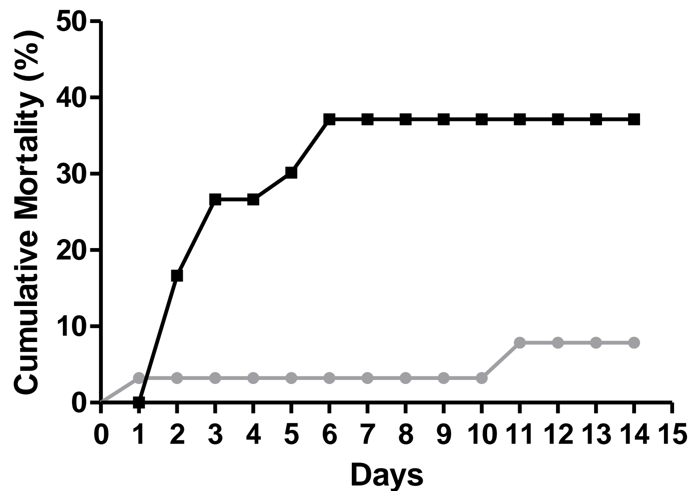



**Figure 7-** Cumulative mortality (%) of gilthead seabream after PBS (●) or *Phdp* (■) intraperitoneal injection (n=60).

## Haematological Analysis

Blood from 6 fish for treatment (12 per time) were sampled at 0, 3, 6, 9, 24 and 48 h post injection. All data is presented as fold increase using time 0 for this normalization.

Regarding heamatological parameters, no differences were found in red and white blood cell counts within different times. However, reduced values on red blood cells were observed in infected animals when compared with control ones and this difference is clearly observable after 48 h. This last result goes in accordance with haematocrit values, where significant differences were registered between different treatment groups

48 h post-infection. Haemoglobin concentration varied within time in the same treatment group, showing higher concentration at 3 h than 24 h in control group, while infected animal values were significally higher 6 and 24 h post infection when compared with fish sampled at 48 h. Although mean corpuscular volume did not change nor on time nor on treatment, significant differences were obtained on mean corpuscular haemoglobin and mean cospuscular haemoglobin concentration (MCHC). While on the first parameter it is only possible to observe a decrease on total values along time regardless treatment, MCHC time values drop within time is followed by treatment discrepancies, putting in evidence the deficit on this parameter on control animals, that are more marked 24 h after i.p injection **(Table III)**.

Differences on peripheral leucocitary population are very strong when blood smears were observed. Neutrophils varied on a waveform, reaching a peak 6 hours after injection with later decrease. Plus, it was also remarkable that infected animals presented higher neutrophil numbers than control group. An interesting finding was observed on monocytes where besides no differences were found on treatment or time isolated, a significant difference was found after 9 hours where infected animals showed lower monocyte values that control and later, on the same infected group, a significant increase on monocyte cells occurred. Lymphocytes and thrombocytes varied on a similar way, with total values increasing with time and control group showing higher cellular values than infected one. However, and although both leucocyte types present the same variation at time 9 h (with both control groups registering higher values than infected fish), lymphocytes also presented this difference 48 h after challenge **(Table IV)**.

### 3.2. Innate Humoral Parameters

Surprisingly, plasma immune parameters have produced no differences on the three protocols performed. Antiproteases activity was the only parameter with statistical differences (resulting on decreased activity along time). Even though it was possible to observe that infected animals registered a tendency for higher peroxidase and antiproteases activities than control ones within the first 24 h **(Table V)**.

**Table III-** Fold increase values of white blood cells (WBC), red blood cells (RBC), heamatocrit, heamoglobin, mean corpuscular volume (MCV), mean corpuscular heamoglobin (MCH) and mean corpuscular heamoglobin concentration (MCHC) in gilthead seabream at 3, 6, 9, 24 and 48 hours after bacterial or placebo challenge.

| Two-Way ANOVA | **Time** | **Treatment** | **Time x Treatment** | **3h** | **6h** | **9h** | **24h** | **48h** | **Control** | **Infected** |
|---|---|---|---|---|---|---|---|---|---|---|
| **WBC** | ns | ns | ns | - | - | - | - | - | - | - |
| **RBC** | ns | 0.02 | ns | - | - | - | - | - | A | B |
| **Hematocrit** | 0.022 | <0.001 | <0.001 | a | ab | ab | b | ab | A | B |
| **Hemoglobin** | <0.001 | ns | 0.001 | a | ab | bc | bc | c | - | - |
| **MCV** | ns | ns | ns | - | - | - | - | - | - | - |
| **MCH** | 0.001 | ns | ns | a | a | a | ab | b | - | - |
| **MCHC** | <0.001 | 0.014 | 0.003 | a | ab | ab | ab | b | B | A |

| One-way ANOVA | **3h** | | **6h** | | **9h** | | **24h** | | **48h** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** |
| **WBC** | 1.89 ± 0.15 | 1.81 ± 0.16 | 1.87 ± 0.06 | 1.66 ± 0.08 | 1.99 ± 0.10 | 1.72 ± 0.39 | 1.79 ± 0.19 | 1.91 ± 0.26 | 1.95 ± 0.14 | 1.89 ± 0.23 |
| **RBC** | 1.89 ± 0.12 | 1.78 ± 0.20 | 1.74 ± 0.17 | 1.71 ± 0.17 | 1.68 ± 0.13 | 1.58 ± 0.11 | 1.71 ± 0.14 | 1.76 ± 0.07 | 1.91 ± 0.22 | 1.61 ± 0.06 |
| **Hematocrit** | 2.13 ± 0.12 | 2.03 ± 0.05$^{A}$ | 2.03 ± 0.10 | 2.02 ± 0.16$^{AB}$ | 2.13 ± 0.15 | 1.85 ± 0.09$^{AB}$ | 1.97 ± 0.09 | 1.82 ± 0.19$^{AB}$ | 2.16 ± 0.11$^{\#}$ | 1.71 ± 0.09$^{B*}$ |
| **Hemoglobin** | 2.36 ± 0.20$^{a}$ | 2.27 ± 0.15$^{A}$ | 1.95 ± 0.22$^{ab}$ | 2.20 ± 0.31$^{A}$ | 2.16 ± 0.33$^{ab}$ | 1.86 ± 0.23$^{AB}$ | 1.73 ± 0.18$^{b}$ | 2.13 ± 0.19$^{A}$ | 1.96 ± 0.25$^{ab}$ | 1.55 ± 0.10$^{B}$ |
| **MCV** | 2.30 ± 0.25 | 2.24 ± 0.15 | 2.55 ± 0.28 | 2.26 ± 0.12 | 2.48 ± 0.27 | 2.58 ± 0.48 | 2.41 ± 0.26 | 2.09 ± 0.22 | 2.37 ± 0.40 | 2.15 ± 0.05 |
| **MCH** | 1.77 ± 0.13 | 1.81 ± 0.17 | 1.64 ± 0.22 | 1.84 ± 0.31 | 1.82 ± 0.22 | 1.70 ± 0.14 | 1.48 ± 0.09 | 1.71 ± 0.12 | 1.53 ± 0.19 | 1.43 ± 0.10 |
| **MCHC** | 1.59 ± 0.15$^{a}$ | 1.59 ± 0.05 | 1.42 ± 0.11$^{ab}$ | 1.62 ± 0.08 | 1.47 ± 0.13$^{ab}$ | 1.48 ± 0.16 | 1.34 ± 0.09$^{*b}$ | 1.57 ± 0.13$^{\#}$ | 1.37 ± 0.07$^{ab}$ | 1.40 ± 0.07 |

Values (Means ± SD) were calculated by dividing each parameter value from challenged fish by the mean value from fish sampled on time 0 plus one (n=6). Two-way ANOVA: ns: non-significant (P>0.05); If interaction was significant, one-way ANOVA was performed. Different superscript lower letters indicate differences betweet control groups through time, different superscript capital letters indicate differences among infected group through time, and different superscript symbols indicate differences between different treatment groups on the same sampling time. Regarding two-way ANOVA, different lower letters indicate differences along time and different capital letters indicate differences between treatments.

**Table IV-** Fold increase values of neutrophils, monocytes, lymphocytes, thrombocytes in gilthead seabream at 3, 6, 9, 24 and 48 h after bacterial or placebo challenge.

| Two-way ANOVA | **Time** | **Treatment** | **Time x Treatment** | **3h** | **6h** | **9h** | **24h** | **48h** | **Control** | **Infected** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Neutrophils** | 0.05 | <0.001 | ns | ab | a | ab | ab | b | B | A |
| **Monocytes** | ns | ns | 0.001 | - | - | - | - | - | - | - |
| **Lymphocytes** | 0.015 | <0.001 | <0.001 | ab | b | ab | a | a | A | B |
| **Thrombocytes** | 0.02 | <0.001 | <0.001 | ab | b | ab | ab | a | A | B |

| One-way ANOVA | **3h** | | **6h** | | **9h** | | **24h** | | **48h** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** |
| **Neutrophils** | 3.72 ± 1.35 | 4.76 ± 1.93 | 4.58 ± 0.66 | 5.34 ± 1.31 | 3.37 ± 1.18 | 4.01 ± 0.80 | 2.78 ± 0.76 | 4.79 ± 2.48 | 2.45 ± 0.53 | 3.09 ± 1.02 |
| **Monocytes** | 2.04 ± 0.56 | $2.32 \pm 0.80^{AB}$ | 2.08 ± 0.31 | $1.69 \pm 0.31^{AB}$ | $2.33 \pm 0.51^{\#}$ | $1.35 \pm 0.10^{*B}$ | 1.77 ± 0.41 | $2.58 \pm 0.69^{A}$ | 2.15 ± 0.42 | $2.82 \pm 0.83^{A}$ |
| **Lymphocytes** | 1.61 ± 0.20 | $1.51 \pm 0.12^{AB}$ | 1.58 ± 0.19 | $1.31 \pm 0.09^{B}$ | $1.75 \pm 0.15^{\#}$ | $1.38 \pm 0.12^{AB*}$ | 1.66 ± 0.12 | $1.62 \pm 0.15^{A}$ | $1.83 \pm 0.18^{\#}$ | $1.50 \pm 0.10^{AB*}$ |
| **Thrombocytes** | 1.81 ± 0.19 | 1.63 ± 0.17 | 1.72 ± 0.11 | 1.47 ± 0.08 | $1.96 \pm 0.07^{\#}$ | $1.52 \pm 0.25^{*}$ | 1.76 ± 0.22 | 1.72 ± 0.14 | 1.96 ± 0.19 | 1.76 ± 0.16 |

Values (Means ± SD) were calculated by dividing each parameter value from challenged fish by the mean value from fish sampled on time 0 plus one (n=6). Two-way ANOVA: ns: non-significant (P>0.05); If interaction was significant, one-way ANOVA was performed. Different superscript lower letters indicate differences betweet control groups through time, different superscript capital letters indicate differences among infected group through time, and different superscript symbols indicate differences between different treatment groups on the same sampling time. Regarding two-way ANOVA, different lower letters indicate differences along time and different capital letters indicate differences between treatments.

**Table V**- Fold increase values of antiproteases activity, peroxidase activity and proteases activity in gilthead seabream at 3, 6, 9, 24 and 48 h after bacterial or placebo challenge.

| Two-way ANOVA | 3h | | 6h | | 9h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** | **Control** | **Infected** |
| **Antiprotease activity** | 2.03 ± 0.05 | 2.04 ± 0.04 | 2.03 ± 0.07 | 1.99 ± 0.07 | 1.99 ± 0.06 | 2.02 ± 0.05 | 2.02 ± 0.05 | 2.07 ± 0.04 | 2.07 ± 0.03 | 2.12 ± 0.02 |
| **Peroxidase activity** | 2.19 ± 0.23 | 2.08 ± 0.37 | 1.58 ± 0.13 | 2.12 ± 0.41 | 1.96 ± 0.27 | 2.03 ± 0.55 | 1.87 ± 0.23 | 2.37 ± 0.53 | 2.57 ± 0.82 | 2.03 ± 0.43 |
| **Protease activity** | 1.99 ± 0.08 | 1.96 ± 0.24 | 1.99 ± 0.14 | 1.90 ± 0.27 | 1.95 ± 0.20 | 2.07 ± 0.03 | 1.81 ± 0.18 | 1.87 ± 0.18 | 1.82 ± 0.13 | 1.71 ± 0.17 |

| One-way ANOVA | **Time** | **Treatment** | **Time x Treatment** | **3h** | **6h** | **9h** | **24h** | **48h** | **Control** | **Infected** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Antiprotease activity** | 0.004 | ns | ns | ab | b | b | ab | a | - | - |
| **Peroxidase activity** | ns | ns | ns | - | - | - | - | - | - | - |
| **Protease activity** | ns | ns | ns | - | - | - | - | - | - | - |

Values (Means ± SD) were calculated by dividing each parameter value from challenged fish by the mean value from fish sampled on time 0 plus one (n=6). Two-way ANOVA: ns: non-significant (P>0.05); If interaction was significant. one-way ANOVA was performed. Different superscript lower letters indicate differences betweet control groups through time, different superscript capital letters indicate differences among infected group through time and different superscript symbols indicate differences between different treatment groups on the same sampling time. Regarding two-way ANOVA, different lower letters indicate differences along time and different capital letters indicate differences between treatments.

### 3.3. Gene Expression Analysis

To evaluate expression of immune genes presented on Figure 8 and 9, cDNA was isolated from head-kidney collected from 6 fish for each time and treatment.

Simultaneous time and treatment effects in fish gene expression were found in non-specific cytotoxic cell receptor protein 1 (NCCRP1), Interleukin 34 (IL-34), Major Histocompatibility Complex class I (MHC-I), Interleukin 1β (IL-1β) and Caspase 1 (Casp1). The most significant result was obtained in IL-1β mRNA expression levels **(Figure 9A)**, where infected groups presented a significant upregulation along all sampling times, when compared with control ones. Casp1 **(Figure 9D)** showed higher transcript numbers among controlled animals after 9 h and, at 24 h, infected animals presented higher mRNA expression than control ones. Similar treatment differences were found on IL-34 **(Figure 8C)** and MHCI **(Figure 8E)**, with a significant upregulation in infected seabream compared to controls after 24 h of pathogen inoculation. NCCRP1 showed differences among different treatments and sampling points **(Figure 8B)**. Transcripts of this gene presented higher expression on control animals on the first 9 h post injection, while infected animals registed its peak at time 48 h, and differences between both treatment groups were significant on the same sampling points.

The mRNA expression of Interleukin 10 (IL-10) and Colony Stimulating Factor-1 receptor (Csf-1r) presented similar patterns in infected animals. While Csf-1r presented increased mRNA expression at time 48 h **(Figure 9B)**, IL-10 expression augmented significally at time 9 h **(Figure 9F)**.

Heat Shok Protein 70 (HSP70), Major Histocompatibility Complex Class II (MHCII), hepcidin (Hep), Transforming Growth Factor β1 (TGF-β1) and β-defensin (β-Def) mRNA expression levels did not change significantly among time and/or treatments **(Figure 8A**

**and 8D).** Although not statistically significant, TGF-β1 and β-Def mRNA expression levels tended to increase in infected animals after 24 and 48 h **(Figures 9C and 9E).**

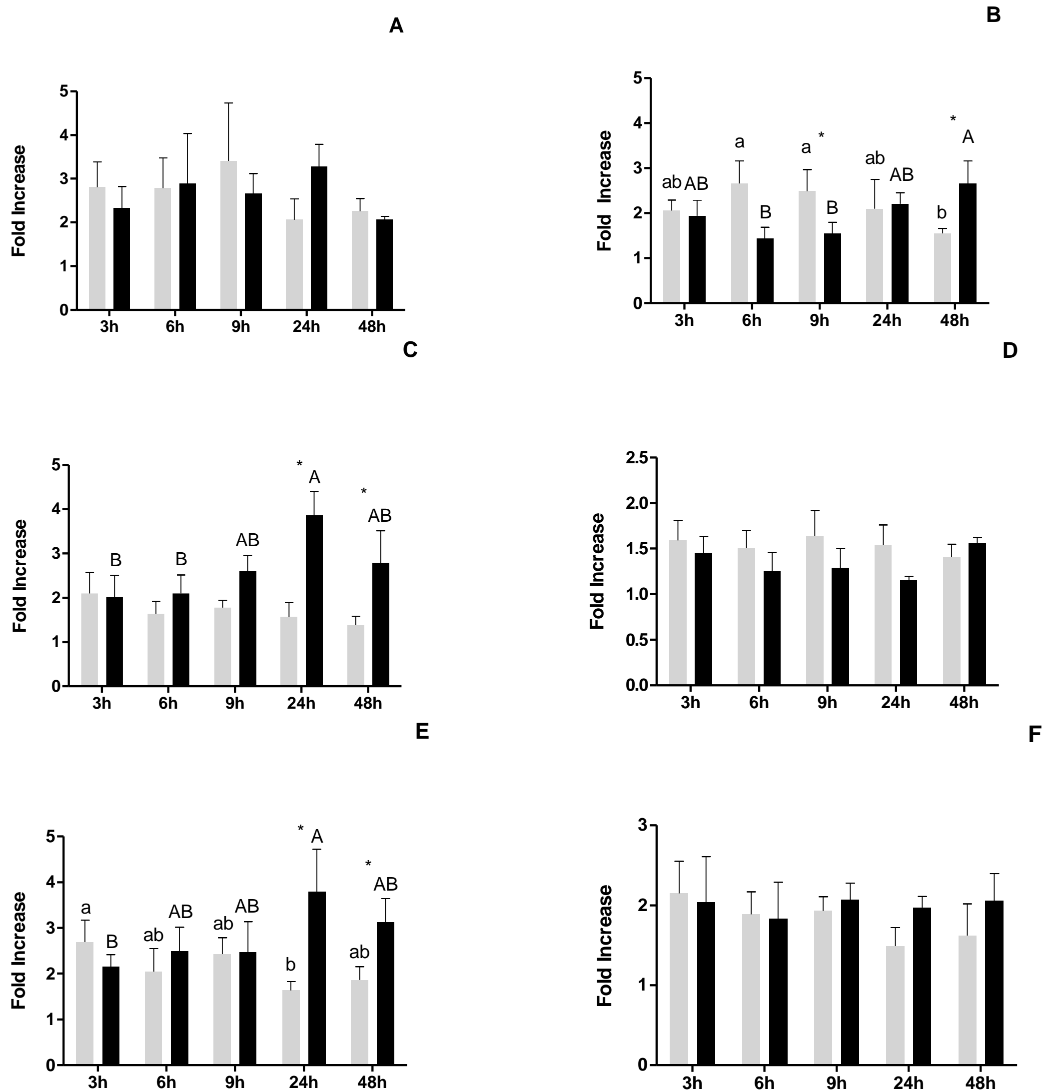



**Figure 8-** Quantitative expression of Heat Shok Protein 70 (**A:** p>0.05), Non-specific Citotoxic Cell Receptor Protein 1 (**B:** p<0.001), Interleukin 34 (**C:** p<0.001), Hepcidin (**D:** p=0.047), Major Histocompatibility Complex I (**E:** p<0.001) and Major Histocompatibility Complex II (**F:** p>0.05) in the head kidney of gilthead seabream juveniles after *Phdp* challenge. Data are expressed as means ± SD (n=6). Bars represent the fold increase in expression as compared to fish prior to infection (Time 0), previously normalized to Elongation Factor 1 (EF1). Different lower case letters stand for significant differences among different times on control animals, while symbol stands for differences between different treatment groups on the same sampling time. Different capital letters indicate differences among different times on infected animals. (Two-way ANOVA; Tukey post-hoc test; p≤0.05).

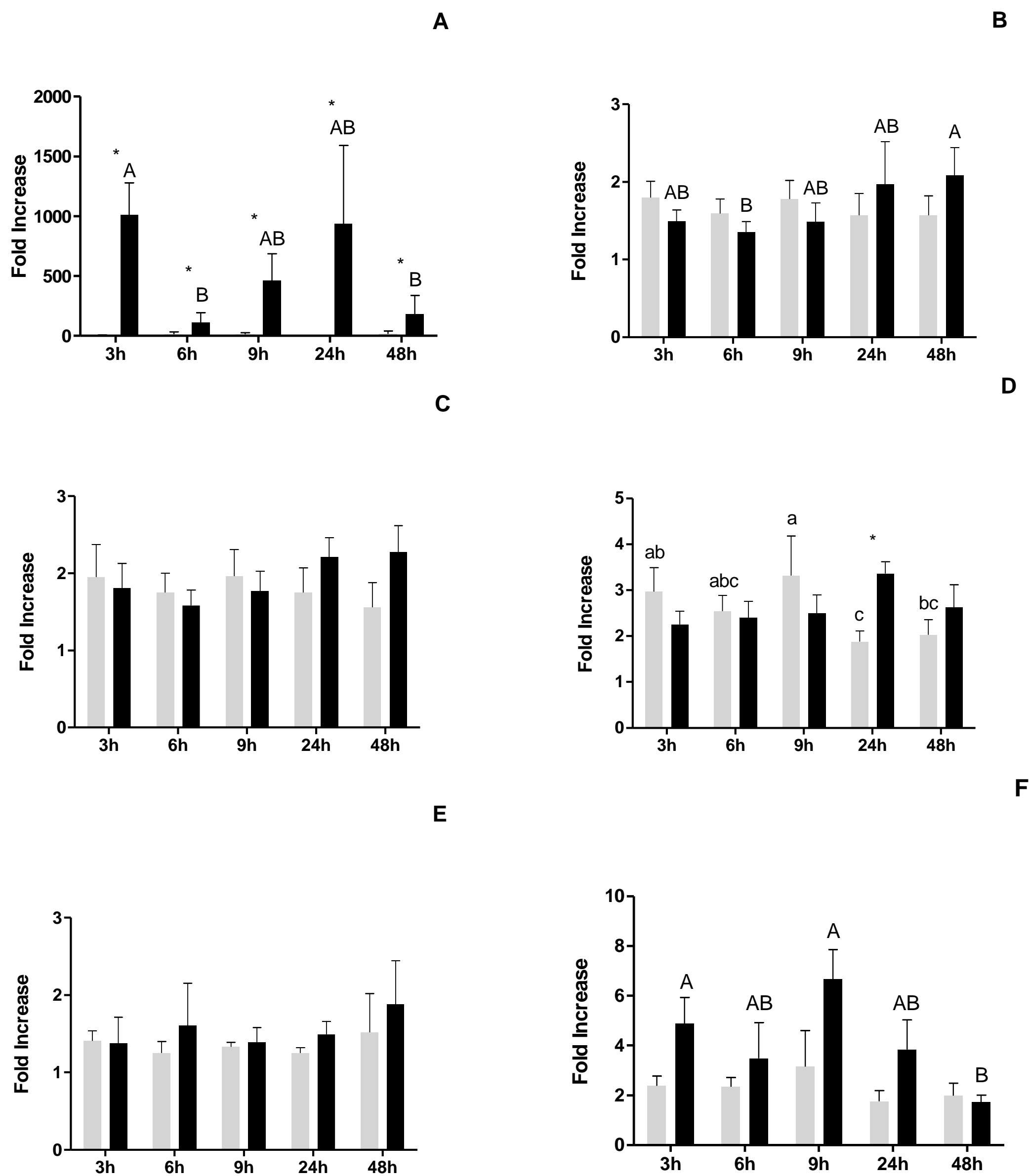



**Figure 9-** Quantitative expression of Interleukin 1β (**A:** <0.001), Colony Stimulating Factor 1 Receptor (**B:** p=0.004), Transforming Growth Factor β1 (**C:** p>0.05), Caspase 1 (**D:** p<0.001), β-Defensin (**E:** p>0.05) and Interleukin 10 (**F:** p=0.02) in the head kidney of gilthead seabream juveniles after *Phdp* challenge. Data are expressed as means ± SD (n=6). Bars represent the fold increase in expression as compared to fish prior to infection (Time 0), previously normalized to Elongation Factor 1 (EF1). Different lower case letters stand for significant differences among different times on control animals, while symbol stands for differences between different treatment groups on the same sampling time. Different capital letters indicate differences among different times on infected animals. (Two-way ANOVA; Tukey post-hoc test; p≤0.05).

# 4- Discussion

The modulation of fish innate immunity after intraperitoneal bacterial insult was here studied. Even though there are several available studies evaluating teleost defence mechanisms in response to infection with *Phdp* **(Acerete *et al.* 2009, Costas *et al.* 2013, Mosca *et al.* 2014, Grasso *et al.* 2015, Nuñez-Diaz *et al.* 2016, Nuñez-Diaz *et al.* 2017, Machado *et al.* 2019)**, the present approach provides a wider and more complete analysis of the mechanisms activated in response to this pathogen using non-infected gilthead seabream juveniles as controls. Moreover, the present time-course study also allows to observed early host responses to *Phdp* infection. For that, all parameters analysed were transformed and presented as fold increase values in order to allow us to compare different treated groups (i.e. sham injected and infected), and therefore increased values due to injection or infection.

Regarding mortality rates evaluation, expected higher values were confirmed on fish injected with bacteria and accumulated death percentage was very close to that observed on studies performed using same route (i.p) and approximated dosis on seabass **(Mosca *et al.* 2014)** and Senegalese sole **(Nuñez-Diaz *et al.* 2016)**. Furthermore, moribund fish were only found on the first six days following pathogen inoculation, with some affected animals exhibiting mild liver and spleen enlargement. Mortality in control groups was low and most likely related to the stressful situation imposed due to handling and PBS injection, since no external or internal disease signs were detected.

Regarding fish haematological data, is possible to observe that fish challenged with bacteria presented an anaemic condition when compared with control ones, possibly due to bacterial enzyme or toxic action that lead to erythrocyte lysis **(Magariños *et al.* 1992a. Naka *et al.* 2007)**. Also haematocrit presented lower values on infected groups regardless time, being this finding yet been described in past challenge experiences performed on seabass and meagre **(Acerete *et al.* 2009. Peixoto *et al.* 2017)**. Haemoglobin values followed the tendency to decrease its concentration in infected animals along time. Comparing with past results with *Renibacterium salmoninarum* infection **(Bruno & Munro 1986)**, is possible to hypotesize that *Phdp* can also produce a positive correlation between haemoglobin and total erythrocyte counts fluctuations. In addiction, studies performed with photobacteriosis suggested that *Phdp* virulence can be increased with previous inoculation of haemin (i.e. an iron-containing porphyrin) and haemoglobin **(Magariños *et al.* 1994)**. Moreover, *Phdp* extracted from infected Senegalese sole showed an increased expression of genes involved in pathogen iron

acquisition such as iron regulatory proteins 1 and 2 (with active roles on the synthesis of siderophore piscidin) and HutB and HutD (encoding for hemic binding protein) **(Nuñez-Diaz *et al.* 2018)**. It was suggested that fish are able to face this bacterial iron uptake strategy by increasing transferrin and haptoglobin concentrations, being transferrin responsible to chelate this metal on host while haptoglobin facilitates iron hemoglobin recycling in liver **(Nuñez-Diaz *et al.* 2017)**. In the present study, an interesting increase in mean corpuscular haemoglobin concentration was found in infected fish 24 h after infection, a result mainly caused by the sharp haemoglobin increase in the same time period. Compiling all data, these blood parameters represent a good opportunity for establishment of disease biomarkers due to its easy and fast evaluation.

In the present study, the total white blood cells population increased slightly in response to infection, while the differential leucocitary populations showed to be significantly influenced by *Phdp*. Results are in line with previous reports that showed clear neutrophilia, monocitosis **(Lamas *et al.* 1994, Afonso *et al.* 2005, Machado *et al.* 2019)** and lymphopenia **(Balfry *et al.* 1997, Costas *et al.* 2013)** in infected animals within the first 24 hours of infection. The neutrophilia observed in the present study was also accompanied by an increase in plasma peroxidase in both control and infected groups in response to the injection. In addition, infected animals also presented reduced thrombocyte values, a result that, although not being very common during photobacteriosis, has yet been described before during infection episodes with other Gram negative bacterial species **(Garcia *et al.* 2007)**, supporting the hypothesis that these cells may have the ability to migrate to the inflammatory focus to cope pathogen invasion. Moreover, sham injected seabream also presented an activated innate immune response to the stimulus and reinforces the importance of having good control treatments for a better understanding of host/pathogen interactions. The slight increase (not significant) in plasma peroxidase and antiproteases activities observed in infected animals 24 h after infection are in line to other findings from european seabass infection **(Machado *et al.* 2015, Machado *et al.* 2018).** It is also plausible that these slight differences were not so clearly seen between control and infected groups due to neutrophil degranulation on the peritoneal cavity, thus decreasing its concentration in plasma. Another explanation for this finding is correlated with the action of bacterial toxin AIP-56 on phagocytic cells **(do Vale *et al.* 2007)**, inducing selective apoptotic destruction of macrophages and neutrophils, ending in reduced pathogen clearance and antimicrobial products release **(do Vale *et al.* 2016)**.

The modulatory effect of bacterial challenge on the expression of pro and anti-inflammatory genes have presented good insights about the mechanisms implied to fight this disease. In the present study, the observed increase in IL-1β expression from

infected gilthead seabream was in line with that already observed in teleost submitted to bacterial diseases. In fact, IL-1β expression evaluation after a bacterial challenge is a common approach and similar studies had yet been performed **(Pelegrín *et al.* 2001, Reyes-Becerril *et al.* 2011, Grasso *et al.* 2015, Kole *et al.* 2017)** resulting on analogous variations. Indeed, IL-1β is a pro-inflammatory cytokine with key role on first stages of inflammation by attracting fish leucocytes **(Zou & Secombes 2016)**. Caspase 1 expression levels followed a similar trend compared to IL-1β variation along time (although with less greatness), since this inflammatory caspase function is to cleave and activate IL-1β, IL-18 and IL-33 **(López-Castejón *et al.* 2008)**. This cleavage occurs at a phylogenethic conserved aspartate residue in seabass **(Reis *et al.* 2012)** and correlation between concentration of both molecules had yet been described in the past using Senegalese sole as infected host **(Nuñez-Diaz *et al.* 2017)**, supporting the hypothesis that this might be a prefered inflammatory pathway in gilthead seabream against *Phdp*.

In order to maintain homeostasis during infection episodes, anti-inflammatory signals are also released. IL-10 is an anti-inflammatory cytokine produced by a high variety of immune cells and takes a pivotal role during inflammatory responses due to its hability to inhibit macrophages and monocytes, leading to decreased pro-inflamatory cytokines release, phagocytosis and host cells damage **(Iyer & Cheng 2013)**. Besides that, IL-10 can also enhance activation and proliferation of all kinds of lymphocytes In the present study, photobacteriosis have modulated this anti-inflammatory cytokine by augmenting its expression on a fast and short time response. In this sense, our results are congruent with the literature **(Pellizzari *et al.* 2013, Tran *et al.* 2019, Machado *et al.* 2019 Elbahnaswy & Elshopakey 2020)**, reinforcing its high importance in the control of inflammation.

As Heat Shock Proteins (HSPs) were yet described before as important chaperones involved in initial stages of the inflammatory process after bacterial infection **(Sung & McRae 2011)**, the present study also focused on the expression levels of HSP70. Even though an augmented expression for HSP70 was expected under a stressful stimulus, no significant differences were found on previous works after infections with *Phdp* in seabream and Senegalese sole **(Mosca *et al.* 2014, Nuñez-Diaz *et al.* 2016)**. Results from the present study are in agreement with the above cited works, and it is here hypothesized that this phenomenon might be triggered with apoptotic stimulation of phagocytes by bacteria, with consequent decreased inflammatory pathway activation.

Another important cellular population contributing to the fast elimination of pathogens are the so called cytotoxic cells. NCCRP is a receptor protein expressed on non-specific cytotoxic cells that are intimately related with the inflammatory response

**(Nuñez-Diaz *et al.* 2016)**. Results from this study showed an upregulation of this gene in infected animals 48 h after infection. Moreover, it was also observed an increase in the mRNA expression of MHCI at 24 h following infection, suggesting that MHCI/CD8+ interaction could be another host strategy used to debelate *Phdp* infection. On the other hand, expression of MHCII remained stable during time among both treatment groups and since this molecule is presented mainly after inflammatory signals on monocytes, macrophages and dendritic cells **(Rock *et al.* 2016)**, there is a possibility that its expression might not be reached due to phagocytic cell apoptosis induced by AIP56. This hypothesis could also be related to the lack of changes observed in TGFβ-1 mRNA expression levels, which are also in line to that found in cobia and European seabass **(Tran *et al.* 2018, Machado *et al.* 2018)**. TGFβ is a multipotent cytokine affecting cell differentiation, proliferation, apoptosis and matrix production **(Taipale *et al.* 1998)**.

Data from the present study also showed an increase in the expression of IL-34 and CSF-1r in infected fish at 48 h. IL-34 is a cytokine which has only recently been described in fish. This cytokine together with CSF-1 have the capacity to bind to CSF-1r resulting into the differentiation, proliferation and survival of monocytes, macrophages and osteoclasts **(Guilloneau *et al.* 2017, Band'huin *et al.* 2010)**. Both CSF-1 and IL-34 bind indististinctly the receptor even though variations in the macrophages secretome obtained by either one or the other molecule ligation were detected **(Boulakirba *et al.* 2018)**. Therefore, it could be hypothesized that both IL-34 and CSF-1r transcripts seem to play a key role on gilthead seabream survival against *Phdp* by improving macrophage differentiation at 48 h after infection with *Phdp*. In fact, this data seems to be correlated to the increased level of circulating monocytes from infected fish at this time.

Hepcidin is an antimicrobial peptide (AMP) that also contributes for iron homeostasis by inhibithing cellular iron efflux from enterocytes, hepatocytes and macrophages through a mechanism that involves ferroportin cell internalization **(Nemeth *et al.* 2004)**. Since hepcidin is easily stimulated by pro-inflammatory cytokines, it was expected that expression on this gene would increase drastically with inflammatory response. However, no differences were found in the present study, and similar results were provided in a study with iron deficitary European seabass **(Rodrigues *et* al. 2006).** Furthermore, acute anemic state has been investigated in myce and related with decreased hepcidin gene expression **(Nicolas *et al.* 2002).**

Defensins are widely studied AMPs with multiple actions on the infectious process. Adding to its antimicrobial role, β-Defensins are also involved on chemotactic task by attracting monocytes, T lymphocytes and immature dendritic cells as well as promoters of dendritic cell's maturation and differentiation **(Lay & Gallo 2009)**. Since β-Defensin mRNA transcription was not significantly affected by *Phdp* infection, it is not

possible to affirm that this AMP enhances immune status during photobacteriosis episodes.

# 5- Conclusion

Intraperitoneal infection by *Photobacterium damselae* subsp. *piscicida* has been proved to interfere in juvenile gilthead seabream innate immune system through several pathways that culminate in inflammatory and phagocytic processes. The importance of this study relies on the fact that few studies were performed within this fish size, that is known to be a critical factor for gross mortalities due to photobacteriosis.

The bacterial challenge produced moderate mortality rates (near 40 %), and allowed to observe an anemic state and increased peripheral monocyte and neutrophil populations in infected animals. Regarding pathogen interaction with host, *Phdp* developed a systemic answer promoted by pro-inflammatory cytokines. On the other hand, and since mass inflammatory processes can result into physiological unbalances, increased anti-inflammatory IL-10 was also observed, contributing to fish's capacity to fight against the pathogen on a sustained form. It is also remarkable that this pathogen has induced phagocyte proliferation and macrophage differentiation through IL-34/CSF1-r stimulation.

Future studies should be performed on the sequence of this project, with emphasis on innate response against other bacterial infections for a better comprehension of the pathological processes and verify if there could be pattern alterations among diseases that could serve as health biomarkers, contributing for a safer and more efficient aquaculture.

# 6- References

Acerete L., Espinosa E., Josa A. & Tort L. (2009) "Physiological response of hybrid striped bass subjected to *Photobacterium damselae* subsp. *piscicida*" **Aquaculture** 298, 16–23.

Afonso A., Ellis A.E. & Silva M.T. (1997) "The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*)" **Fish & Shellfish Immunology** 7, 335–348.

Afonso A., Gomes S., da Silva J., Marquesa F. & Henrique M. (2005) "Side effects in sea bass (*Dicentrarchus labrax L.*) due to intraperitoneal vaccination against vibriosis and pasteurellosis" **Fish & Shellfish Immunology** 19 (1), 1-16.

Afonso A., Lousada S., Silva J., Ellis A.E. & Silva M.T. (1998) "Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*: A light and electron microscopic cytochemical study" **Disease Aquatic Organisms** 34, 27-37.

Amagliani G., Omiccioli E., Andreoni F., Boiani R., Bianconi I., Zaccone R. & Mancuso M. (2009) "Development of a multiplex PCR assay for *Photobacterium damselae* subsp. *piscicida* identification in fish samples" **Journal of Fish Diseases** 32(8), 645-653.

Andreoni F. & Magnani M. (2014) "Photobacteriosis: Prevention and Diagnosis" **Research Journal of Immunology** 2014, 793817.

Ashfaq H., Soliman H., Saleh M. & El-Matbouli M. (2019) "CD4: a vital player in the teleost fish immune system" **Veterinary Research** 50(1), 1.

Austin B. & Austin D.A. (2007) "Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish" **Springer Praxis Books** 4th edition, Netherlands, pp 2.

Balfry S.K., Shariff M. & Iwama G.K. (1997) "Strain differences in non-specific immunity of tilapia *Oreochromis niloticus* following challenge with *Vibrio parahaemolyticus*" **Diseases of Aquatic Organisms** 30, 77–80.

Baptista T., Romalde J.L. & Toranzo A.E. (1996) "First occurence of pasteurellosis in Portugal affecting cultured gilthead seabream (*Sparus aurata*)" **Bulletin of the European Association of Fish Pathologists** 16(3), 92-95.

Barton G.M. (2008) "A calculated response: control of inflammation by the innate immune system" **Journal of Clinical Investigation** 118, 413-420.

Bayne C.J. & Gerwick L. (2001) "The acute phase response and innate immunity of fish" **Developmental & Comparative Immunology** 25, 725-743.

Band'huin M., Renault R., Charrier C., Riet A., Moreau A., Brion R., Gouin F., Duplomb L. & Heymann D. (2010) "Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis" **The Journal of Pathology** 221(1), 77-86.

Borrego J.J., Labella A.M., Castro D., Ortiz-Delgado J.B. & Sarasquete C. (2017) "Updated of the pathologies affecting cultured gilthead seabream, *Sparus aurata*" **Annals of Aquaculture and Research** 4(1), 1033.

Boulakirba S., Pfeifer A., Mhaidly R., Obba S., Goulard M., Schmitt T., Chaintreuil P., Calleja A., Furstoss N., Orange F., Lacas-Gervais S., Boyer L., Marchetti S., Verhoeyen E., Luciano F., Robert G., Auberger P. & Jacquel A. (2018) "IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential" **Scientific reports** 8(1), 1-11.

Breloer M., Marti T., Fleischer B. & von Bonin A. (1998) "Isolation of H2K(b) – binding ovoalbumin-derived peptides associated with the stress proteins HSP70 and GP96" **European Journal of Immunology** 28, 1016-1021.

Bruno D.W. & Munro L.S. (1986) "Hematological assessment of rainbow trout *Salmo gairdneri Richardson*, and Atlantic salmon, *Salmo salar L.*, infected with *Renibacterium salmoninarum*" **Journal of Fish Diseases** 9, 195–204.

Cabello F.C. (2006) "Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment" **Environmental Microbiology** 8(7), 1137-1144.

Carraro R., Rovere G.D., Ferraresso S., Carraro L., Franch R., Toffan A., Pascoli F., Patarnello T. & Bargelloni L. (2017) "Development of a real-time PCR assay for rapid detection and quantification of *Photobacterium damselae* subsp. *piscicida* in fish tissues" **Journal of fish diseases** 41, 247-254.

Chaplin D. (2010) "Overview of the immune response" **The Journal of Allergy and Clinical Immunology** 125, S3-23.

Chang J. & An J. (2007) "Cytokines, Inflammation and Pain" **International Anesthesiology Clinics** 45(2), 27-37.

Corbel M.J. (1975) "Immune-Response in Fish – Review" **Journal of Fish Biology** 7, 539-563.

Costa J.Z., McCarthy Ú., Perez O., Ramos E., Rodriguez M., Monterroso O. & Riera R. (2017) "Occurrence of *Photobacterium damselae* subsp. *piscicida* in Sea-Cage Farmed Meagre (*Argyrosomus regius*) in Tenerife, Canary Islands, Spain" **Thalassas: An international Journal of Marine Sciences** 33, 65-71.

Costas B., Couto A., Azeredo R., Machado M., Krogdahl A., Oliva-Teles A. (2014) "Gilthead seabream (*Sparus aurata*) immune responses are modulated after feeding with purified antinutrients" **Fish & Shellfish Immunology** 30, 173-181.

Costas B., Rêgo P.C.N.C., Simões I., Marques J.F., Castro-Cunha M. & Afonso A. (2013) "Cellular and humoral immune responses of Senegalese sole, *Solea senegalensis* (Kaup), following challenge with two *Photobacterium damselae* subsp. *piscicida* strains from different geographical origins" **Journal of Fish Diseases** 36, 543–553.

Dogget T.A. & Harris J.E. (1989) "Ultrastructure of the peripheral blood leucocytes of *Oreochromis mossambicus*" **Journal of Fish Biology** 33, 747-756.

do Vale A., Costa-Ramos C., Silva A., Silva S.P.D., Gärtner F., dos Santos N.M.S. & Silva M.T. (2007) "Systemic macrophage and neutrophil destruction by secondary necrosis induced by a bacterial exotoxin in a Gram-negative septicaemia" **Cellular Microbiology** 9, 988–1003.

do Vale A., Cabanes D. & Sousa S. (2016) "Bacterial Toxins as Pathogen Weapons Against Phagocytes" **Frontiers in Immunology** 7,42.

Elbahnaswya S. & Elshopakey G.E. (2020) "Differential gene expression and immune response of Nile tilapia (*Oreochromis niloticus*) challenged intraperitoneally with *Photobacterium damselae* and *Aeromonas hydrophila* demonstrating immunosuppression" **Aquaculture** 526, 735364.

Ellis A.E. (1981) "Non-specific defence mechanisms in fish and their role in disease processes" **Development of Specifications for Biotechnology Pharmaceutical Products** 49, 337-352.

Ellis A.E. (1990) "Serum antiproteases in fish" **Techniques in Fish Immunology** edition 1 FairHaven: SOS, 95-99.

Ellis A.E. (1999) “Immunity to bacteria to fish” **Fish & Shellfish Immunology** 9, 291-308.

Ellis A.E. (2001) “Innate host defence mechanisms of fish against viruses and bacteria” **Developmental & Comparative Immunology** 25, 827-839.

FAO (2016) “World review of fisheries and aquaculture” **The State of World Fisheries and Aquaculture 2016** Rome, 3-4.

FAO (2018) "Meeting the sustainable development goals," **The State of World Fisheries and Aquaculture 2018** Rome, 2-3.

FAO (2020) ”Sustainability in action” **The State of World Fisheries and Aquaculture 2020** Rome, 4-5.

FAO (2020b), “Cultured Aquatic Species Information Programme, Sparus aurata”, http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en accessed on 9/10/2020.

Fischer U., Katrin U., Somanoto T., Köllner B., Ototake M. & Nakanishi T. (2006) “Cytotoxic activity in fish leucocytes” **Fish and Shellfish Immunology** 20(2), 209-226.

Garcia F., Pilarski F., Onaka E.M., Moraes F.R. & Martins M.L. (2007) “Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*” **Aquaculture** 271, 39–46.

Grasso V., Padilla D., Bravo J., Román L., Rosario I., Acosta B., Vega B., El Aamri F., Escuela O., Ramos-Vivas J. & Acosta F. (2015) “Immunization of sea bream (*Sparus aurata*) juveniles against *Photobacterium damselae* subsp. *piscicida* by short bath: Effect on some pro-inflammatory molecules and the Mx gene expression” **Fish and Shellfish Immunology** 46, 292-296.

Griffin B.R. (1984) “Random and directed migration of trout (*Salmo gairdneri*) leukocytes: activation by antibody, complement and normal serum components” **Developmental and Comparative Immunology** 8, 589-597.

Guillonneau C., Bezie S. & Anegon J. (2017) “Immunoregulatory properties of the cytokine IL-34” **Cellullar and Molecular Life Sciences** 74, 2569- 2586.

Hogkinson J.W., Grayfer L. & Belosevic M. (2015) “Biology of bony fish macrophages” **Biology** 4(4), 881-906.

Instituto Nacional de Estatística (2020). “Aquicultura e salicultura” **Estatísticas da Pesca 2019** 77-81.

Ingram G.A. (1980) “Substances involved in the natural resistance of fish to infection – a review” **Journal of fish biology** 16, 23-60.

Iyer S.S. & Cheng G. (2013) “Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease” **Critical Reviews in Immunology** 32(1), 23-63.

Junger W.G. (2008) “Purinergic regulation of neutrophil chemotaxis” **Cellular and Molecular Life Sciences** 65, 2528-2540.

Kaplow L.S. (1965) “Simplified Myeloperoxidase Stain Using Benzidine Dihychloride” **The Journal of Haematology** 26, 215.

Kibenge F.S.B., Godoy M.G., Fast M., Workenhe S. & Kibenge M.J.T. (2012) “Countermeasures against viral diseases of farmed fish” **Antiviral Research** 95, 257-281.

Kinne O. (1980) “Diseases of Marine Animals, Vol, 1, General Aspects, Protozoa to Gastropoda” **John Wiley & Sons** Chichester, U.K. pp. 15.

Kiron V. (2012) “Fish immune system and its nutritional modulation for preventive health care” **Animal Feed Science and Technology** 173, 111-133.

Klebanoff S.J. & Clark R.D. (1978) “The Neutrophil: Function and Clinical Disorders” **North Holland Publishing Company**, Amsterdam, 409-466.

Kole S., Anand D., Sharma R., Tripathi G., Makesh M., Rajendran K.V. & Bedekar M.K. (2017) “Tissue specific expression profile of some immune related genes in *Labeo rohita* to *Edwardsiella tarda* infection” **Fish and Shellfish Immunology** 66, 575-582.

Lamas J., Santos Y., Bruno D.W., Toranzo A.E. & Anadón R. (1994) “Non-Specific cellular responses of rainbow trout to *Vibrio anguillarum* and its extracellular products (EPCs)” **Journal of Fish Biology** 45(5), 839-854.

Lai Y. & Gallo R.L. (2009) “AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense” **Trends in Immunology** 30(3), 131–141.

Ley K. (2017) “M1 Means Kill; M2 Means Heal” **The Journal of Immunology** 199, 2191-2193.

Li Y., Li Y., Cao X., Jin X. & Jin T. (2016) “Pattern recognition receptors in zebrafish provide functional and evolutionary insight into innate immune signaling pathways” **Cellular and Molecular Immunology** 14, 80-89.

Liu T., Zhang L., Joo D. & Shao-Cong S. (2017) “NF-κB signaling in inflammation” **Signal Transduction and Targeted Therapy** 2, 17023.

López-Castejón G., Sepulcre M.P., Mulero I., Pelegrín P., Meseguer J. & Mulero V. (2008) “Molecular and functional characterization of gilthead seabream *Sparus aurata* caspase-1: The first identification of an inflammatory caspase in fish” **Molecular Immunology** 45(1), 49-57.

Machado M., Azeredo R., Díaz-Rosales P., Afonso A., Peres H., Oliva-Teles A. & Costas B. (2015) “Dietary tryptophan and methionine as modulators of European seabass (*Dicentrarchus labrax*) immune status and inflammatory response” **Fish & Shellfish Immunology** 42 (2), 253-362.

Machado M., Azeredo R., Domingues A., Fernandez-Boo S., Dias J., Conceição L.E.C. & Costas B. (2019) “Dietary tryptophan deficiency and its supplementation compromises inflammatory mechanisms and disease resistance in a teleost fish” **Scientific Reports** 9(1), 7689,

Machado M., Azeredo R., Fontinha F., Fernández-Boo S., Conceição L.E.C., Dias J. & Costas B. (2018) “Dietary Methionine Improves the European Seabass (*Dicentrarchus labrax*) Immune Status. Inflammatory Response, and Disease Resistance” **Frontiers in immunology** 9, 2672.

Magariños B., Couso N., Noya M., Merino P., Toranzo A.E. & Lamas J. (2001), “Effect of temperature on the development of pasteurellosis in carrier gilthead seabream (*Sparus aurata*)” **Aquaculture** 195, 17– 21.

Magariños B., Romalde J.L., Bandín I., Fouz B. & Toranzo A.E. (1992b) “Phenotypic, Antigenic, and Molecular Characterization of *Pasteurella piscicida* Strains Isolated from Fish” **Applied and Environmental Microbiology** 58(10), 3316-3322.

Magariños B., Romalde J.L., Barja J.L., Núñez S. & Toranzo A.E. (1999) “Protection of gilthead seabream against pasteurellosis at the larval stages” **Bulletin of the European Association of Fish Pathologists** 19, 159-161.

Magariños B., Romalde J.L., Lemos L.M., Barja J.L. & Toranzo A.E. (1994) “Iron uptake by *Pasteurella piscicida* and its role on the pathogenicity for fish” **Applied and Environmental Microbiology** 60(8), 2990-2998.

Magariños B., Romalde J.L., Noya M., Barja J.L. & Toranzo A.E. (1996b) "Adherence and invasive capacities of the fish pathogen *Pasteurella piscicida*" **FEMS Microbiology Letters** 138, 29-34.

Magariños B., Santos Y., Romalde J.L., Rivas C., Barja J.L. & Toranzo A.E. (1992a) "Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida*" **Journal of General Microbiology** 138, 2491-2498.

Magariños B., Toranzo A.E. & Romalde J.L. (1996a) "Phenotypic and pathobiological characteristics of P*asteurella piscicida*" **Annual Review of Fish Diseases** 6, 41-64.

Magnadóttir B. (2006) "Innate immunity of fish (overview)" **Fish & Shellfish Immunology** 20, 137-151.

Magnadóttir B. (2010) "Immunological control of fish diseases" **Marine Biothecnology** 12, 361-379.

Martins R. & Carneiro M. (2018) "Manual de identificação de peixes ósseos da costa continental portuguesa – Principais características diagnosticantes" **IPMA** I.P. pp.204.

Miccoli A., Saraceni P.R. & Scapigliati G. (2019) "Vaccines and immune protection of principal Mediterranean marine fish species" **Fish & Shellfish Immunology** 94, 800-809.

Mitcheson S. & Liu M. (2008) "Functional hermaphroditism in teleosts" **Fish and Fisheries** 9(1), 1-43.

Moretti A., Fernandez-Criado M.P., Cittolin G. & Guidastri R. (1999) "Manual on hatchery production of seabass and gilthead seabream" **FAO** vol,1, Rome, Italy, pp. 194.

Mosca F., Ciulli S., Volpatti D., Romano N., Volpe E., Bulfon C., Massimini M., Caccia E., Galeotti M. & Tiscar P.G. (2014) "Defensive response of European sea bass (*Dicentrarchus labrax*) against *Listonella anguillarum* or *Photobacterium damselae* subsp. *piscicida* experimental infection" **Veterinary Immunology and Immunopathology** 162, 83-95.

Naka H., Hirono I. & Aoki T. (2007) "Cloning and characterization of *Photobacterium damselae* subsp. *piscicida* phospholipase: an enzyme that shows haemolytic activity" **Journal of Fish Diseases** 30, 681–690.

Nemeth E., Tuttle M.S., Powelson J., Vaughn M.B., Donovan A., Ward D.M., Ganz T. & Kaplan J. (2004) "Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization" **Science** 306(5704), 2090-2093.

Nicolas G., Chauvet C., Viatte L., Danan J.L., Bigard X., Devaux I., Beaumont C., Kahn A. & Vaulont S. (2002) "The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation" **The journal of Clinical Investigation** 110(7), 1037-1044.

NOAA (2019) "What is aquaculture?"**https://oceanservice,noaa,gov/facts/aquaculture,html** acessed on 9/10/2020.

Núñez-Díaz J.A., Fumanal M., Mancera J.M., Moriñigo M.A. & Balebona M.C. (2016) "Two routes of infection with *Photobacterium damselae* subsp. *piscicida* are effective in the modulation of the transcription of immune related genes in *Solea senegalensis*" **Veterinary Immunology and Immunopathology** 179, 8-17.

Núñez-Díaz J.A., García de la Banda I., Lobo C., Moriñigo M.A. & Balebona M.C. (2017) "Transcription of immune related genes in *Solea senegalensis* vaccinated against *Photobacterium damselae* subsp. *piscicida*, Identification of surrogates of protection" **Fish and Shellfish Immunology** 66, 455-465.

Núñez-Díaz J.A., Fumanal M., do Vale A., Fernández-Díaz C., Moriñigo M.A. & Balebona M.C. (2018) "Transcription of IVIAT and Virulence Genes in *Photobacterium damselae* subsp. *piscicida* Infecting *Solea senegalensis*" **Microorganisms** 6(3), 67.

Parin U., Erbas G., Savasan S. & Yuksel H.T., Gurpinarand S. & Hirkan S. (2016) "Investigation of antimicrobial resistance of *Photobacterium damselae* subsp. *piscicida* strains in sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*)" **International journal of recent scientific research** 7(9), 10882-10886.

Parkin J. & Cohen B. (2001) "An overview of the immune system" **The Lancet** 357, 1777-1789.

Peixoto M.J., Salas-Leitón E., Brito F., Pereira L.F., Svendsen J.C., Baptista T., Pereira R., Abreu H., Reis P.A., Gonçalves J.F.M. & Ozório R.O.A. (2017) "Effects of dietary *Gracilaria* sp. and *Alaria* sp. supplementation on growth performance, metabolic rates and health in meagre (*Argyrosomus regius*) subjected to pathogen infection" **Journal of Apllied Phycology** 29, 433-447.

Pelegrín P., García-Castillo J., Mulero V. & Meseguer J. (2001) "Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines" **Cytokine** 16(2), 67-72.

Pellizzari C., Krasnov A., Afanasyev S., Vitulo N., Franch R., Pegolo S., Patarnello T. & Bargelloni L. (2013) "High mortality of juvenile gilthead sea bream (*Sparus aurata*) from photobacteriosis is associated with alternative macrophage activation and anti-inflammatory response: Results of gene expression profiling of early responses in the head kidney" **Fish & Shellfish Immunology** 34, 1269-1278.

Pfaffl M.W. (2001) "A new mathematical model for relative quantification in real-time RT–PCR" **Nucleic Acids Research** 29(9), e45.

Quade M.J. & Roth J.A. (1997), "A Rapid, direct assay to measure degranulation of bovine neutrophil primary granules" **Veterinary Immunology and Immunopathology** 58, 239-248.

Rajan P., Lin J., Ho M. & Yang H. (2003) "Simple and rapid detection of *Photobacterium damselae* ssp. *piscicida* by a PCR technique and plating method" **Journal of Applied Microbiology** 95, 1375-1380.

Reis M.I.R, do Vale A., Pereira P.J.B., Azevedo J.E. & Santos N.M.S. (2012) "Caspase-1 and IL-1β Processing in a Teleost Fish" **PLoS ONE** 7(11), e50450.

Reyes-Becerril M., López-Medina T., Ascencio-Valle F. & Esteban M.Á. (2011) "Immune response of gilthead seabream (*Sparus aurata*) following experimental infection with *Aeromonas hydrophila*" **Fish & Shellfish Immunology** 31, 564-570.

Roberts R.J. (2012) **Fish Pathology** Wiley-Blackwell, pp. 62-65.

Roberts R.J. & Bullock A.M. (1980) "The skin surface ecosystem of teleost fishes" **Royal Society of Edinburgh** 79B, 87-91.

Roca F.J., Sepulcre M.P., López-Castejón G., Meseguer J. & Mulero V. (2006) "The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream" **Molecular Immunology** 43(9), 1418-1423.

Rock K.L., Reits E. & Neefjes J. (2016) "Present Yourself! By MHC Class I and MHC Class II Molecules" **Trends in immunology** 37(11), 724-737.

Rodrigues P.N.S., Vásquez-Dorado S., Neves J.V. & Wilson J.M. (2006) "Dual function of Fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*) **Developmental & Comparative Immunology**, 30(12), 1156-1167.

Romalde J.L. (2002) "*Photobacterium damselae* subsp. *piscicida*: an integrated view of a bacterial fish pathogen" **International Microbiology** 5, 3-9.

Sasaki Y., Maita M., & Okamoto N. (2002) "Rainbow trout are responsible for non-specific cytotoxicity" **Fish & Shellfish Immunology** 12, 243-252.

Schluter S.F., Bernstein R.M., Bernstein H. & Marchalonis J.J. (1999) "Big Bang emergence of the combinatorial immune system" **Developmental & Comparative Immunology** 23, 107–111.

Ségaliny A.I., Brion R., Brulin B., Maillasson M., Charrier C., Téletchéa S. & Dominique H. (2015) "IL-34 and M-CSF form a novel heteromeric cytokine and regulate the M-CSF receptor activation and localization" **Cytokine** 76, 170-181.

Snieszko S.F. (1974) "The effects of environmental stress on outbreaks of infectious diseases of fishes," **Journal of Fisheries Biology** 6, 197-208.

Sniezko S.F., Bullock G.L., Hollis E. & Boone J.G. (1964) "*Pasteurella* sp. from an epizootic of white perch (*Roccus americanus*) in Chesapeake Bay tidewater areas" **Journal of Bacteriology** 88(6), 1814-1815.

Sung Y.Y. & MacRae T.H. (2011) "Heat shock proteins and disease control in aquatic organisms" **Journal of Aquaculture Research & Development** 3, 1–10.

Taipale J., Saharinen J. & Keski-Oja J. (1998) "Extracellular matrixassociated transforming growth factor-beta: role in cancer cell growth and invasion" **Advances in Cancer Research** 75, 87 - 134.

Toranzo A.E., Barreiro S., Casal J.F., Figueras A., Magariños B. & Barja J.L. (1991) "Pasteurellosis in cultured gilthead seabream (*Sparus aurata*): first report in Spain" **Aquaculture** 99, 1-15.

Toranzo A.E., Baya A.M., Roberson B.S., Barja J.L., Grimes D.J. & Hetrick F.M. (1987) "Specificity of Slide Agglutination Test for Detecting Bacterial Fish Pathogens" **Aquaculture** 61, 81-97.

Toranzo A.E., Magariños B. & Romalde J.L. (2005) "A review of the main bacterial fish diseases in mariculture systems" **Aquaculture** 246, 37-61.

Tort L., Balasch J.C. & Mackenzie S. (2003), "Fish immune system, A crossroad between innate and adaptative responses" **Immunología** 22(3), 277-286.

Tran H.B., Chen S., Chaung H. & Cheng T. (2019) "Molecular cloning of IL-6, IL-10, IL-11, IFN-ϒ and modulation of pro- and anti-inflammatory cytokines in cobia (*Rachycentron canadum*) after *Photobacterium damselae* subsp. *piscicida* infection" **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology** 230, 10-18.

Tran H.B., Lee Y., Guo J. & Cheng T. (2018) "De novo transcriptome analysis of immune response on cobia (*Rachycentron canadum*) infected with *Photobacterium damselae* subsp. *piscicida* revealed inhibition of complement components and involvement of MyD88-independent pathway" **Fish and Shellfish Immunology** 77, 120-130.

Uribe C., Folch H., Enriquez R. & Moran G. (2011) "Innate and adaptative immunity in teleost fish: a review" **Veterinarni Medicina** 56, 486- 503.

Uribe-Querol E. & Rosales C. (2017) "Control of Phagocytosis by Microbial Pathogens" **Frontiers in immunology** 8, 1368.

Valero Y., Saraiva-Fraga M., Costas B. & Guardiola F. (2020) "Antimicrobial peptides from fish: beyond the fight agains pathogens" **Reviews in Aquaculture** 12(1), 224-253.

Yoon S., Mitra S., Wyse C., Alnabulsi A., Zou J., Weerdenburg E.M., Van der Sar A.M., Wang D., Secombes C.J. & Bird S. (2016) "Correction: First Demonstration of Antigen Induced Cytokine Expression by CD4-1+ Lymphocytes in a Poikilotherm: Studies in Zebrafish (*Danio rerio*)" **PLoS ONE** 11(12), e0169149.

Zohar Y. (1989) “Fish reproduction: its physiology and artificial manipulation” **Fish culture in warm water systems: problem and trends**, CRC Press, Boca Raton, FL, 65-119.

Zou J. & Secombes C.J. (2016) “The Function of Fish Cytokines” **Biology**, 5(2), 23.