U.PORTO

MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS

BIOLOGIA E ECOLOGIA MARINHAS

Mucosal immune response in skin mucus from ocular and blind sides of Senegalese sole (*Solea senegalensis* Kaup) after bacterial challenge

Maria del Pilar Escribano Rodríguez

M

2018

U.PORTO

Maria del Pilar Escribano Rodríguez. Mucosal immune response in skin mucus from ocular and blind sides of Senegalese sole (*Solea senegalensis* Kaup) after bacterial challenge

Mucosal immune response in skin mucus from ocular and blind sides of Senegalese sole (*Solea senegalensis* Kaup) after bacterial challenge

Maria del Pilar Escribano Rodríguez

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

M.ICBAS 2018

MARIA DEL PILAR ESCRIBANO RODRÍGUEZ

MUCOSAL IMMUNE RESPONSE IN SKIN MUCUS FROM OCULAR AND BLIND SIDES OF SENEGALESE SOLE (*Solea senegalensis* KAUP) AFTER BACTERIAL CHALLENGE

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutor Benjamín Costas.
Categoria – Investigador Auxiliar.
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental.

Co-orientador – Doutor Francisco Guardiola.
Categoria – Investigador Post-doctoral.
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental.

“Reserve your right to think, for even to think wrongly is better than not to think at all”.

Hypatia

Acknowledgments

Now, when the moment that seemed unachievable is finally here, I can only feel thankful. Studying such a complete master's degree in another country, in a different language, far from my comfort zone, has been a great challenge that now comes to an end.

I want to start by thanking ICBAS-UP for being the university that has welcomed me so warmly for the last three years. To the director of the master, Dr. Eduardo Rocha, for his endless patience and great support in all the moments of struggle. To Mrs. Zélia Lopes for helping me with all the bureaucratic aspects, your advice has been essential. Of course, I cannot forget all my classmates and teachers and give them credit for all the support during this long journey.

On the other hand, I would like to thank the laboratory of nutrition and immunobiology - NUTRIMU- of CIIMAR for opening to me the doors to work in a laboratory from which I have learned as I couldn't have imagined. I deeply appreciate the help of Marina, Sergio, Lourenço, Rita and Filipa; your assistance with the laboratory techniques and your advice have been vital for my thesis. I learnt a lot from you. Thank you. A special mention to Professor Afonso for recommending me the group. To Dr. Benjamín Costas for all your help and patience during all this time and for all the advice and help with my writing. I want to make a special mention to Dr. Francisco Guardiola, thanks for all the support and enthusiasm, it's nice to work so close to people that find science as exciting as you do. Thanks for your guidance in my first steps in the aquaculture world, making me enjoy even more this amazing field of research. It has been a great pleasure to have the support of two great investigators like Fran and Benjamín. I'm grateful for your guidance, always with optimism and a familiar spirit, which you transmitted and is seen in your amazing lab group. I will be eternally grateful.

A todas las personas que han vivido a mi lado todo el proceso de elaboración de la tesis, muchos saben que he pasado momentos difíciles, que posiblemente no habría superado tan fácilmente sin vuestra presencia. A mis amanitas por entender a mi lado lo difícil que es el mundo de la ciencia, a Celia por sus correcciones y amistad, a todas las amigas (Marta, Ruth & Co.) que han aguantado oírme hablar de peces durante tanto tiempo sin cansarse (o al menos disimulándolo muy bien), a todos aquellos amigos de diversos lugares que siempre han creído en mí y en mis posibilidades. Y lo más importante, gracias a mi familia, a mis padres por hacer posible absolutamente TODO lo que tengo y lo que soy, a mis hermanas por ser incondicionales, y a ti Borja, por ser el pilar

fundamental y complemento que lo mueve todo, por confiar en mí cuando ni yo lo hago y acompañarme en cada paso que doy. Eres mi luz.

Por último, mas não menos importante, obrigada à cidade do Porto por me receber de uma forma tão especial, por me fazer apaixonar pelas suas ruas, sons e pessoas incríveis, por me fazer crescer em aspetos tão diversos da vida que não conseguiria descrever.

Home is where your heart is.

This work was supported by Project PEst-C/MAR/LA0015/2013 under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF), and through the COMPETE and POPH Programmes and national funds through FCT-Foundation for Science and Technology, respectively. L. Ramos-Pinto, B. Costas and F.A. Guardiola benefited from grants by Fundação para a Ciência e Tecnologia, Portugal (PD/BDE/114436/2016, IF/00197/2015 and SFRH/BPD/104497/2014, respectively).

Abstract

Most pathogens start the process of infection at the mucosal fish surfaces and therefore the mucosal immune response plays an essential role in the course of the infection. Due to their condition of flatfish, the present comparative study aimed to analyse several immune-related enzymes as well as the bactericidal activity (against fish pathogens *Vibrio anguillarum, Photobacterium damselae piscicida*) between the skin mucus from ocular and blind sides of Senegalese sole (*Solea senegalensis* Kaup) bath challenged with *Tenacibaculum maritimum* during 24 hours at 1, 2 and 3 weeks. The haematological profile and immune-related parameters were also measured in plasma in order to evaluate the systemic immune response after *T. maritimum* challenge. In a second study, the same immune-related enzymes and bactericidal activities were assessed in skin mucus (from both sides) and plasma at 4 weeks following a 24h non-lethal bath challenge with *T. maritimum.* In the later study, the survival rates after a lethal bath challenge with *T. maritimum* (ten times higher – LD_{50}) at 5 weeks of first bath challenge were measured to assess possible resistance to the disease. Our results showed that most of the parameters tested were increased in skin mucus of bath challenged fish compared to unchallenged ones. Contrarily, the sublethal dose tested did not influence the haematological profile as well as the absolute numbers of the different leucocyte types studied. Moreover, no variations were observed in plasma lysozyme, peroxidase, protease and haemolytic complement activities between unchallenged and bath challenged fish. This study demonstrates that all the studied innate immune-related molecules were constitutively present in both skin mucus sides but at different levels. Interestingly, the levels of most parameters measured were higher on the ocular side than on the blind side, possibly due to the higher exposure to invasion by waterborne microorganisms on this side. In addition, the results showed that there is a response to the first contact with *T. maritimum* which makes Senegalese sole more resistant to a new contact with the pathogen. Therefore, the present study brings some insights regarding local immune responses after bacterial challenge in skin mucus from the ocular and blind sides in one of the most valuable flatfish species in Southern Europe.

Keywords: Cell-mediated immunity, Humoral and mucosal immunity, Senegalese sole (*Solea senegalensis*), *Tenacibaculum maritimum.*

Resumo

A maioria dos agentes patogénicos iniciam o processo de infeção na mucosa da superfície dos peixes e, assim, a resposta imune desta mucosa desempenha um papel essencial no decurso das infeções. Devido à sua condição de peixe plano, o presente estudo comparativo teve como objetivo analisar várias enzimas relacionadas com o sistema imunológico, assim como a atividade bactericida (contra os organismos patogénicos *Vibrio anguillarum, Photobacterium damselae* subsp. *piscicida*) entre o muco epitelial dos lados ocular e cego do linguado senegalês (*Solea senegalensis* Kaup), colocados num banho de *Tenacibaculum maritimum* durante 24 horas nas semanas 1, 2 e 3. Paralelamente, o perfil hematológico e os mesmos parâmetros imunológicos foram medidos no plasma de modo a avaliar a resposta imunológica após o desafio de *T. maritimum*. Posteriormente, as mesmas enzimas e atividades bactericidas foram avaliadas no muco da pele (de ambos os lados) e plasma após 4 semanas de banho com *T. maritimum* numa nova experiência, bem como as taxas de sobrevivência após um segundo banho com *T. maritimum* (dez vezes superior) às 5 semanas do primeiro banho, para avaliar a possível resistência ao ensaio com banho. Os nossos resultados demonstraram que a resposta da maioria dos parâmetros testados aumentou no muco da pele dos peixes colocados em banho, comparativamente com os não testados. Contrariamente, a dose subletal testada não influenciou o perfil hematológico, assim como o número absoluto dos diferentes tipos de leucócitos estudados. A nível sistémico, não foram observadas variações nas atividades de lisozima, peroxidase, protease e complemento hemolítico plasmático entre peixes não testados e testados em banho. Este estudo demonstra que todas as moléculas inatas relacionadas ao sistema imunológico estavam constitutivamente presentes em ambos os lados do muco da pele, mas em diferentes níveis. Curiosamente, os níveis da maioria dos parâmetros medidos foram maiores no lado ocular do que no lado cego, possivelmente devido à maior exposição à invasão por microrganismos aquáticos deste lado. Além disso, os resultados mostraram que existe uma resposta ao primeiro contato com *T. maritimum* e que torna o linguado senegalês mais resistente a um novo contato com o patogénio. Portanto, o presente estudo traz algumas informações sobre o funcionamento das respostas imunes locais após o desafio bacteriano no muco da pele dos lados ocular e cego numa das espécies de peixes planos mais valiosas do sul da Europa.

Palavras-chave: Imunidade mediada por células, imunidade humoral e mucosa, Senegalese sole (*Solea senegalensis*), *Tenacibaculum maritimum.*

TABLE OF CONTENTS

LIST OF FIGURES & TABLES

1. INTRODUCTION

Aquaculture is an emerging industry which has undergone strong growth during the recent years. However, issues such as the introduction of new culture species for diversification and the control of bacterial fish diseases, which constitute one of the main causes of economic losses, still pose major challenges. Due to the signs of market saturation by dominant species in the context of marine aquaculture, new alternatives have been sought in the last years for the cultivation of marine fish. From this point of view, the cultivation of flatfish worldwide has increased in the last years constituting also one of the groups of species with greater commercial value (FAO, 2018).

Consequently, *Solea senegalensis* (Kaup, 1858) is one of the fish species that have most recently been incorporated to large-scale aquaculture production in Europe being farmed in the south-western and Mediterranean areas of Spain and Portugal (Dinis *et al.,* 1999). The success is based on the control of the reproduction, a good knowledge of the fish biology, technology innovation, the development of a specific feed, the animal response to stressful conditions and the control of the pathogens (Peatman & Beck, 2015). It is known that produce more disease-resistant fish is a great goal to the aquaculture industry (van der Marel *et al.,* 2010). Bacterial diseases are the main source of economic losses in aquaculture and especially in flatfish, far surpassing those caused by other problems such as mortality due to sudden changes in oxygen concentration, chemical pollution or predation (Austin & Austin, 1999). Water in aquaculture systems may support a wide range of microorganisms which bombard the mucosal epithelial barriers of aquatic animals (Van der Marel *et al.,* 2010; Salinas, 2015).

Tenacibaculosis is one of the most threatening bacterial infections limiting the culture of many species of commercial value in distinct geographical areas of the world (see review by Toranzo *et al.* 2005). *Tenacibaculum maritimum* is the aetiological agent of this ulcerative disease and its eradication is of considerable economic significance to aquaculture producers. Classically, control of pathogens is achieved by the administration of antimicrobial agents. Nevertheless, according to WHO fact sheet 194 (World Health Organisation Antimicrobial Resistance Fact Sheet 194 2017, http://www.who.int/mediacentre/factsheets/fs194/en/), the excessive use of them can produce the transference of resistance genes and their application in the aquaculture industry is limited due to legal issues (i.e. environmental and health impacts). This problem was discussed by Austin in 1983, but it has become far worse with the major increase in farming that has occurred since then. The modern aquaculture demands alternatives to maintain the animal welfare as well as a healthy environment. A better knowledge of the fish immune system will help to achieve these aims (Esteban, 2012). It offers the possibility to develop health management tools to support a growing finfish

aquaculture industry, the development of novel vaccination strategies in fish and the information about questions concerning origins and evolution on immunity on vertebrates (Esteban, 2012; Rakers *et al.,* 2013).

The immune system of vertebrates involves both innate and acquired immune responses, therefore the studies of both responses are important to develop new strategies to reduce the attack by pathogens in aquaculture. Actually, the use of innate immune components may help to reduce the antimicrobial agents and improve the productivity and the economic gains for the aquaculture industry (Ewart *et al.,* 2001; Chabrillón *et al.,* 2005b). For example, even in eurythermal fishes, acquired (antibody-mediated) immunity is reduced at low temperatures. Some works determined that innate immune components are less affected by lower temperatures and obstruct the transference of resistance genes (Magnadóttir *et al.,* 1999). For these and other reasons, the studies about innate immune components are increasing. The initial cue of the innate immune response is essential for the later establishment of specific adaptive immunity based on B and T cells (Salinas, 2015). While an acquired immune system is mostly related to the antibodies, an innate immune system is independent of them and constitute the first line of defence against an infection (Ewart *et al.,* 2001). On the one hand, antibodies are immunoglobulins used to develop vaccines against pathogens (Salinas, 2015). On the other, several molecules are involved in the innate immune system with their antibacterial activity and stimulate the pathogens destruction by macrophages or the complement (Yano, 1996; Castro & Tafalla, 2015).

The skin mucus is the first barrier of teleost fish and; together with the skin, plays a critical role in the defence mechanisms acting as a natural, dynamic, physical, chemical, and biological barrier that protects the animal from pathogens, potent harmful chemicals and physical factors in the water where it is constantly submerged (Raj *et al.,* 2011; Gómez *et al.,* 2013). Moreover, composition and functional characterization of fish skin mucus has recently received significant interest since it contains many mechanisms (including important enzymes) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment (Shephard, 1994; Van der Marel *et al.,* 2010). However, the most available studies are focused on gut (GALT) and there is insufficient information about the state of the mucus on the epidermis (SALT) and its functions in a lot of fish species as the Senegalese sole (Rombout *et al.,* 2011; Salinas *et al.*, 2011; Esteban, 2012).

The role of the mucus layer in fish health is particularly relevant in farmed fish due to the large number of pathogens that are involved in aquaculture (Benhamed *et al.,* 2014;

Castro & Tafalla, 2015). Susceptibility to different diseases among related species is variable. To develop a better understanding of the basis for species variability, several important non-specific humoral parameters were examined in the economically important species. Consequently, the characterization of the mucus from fish skin has been approached from different perspectives and has focused on fish species of economic interest to aquaculture (Jurado *et al.,* 2015).

Mucosal surfaces are armed with cellular and humoral defenses (Salinas *et al.,* 2011; Castro & Tafalla, 2015). The distribution of some components and their possible role in defence have been reported from skin mucus of several species such as coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhyncus mykiss*), Atlantic salmon (*Salmo salar* L.), Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), koi carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), haddock (*Melanogrammus aeglefinus*), Atlantic cod (*Gadus morhua*) or hagfish (*Myxine glutinosa*) (Fast *et al.,* 2002; Subramanian *et al.,* 2007, 2008). After that, Palaksha *et al.* (2008) demonstrated the presence of 15 enzymes in skin mucus of olive flounder (*Paralichthys olivaceus*) and Narvaez *et al.* (2010) provided the first evidence for quantifying the presence of active AMPs in the skin mucus of Atlantic salmon using an immunological method. Changes in protein composition of epidermal mucus in turbot (*Scophthalmus maximus* L.) were studied in Ai-Jun *et al.* (2013). More recently, it was identified and characterized different constitutive humoral defence mechanisms of the skin mucus of five marine teleosts: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*) and dusky grouper (*Epinephelus marginatus*) (Guardiola *et al.,* 2014). Furthermore, it was identified and characterized for the first time the proteome map of the skin mucus of farmed gilthead seabream and from European seabass (Cordero *et al.,* 2015; Jurado *et al.,* 2015). All of them are considered a valuable source of food for both consumers taste and preference.

Even though several teleost species have been studied in the last two decades from an immunological point of view (Peres *et al.,* 2014; Medina *et al.,* 2015; Roman-Padilla *et al.,* 2016), there is little available information about the skin mucus immunity of others as the flatfish Senegalese sole (*Solea senegalensis* Kaup). For instance, Guardiola *et al.* (2017) examined the abundance of terminal carbohydrates, several enzymes related to immunity, bactericidal activity and different physicochemical parameters in the skin mucus of Senegalese sole. Nevertheless, there is not much more information about the rest of the immunological parameters in this species and none comparing information between its upper and lower face. In addition, despite the important roles of mucus in

the immunity of fish, knowledge of the detailed events occurring during the infection process against one of its main parasites as *T. maritimum* is still limited.

2. STATE OF THE ART

2.1. The host and the pathogenic agent

2.1.1. Senegalese sole *(Solea senegalensis*, Kaup, 1858*)*

After years of scientific research and technological development, the culture of flatfish species constitutes one of the great bets in aquaculture in the last decades (FAO, 2018). Outside Europe, the main cultivated species were the Japanese flounder or hirame in Japan and Korea, the summer flounder (*Paralichthys dentacus*) and the Atlantic halibut (*Hippoglossus hippoglossus)*, almost endangered in America north. On the other hand, in Europe the cultivation is dominated by the turbot and the Atlantic halibut, followed by other species such as flounder (*Platichthys flesus*), plaice (*Pleuronectes platessa*) or sole (*Solea spp.*).

The European sole farming began in Portugal in the late 1970s with only 2 tonnes per year. In the mid-1980s, Spain began sole production, by which time Portuguese sole farming continued to expand, fluctuating between 10 and 70 tonnes until 2007. Italy entered the sole market in 2008 with 20 tonnes but stopped production in 2012. The greatest impulse in sole farming was in 2010 when French production started. Currently, Portugal, France and Spain are the leading sole producers (FEAP, 2014). Its culture has opened up new opportunities for business and job creation, becoming in one of the most promising species for European aquaculture. In 2016 the world aquaculture production of Senegalese sole was 1,468 tons, practically the same harvest as the previous year and it is expected to exceed 1600 tons in 2017 as shown in Fig. 1 (APROMAR, 2017).

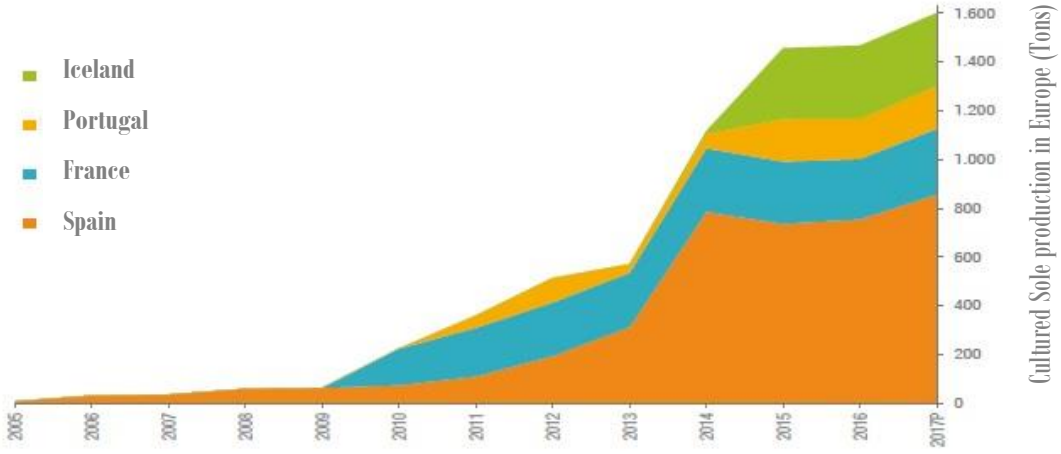


Figure 1. Evolution of cultured Senegalese sole (*Solea senegalensis*) production in Europe between 2005-2016 and forecast for 2017 (On FAO, FEAP and APROMAR).

Senegalese sole (*Solea senegalensis* Kaup) is one of the fourth economically most valuable flatfish species in Southern Europe along with the common sole (*Solea solea)*, the wedge sole (*Dicologoglossa cuneata*) and brill (S*cophthalmus rhombus*) (Bjørndal *et al.*, 2016). Senegalese sole belongs to a Soleidae family reaching a size of about 60 cm in length. Its distribution goes from the Bay of Biscay (Spain) to the coasts of Senegal, being also present in some areas of the Mediterranean Sea. The works to obtain their production at commercial level began in in the 80s and nowadays is an expanding industry in Spain and Portugal due to its possibility to develop their complete biological cycle (Rodriguez, 1984; Dinis *et al.,* 1999, Chabrillon *et al.,* 2005a). *S. senegalensis* is more suitable for production than *S. solea* due to be better adapted to the warmer water of temperate climates (Dinis *et al.,* 1999). However, it is not possible to affirm that at present the Senegalese sole is a species consolidated at the level of industrial production due to the high incidence of pathologies, in many cases opportunistic that affects them, especially in the phases of pre-fattening and fattening (Imsland, 2003; Padros *et al.,* 2003; Toranzo *et al.,* 2003; Cañavete, 2005).

Nonetheless, important progress has been made in the last decade towards developing a stronger and sustainable aquaculture industry for Senegalese sole, as a result of a consistent research effort in several biological disciplines (as reproductive biology, behavior, physiology, nutritional requirements, modulation of the immune system in response to environmental parameters and stress or characterization and mitigation of the main disease threats), a better management and technical improvements (Morais *et al.,* 2014). For example, in the last years, its production has increased significantly above all in Spanish areas with a production of 775 t in 2016 such as Galicia, the Canaries islands or Andalucía and studies to avoid the incidence of pathogens are increasing due to its great commercial value (APROMAR, 2017). Overall, the most important factor for sole aquaculture is that high juvenile costs are likely to come down. Consequently, the prospects for expansion in sole farming in the coming years are good (Bjørndal *et al.,* 2016).

2.1.2. Challenges in Solea senegalensis culture

Despite being relatively warm water species, temperature control used to be one of the problems in the sole culture that affected the most north-west farms of Europe. Today technological advances have mitigated this problem and the major cause of mortality is the infection by pathogenic agents (Reviewed in Imsland *et al.,* 2003).

The main problem in the sole culture over the last decades is the occurrence of the Black Patch Necrosis (BPN). BPN was first described in common sole by McVicar and White (1979) and later confirmed to be caused by the bacterium *Flexibacter maritimus*, nowadays called *Tenacibaculum maritimum* (Bernadet *et al.,* 1990). However, it is now known that one of the main reasons of the disease was the poor nutrition diets fed to the sole (Baynes & Howell, 1993). Flexibacteriosis, fin rot or black patch necrosis are currently called as tenacibaculosis disease.

Unfortunately, this is not the only pathogen that affects sole production. Zorrilla *et al.* (1999) reported another disease that affected the cultivation of *S. senegalensis* in the southwestern of Spain, the Pasteurellosis or Photobacteriosis. Infected fish showed no apparent large lesions other than swelling in the abdominal cavity and some dark pigmentation. The affected samples exhibited pallor of liver and kidney and typical white tuberculosis of 1-2 mm in diameter in the spleen. Microbiological analysis of these fish revealed the presence of a bacterial colony in all organs examined characterized as *Photobacterium damselae* subsp. *piscicida*. This bacterium is the causative agent of a disease that causes massive mortalities in marine aquaculture.

Years later, a new outbreak with moderate mortalities of 20% of the production of *S. senegalensis* in southern Spain was observed (Zorrilla *et al.,* 2003). The bacteria of the outbreak were identified as *Vibrio harveyi* and *Vibrio. parahaemolyticus*. For both species, vaccination with sublethal doses of extracellular products (ECP) reduced the mortality by 32-37% (*V. parahaemolyticus*) and 76-83% (*V. harveyi*) compared to unvaccinated fish. It was concluded that ECP could be considered as a protective antigen to design potential vaccines against vibriosis *in* Senegalese sole *(*Reviewed in Imsland *et al.,* 2003*)*.

Aeromonas salmonicida subsp. *salmonicida* was reported as the causative agent of an 'atypical' furunculosis outbreak in cultured sole in a marine farm operating in a recirculation system in Galicia (Magariños *et al.*, 2011). In this particular case, soles were grown in a farm which also produced turbot, which pointed towards a potential crossed infection of this bacterium from one fish species to another. Similarly, Castro *et al.* (2012) isolated *Edwarsiella tarda* in Senegalese sole growing in a farm which also produced turbot, which again pointed towards a potential crossed infection named Edwardsielosis.

Despite transmission of mycobacteria in fishes is poorly understood, Ziehl-Neelsen-positive stain was observed in some fish species as Senegalese sole (Padrós *et al.*, 2001). These bacteria, resembling *Mycobacterium* spp., could represent a potential new

hazard for cultured sole and therefore recirculation systems may play an important role in its transmission in aquaculture.

As viral diseases, Rodriguez *et al.* (1997) isolated and characterized a *Birnavirus* (genus *Aquabirnavirus*) named solevirus from the skin and internal organs of dead or dying soles. External signs of this pathology showed fish with a dark coloration, erratic swimming and uncoordinated behaviour. It was described as the agent causing 100% mortality in wild Senegalese sole broodstock introduced into a culture facility in southwest Spain. Comparing the virus, it was defined as Sp Serotype, the most common serotype in Spain (Perez-Prieto *et al.*, 2001). *Nodavirus* (genus *Betanodavirus*), the aetiological agents of the viral nervous necrosis or viral encephalopathy and retinopathy (VER or VNN) and lymphocystis have also been detected in cultured sole (Starkey *et al.,* 2001; Toranzo *et al.,* 2003; Thiéry *et al.,* 2004; Alonso *et al.,* 2005; Cutrín *et al.,* 2007; Olveira *et al.,* 2009; Cano *et al.,* 2010; Hodneland *et al.,* 2011). Lymphocystis disease is characterised by papilloma-like lesions typically on the skin, fins and tail and is caused by an *Iridovirus* with a worldwide geographical distribution that involves a chronic disease (Walker & Hill, 1980). Although these viruses can be transmitted horizontally by contact between diseased and healthy fish, the main transmission route is vertical (Toranzo *et al.,* 2004), which highlights the importance of detecting broodstock carriers that could transmit the virus to the larvae through fertilised eggs.

In recent years, parasitic problems have been observed in cultured Senegalese sole due to the presence of amoebas as *Endolimax piscium (Archamoeba*) (Constenla *et al.,* 2014). Although the condition was not associated with high mortalities, reduced growth and high morbidity were noted. In fact, protuberances on the skin surface in addition to unspecific signs of diseases due to a lethargy with sporadic and erratic swimming were shown (Constenla & Padrós, 2010). Amoeboid organisms like *Neoparamoeba* sp. have also been observed in cultured Senegalese sole causing a chronic proliferative mucoid inflammation in gills. In addition, some sporadic infections by protist parasites like flagellates or ciliates (*Amyloodinium, Cryptobia* and *Cryptocaryon*) have also been described usually in cases where sole were reared in ponds (Padrós *et al.,* 2003).

In general, cultured sole are extremely susceptible to a host of diseases that commonly affect other cultured flatfish and finfish species. In most cases, the severity of the disease seems to be linked with increasing intensification of production. The results given in Machado *et al.,* (2018) indicate that situations of acute hyperoxia exposure in RAS systems may result in negative consequences to the host over a longer time frame, such as a decrease in growth or lower disease resistance as a harmful outcome.

At the present, photobacteriosis (*Photobacterium damselae* subsp. *piscicida*), vibriosis (*Vibrio sp.*) and flexibacteriosis (*Tenacibaculum maritimum*) are considered the most important pathogens affecting sole culture and limiting successful expansion (FAO, 2018). Tenacibaculosis can cause significant mortality in fish farms in many countries, limiting the culture of economically important marine fish species (Santos *et al.,* 1999). For this and other reasons is the pathogen in which our study was focused.

2.1.3. Tenacibaculum maritimum

Tenacibaculum maritimum, formerly known as *Flexibacter maritimus* or *Cytophaga marine* (Wakabayashi *et al.,*1986; Holmes, 1992; Suzuki *et al.,* 2001), is a Gram-negative filamentous bacterium with oxidative metabolism belonging to the Bacteroidetes filus, family Flavobacteriaceae. It is a global distribution agent that produces tenacibaculosis or flexibacteriosis, an ulcerative disease that causes massive mortalities and important economic losses in cultures of many marine species (Toranzo *et al.*, 2005; Avendaño-Herrera *et al.,* 2006).

The disease was first described in 1977 by Masumura and Wakabayashi in Japan, causing high mortalities in cultured marine fish. However, *T. maritimum* was not only reported in Senegalese sole, but also in turbot, common sole, European seabass, Japanese flounder, European flounder, gilthead seabream, white sea bass, red seabream, black seabream, Tub gurnard (*Chelidonichthys lucerna*), rainbow trout, striped trumpeter (*Latris lineata*), greenback flounder (*Rhombosolea tapiriña*), yellow-eye mullet (*Aldrichetta forsteri*) and pacific sardine (*Sardinops sagax*) (Reviewed in Avendaño-Herrera, 2006). It is considered a potential limiting factor for the culture of economically important marine fish species causing serious mortalities in farms in many countries (Santos *et al.*, 1999).

The main symptoms are lesions on the body surface (ulcers, necrosis), erosion of the mouth and frayed or rotten fins as can be seen in Fig. 2. Septicemic may occasionally occur. The loss of epithelial fish surface, typical of this disease, is also a portal of entry for other bacterial or parasitic pathogens. Since these lesions favor the entry of other saprophytic and pathogenic bacteria, as well as protozoa, *T. maritimum* often appears in mixed infections (Toranzo *et al.,* 2005). The disease presents a higher prevalence and severity with temperatures above 15°C, and when stress situations occur and with host-related factors (skin surface condition) (Magariños *et al.,* 1995).

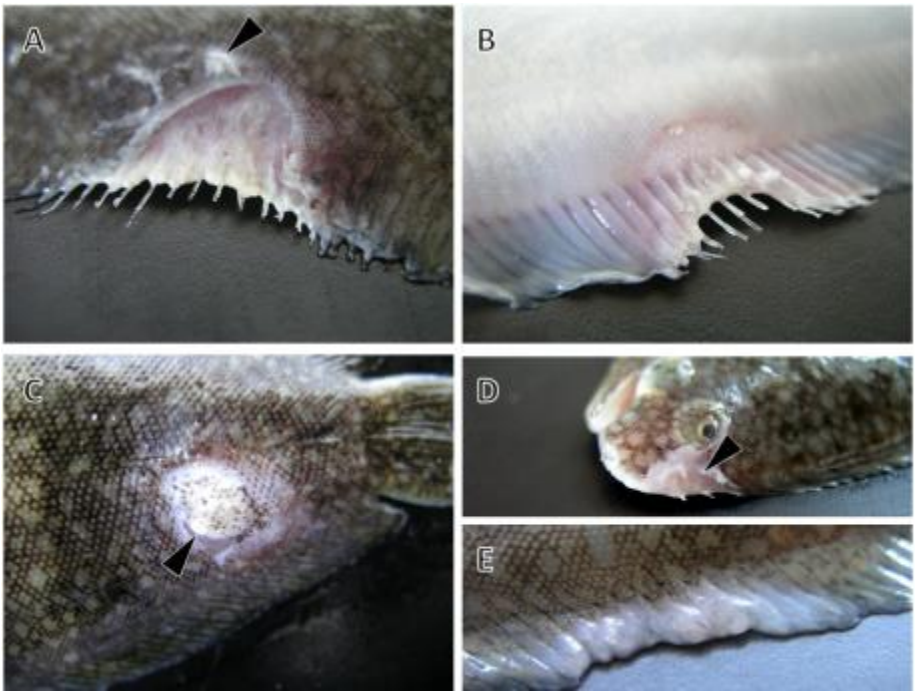


Figure 2. Macroscopic images of lesions in affected soles. A. Wedge-shaped ulcer located in the dorsal fin. Flap of necrotic epidermis associated with the damage (arrowhead). B. Ventral side with a wedge-shaped ulcer on the dorsal fin. C Circular ulcer located on the trunk of the fish surrounded by evident depigmentation. Flap of necrotic epidermis associated with the damage (arrowhead). D Small ulcer located on the dorsal fin close to eye and mouth (arrowhead). E. Mild erosion and thickening of the dorsal fin. (From Vilar *et al.,* 2012)

Typical colonies of *T. maritimum* are pale-yellow, flat with uneven edges. Although the bacterium is biochemically homogeneous, at least two major O-serogroups can be detected which seem to be related to the host species (Avendaño-Herrera *et al.,* 2004a). Thus, one of the major problems in the study of this bacterium is the difficulty of distinguishing it from those of the genera *Flavobacterium* and *Cytophaga.* Therefore, the identification of morphological and biochemical characteristics or different molecular methods based on the PCR technique can be carried out for the identification of the pathogen (Bader & Shotts, 1998; Toyanna *et al.,* 1996; Wilson *et al.*, 2003; Warsen *et al.*, 2004). Cepeda and Santos (2002) isolated for the first time *T. maritimum* from Senegalese sole in southwest Spain, where it caused almost 100% mortality of the affected stocks. Recently, Vilar *et al.,* (2012) described particularly severe ulcerative disease outbreaks in cultured Senegalese sole associated with *T. maritimum*.

Despite efforts made in the past 10 years to deal with the problems discussed above, it is obvious that the pathogenesis of *T. maritimum* is a complex, multifactorial process not yet fully understood. The first data on tenacibaculosis control using drugs was reported

by McVicar & White (1979) in Scotland. Studies have now been carried out from the point of view of the acquired immune system so that effective commercial vaccines are available since a flexibacteriosis vaccine (FM 95) was patented by the University of Santiago (Spain) to prevent mortalities caused by *F. maritimus* in turbot (Santos *et al.,* 1999). Nevertheless, the excessive use of vaccines can produce the transfer of resistance genes due to a transferable R-plasmid.

Additionally, studies from the point of view of the innate response of several fish species are increasing in the last years (Avendaño-Herrera *et al.*, 2006; Magariños *et al.* 1995). The few existing reports on the incidence of specific pathogens in aquaculture (Frans *et al.*, 2011; Silva *et al.*, 2014) reinforce the idea that opportunistic pathogens are the main cause of mortality. However, the first information about the response of Senegalese sole mucus and plasma against *T. maritimum* was reported in Mabrok *et al.* (2016). In that study, both mucus and plasma samples presented a relatively low bactericidal capacity, suggesting that Senegalese sole does not contain adequate compounds with potent bactericidal activity to kill *T. maritimum*, which require further investigations. These results can help to understand the mechanism of *T. maritimum* infection and assist future studies to increase vaccine efficiency in this species.

2.2. Fish immune defence

Fish live in aquatic environments, which are an ideal medium for the growth of the pathogenic organisms (Magnadottir, 2010). Additional challenges can be raised to the immune system of aquatic vertebrates versus their terrestrial counterparts in these conditions (Gómez *et al.,* 2013; Esteban & Cerezuela, 2015; Salinas, 2015).

The fish immune system, comprised of numerous distinct and interdependent immune components and immune organs, is necessary for organisms to defend themselves against invading microorganisms (Esteban, 2012). Fish possess both innate immune system as well as an adaptive immune system. Nevertheless, the innate immunity is stronger than adaptive immunity (Rauta *et al.,* 2012). In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response (Litman *et al.,* 2005). If pathogens successfully evade the innate response, it activates the adaptive immune system, which is more adapted by its specific response and its ability to retain the response in the form of immunological memory (Uribe *et al.,* 2011). However, in fish, the innate response has been considered an essential component in combating pathogens due to limitations of the adaptive immune system, their poikilothermic nature, their limited repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007). It is commonly divided into three compartments: the epithelial/mucosal barrier, the humoral parameters and the cellular components.

The spleen, thymus and head-kidney are the major lymphoid organs in fish (Zapata *et al.,* 2006). Teleost lack lymph nodes and the spleen, with the kidney, form the two major filtering organs removing foreign agents and effete blood cells from the vascular system. Basically, the thymus can be considered as an aggregation of macrophages that promote the encapsulated proliferation of T cells, as it does in mammals, involved in cell-mediated immunity (Davis *et al.,* 2002). The head-kidney in teleost fish is the equivalent of the bone marrow in vertebrates and is the largest site of haematopoiesis until adulthood (Zapata *et al.,* 2006). Thymus and head-kidney are considered the primary lymphoid organs (lymphocyte-generating) in fishes working along with the secondary lymphoid tissue (immune response-generating), the spleen (Rauta *et al.,* 2012). Furthermore, evidence reveals that teleost fish possess an adaptive immune system associated with each of their mucosal body surfaces: the mucosa-associated lymphoid tissue (MALT) (Salinas, 2015).

Mucosa immune system is the first barrier against pathogens because there is an intimate contact between these animals and the aquatic environment (Esteban, 2012; Parra *et al.,* 2015; Salinas *et al.,* 2011). Mucosa-associated lymphoid tissue is predominantly on the general skin surface (SALT), the gills (GIALT) and the gut lining (GALT); but also, has been identified a functional nasal immune system defined as NALT as shown in figure 3 (Tacchi *et al.,* 2014; Salinas, 2015). Composition and functional characterization of fish skin mucus has recently received significant interest since it contains many defence mechanisms (including important enzymes) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment (Shephard, 1994; Van der Marel *et al.,* 2010). Moreover, mucus plays a critical role in the defence mechanisms acting as a natural, dynamic, physical, chemical, and biological barrier as it will be detailed in later chapters (Raj *et al.,* 2011). This importance was clearly demonstrated when transcript analysis mucus of common carp revealed 82 orthologous of genes with immune relevance in other organisms (Gonzalez *et al.,* 2007). However, excluding some studies as in Guardiola *et al.* (2016), the most available studies are focused on gut (GALT) and there is insufficient information about the state of the mucus on the epidermis (SALT) and its functions in a lot of fish species as the flatfish Senegalese sole (Esteban, 2012; Rombout *et al.,* 2011; Salinas *et al.,* 2011;).

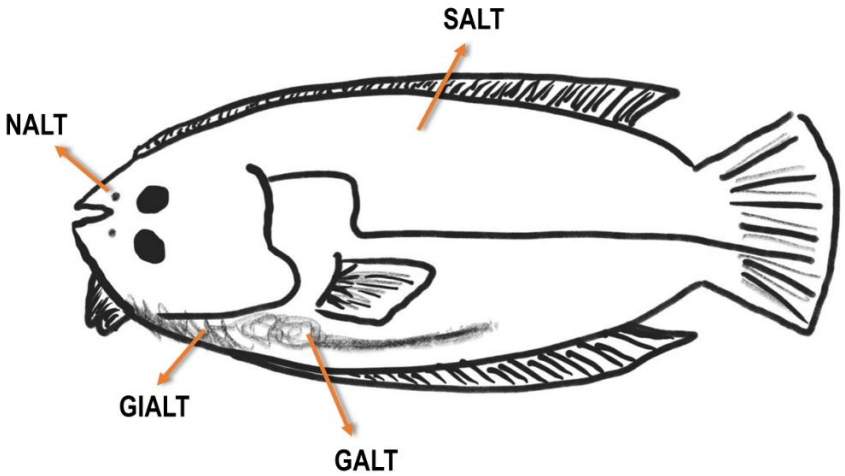


Figure 3. Schematic representation of the four teleost main mucosa-associated lymphoid tissues (MALT) described so far and their anatomical localization. GALT: gut-associated lymphoid tissue; SALT: skin-associated lymphoid tissue; GIALT: gill-associated lymphoid tissue; and NALT: nasopharynx-associated lymphoid tissue. Adapted from (Salinas, 2015).

2.3. Teleost skin

Skin is the structure that covers the body surface and protects the entry of pathogens, allergens, solutes or ions from the leakage of water (Castro & Tafalla, 2015). These barrier functions are dependent on the epidermis, a stratified cellular sheet. While a differentiated cornified cellular sheets (stratum corneum) covers the epidermis in amphibian adults, reptiles, birds and mammals; mucus covers the epidermis in fish and amphibian tadpoles. In fish, the outermost layer of cells is alive and with capacity to divide (Salinas *et al.*, 2011). Teleost skin secretes mucus when is involved in immune functions as adaptation to aquatic environmental (Esteban, 2012).

Skin not only separates and protects the fish, also represents a metabolically active tissue (Bullock & Roberts, 1974). Fish tegument has roles in protection, communication, sensory perception, locomotion, respiration, ion regulation, excretion and thermal regulation (Elliott, 2000). These functions are possible due to skin's complex structure and cell composition as shown in figure 4.

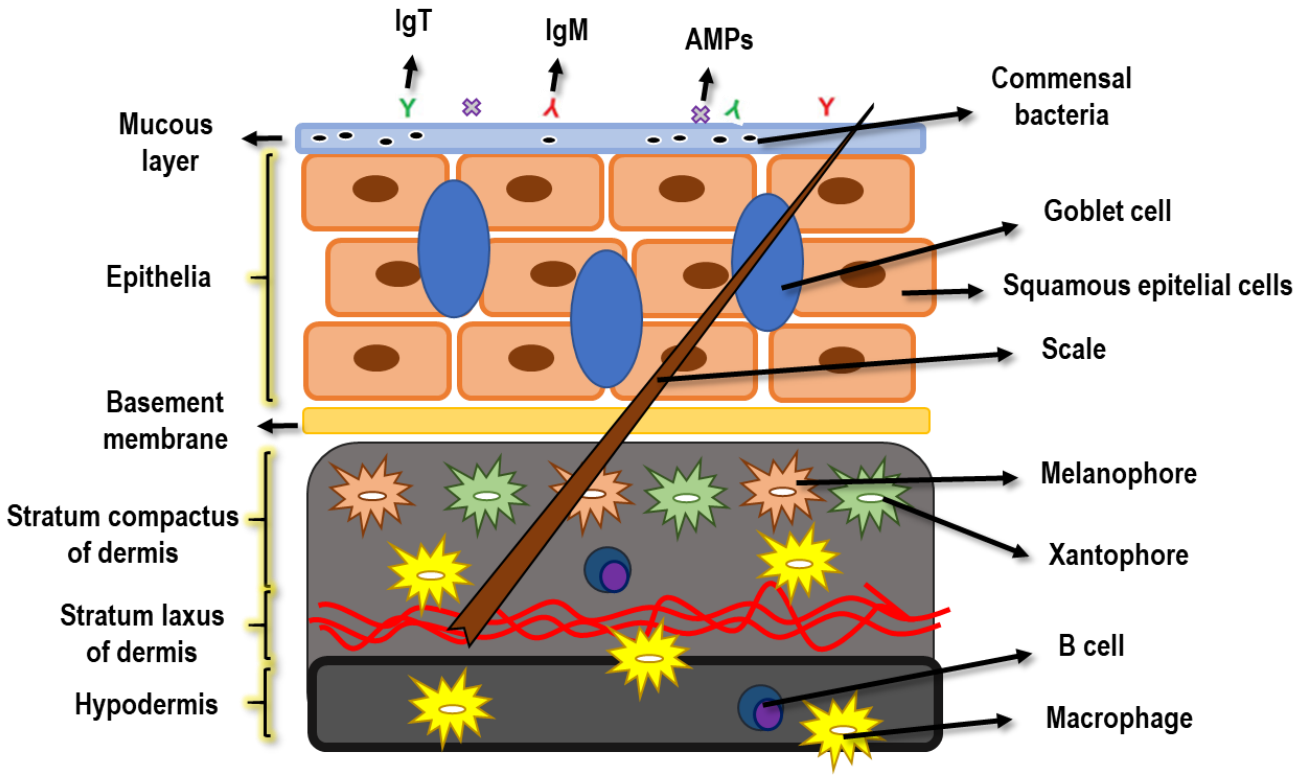


Figure 4. Schematic depiction of teleost fish skin highlighting the general structure, components, and the main cell types present. Adapted from (Gómez *et al.,* 2013).

Some of these functions are very important in fish larvae. Larval skin is a thin two-cell layer lying on a basal membrane and overlying an extensive haemocoel, according to the existing data (Esteban, 2012). Although a lot of studies have focused on the histology and hydrochemistry of the adult teleost epidermis (Bullock & Roberts, 1974; Esteban, 2012), only a few studies have focused on the structure on the larval skin (Ottesen & Olafsen, 1997). In *Solea senegalensis* goblet cells were evident on days 15-20 of larval development and contain N-acetyl glucosamine and/or sialic acid (Sarasquete, 1998).

2.3.1. General tegument in adults: complex composition

The integument or skin forms the external covering of the body. The skin in teleost shows some inter-species differences; some species have no scales while others have special large epidermal alarm substance cells (club cells). The skin is composed of two layers, an outer epidermis and an underlying dermis as is shown in figure 5.

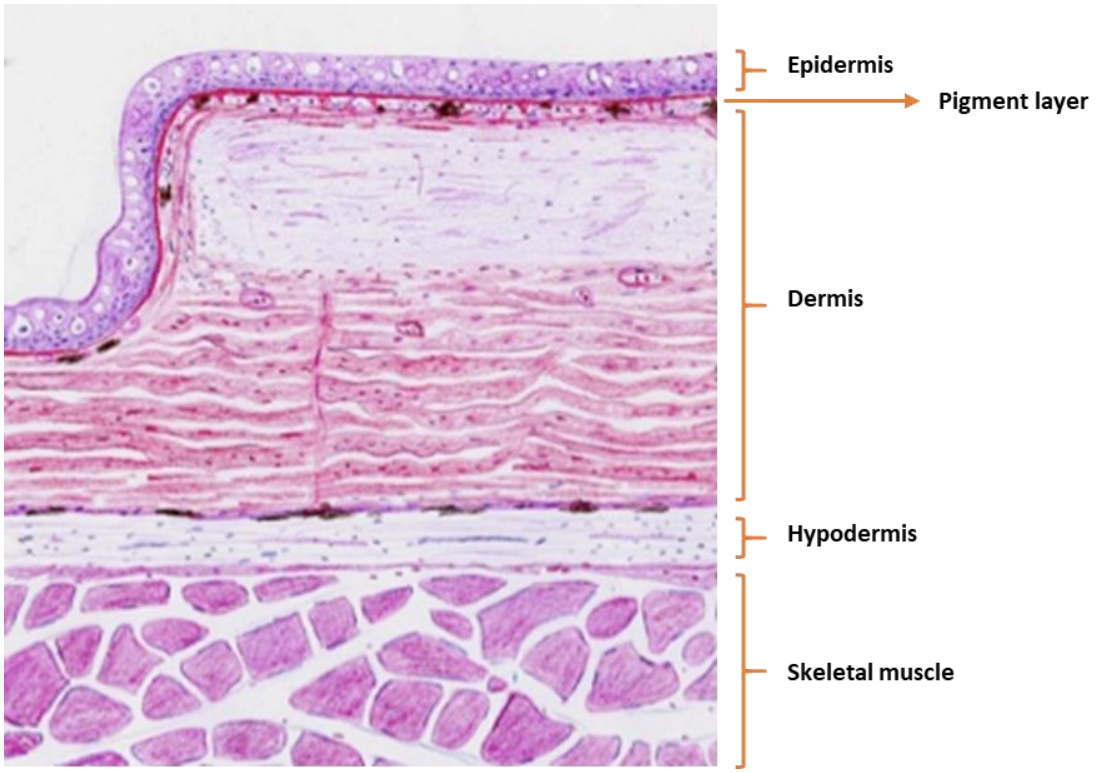


Figure 5. Drawing of a cross section of a flatfish (from Bermúdez, 2012).

- ***Bacteria-microbiota –*** fish mucus harbours an abundant and diverse microbial community with bacteria and fungi. It is no clearly how this community affects to the SALT (Austin, 2006).

- ***Epidermis –*** a squamous but non-keratinized stratified epithelium with goblet cells (Salinas *et al.,* 2011). The major difference from mammals is that in the teleost, the outermost epidermal fusiform cells remain viable, and retain the capacity to divide (Genten & Terwinghe, 2009).

- The layer is about 5-10 cells thick, of which the majority are squamous cells, characterized by numerous desmosomes and associated cytoplasmic filaments with minimal quantities of keratin. Squamous cells of the superficial layer show microridges that contain mucus and antibacterial substances secreted to the surface from mucous goblet cells located in the intermediate stratum of the epidermis. The minority composition of the layer are mucous cells (Zhao *et al.,* 2008).

- ***Dermis –*** mainly composed of dense connective tissue with a large number of collagen fibres (it contains relatively little of the connective tissue found in tetrapods), with blood and lymphatic supply. The dermis contains two strata:

- **Hypodermis or stratum spongiosum** - a frequent site of development of infectious processes.

- **Innermost layer or stratum compactum**.

2.3.2. Immune cells in fish skin

The different leucocyte types derived from the lymphoid and myeloid lineages known from mammals have also been recognized in fish (Castro & Tafalla, 2015). In contrast to mammals, MALT contains the major lymphoid accumulations. It is possible to find:

- ***Leucocytes:*** They can be lymphocytes (T and B cells), plasma cells, macrophages or granulocytes. Plasma cells and plasmablasts are responsible to produce secretory immunoglobulins. B cells are secreted by the head-kidney

while T cells are produced by the thymus (Zapata *et al.*, 2006). Teleost T cell have similar characteristics to mammals. Two major T cells receptors (TCR) have been described: TCRαβ and TCRγδ. In addition, two co-stimulatory molecules define the T cells: $CD4^+$ and $CD8^+$. $CD4^+$ T cells are the main component of adaptive immune system of vertebrates (Salinas, 2015). The presence of this T cell population is modulated by cytokines. Unfortunately, there is no much information about regulatory T cells (Tregs) related homeostasis in teleost except in common carp and in rainbow trout (Yang *et al.,* 2012; Salinas, 2015).

- ***Antibody-secreting cells (ASCs):*** including plasmablasts and non-replicating plasma cells. It is known that the number of ACSs depends of the parasite exposure.

- ***Mast cells or eosinophilic granular cells (EGCs):*** present in a lot of tissues as gills, skin, gut or brain and in blood.

However, little information is available about the function of each one during the immune response in fish (Salinas *et al.,* 2011; Salinas, 2015).

2.4. The cutaneous mucus layer

2.4.1. Functions

Different functions have been suggested for the fish mucus and its role in the immune system of the fishes. Functions very specific were defined in some species as to glue nest material collected to predation at night or to inhibit discharge nematocysts by anemone fishes (Shephard, 1994). However, these proposed roles for mucus have a very little supporting information on the relevant actions of mucus. Functions attributed to this mucus include the predator elusion, the improvement of the locomotion reducing the fluid friction (Guardiola *et al.,* 2015a), the drag reduction and the isolation of superficial epithelial cells from bacteria (Genten & Terwinghe, 2009; Shephard, 1994). The fish mucus acts as a physical, biochemical, biological and semipermeable barrier that allows the exchange of nutrients, water, gases, odorants, hormones or gametes acting as an osmotic, ionic and acid-base regulator (Shephard, 1994; Cone, 2009; Guardiola *et al.,* 2015a). In fact, there is evidence to suggest that many teleost fishes filter feed in a process assisted by the mucus (Sanderson & Cech, 1991).

As it was mentioned before, the mucosal surface of the fishes or MALT (gill, skin and gastrointestinal tract) are the first robust barrier of defence against infections between the external environment and the internal milieu. In fact, the mucus has the power of trap and immobilizes organisms as pathogens, viruses or other nanoparticles (NP) that are removed from the mucosa by the water current before they can contact epithelial in the surfaces. Fish mucus also, on occasions, contains substances as lysozyme able to lyse cells. Lysozyme is able to lyse Gram-negative bacteria and may have an influence on fungal cell walls. In addition, mucus has a defensive role providing a medium in which antibacterial mechanism can act due to the presence of endogenous peroxidase and alkaline phosphatase in the goblet cells (Shephard, 1994). This antimicrobial property has been tested in different fish species (Narvaez *et al.,* 2010; McGuckin, 2011). Immunoglobulins, leucocytes and macrophages, also present in mucus, providing an additional protection against infections (Genten & Terwinghe, 2009).

Another aspect of the defence that is possible to find in some fish is that of resistance to abrasion. Abrasion resistance appears to depend on the lubrication qualities of the mucus based on the “hydrodynamic lubrication” (Hills, 1988). When there are abrasive materials suspended in the water the fish swims against, it is assumable that mucus

provides such a defence. By last, a function related to defence is the production of toxic materials in some fish species (Shephard, 1994).

2.4.2. Properties and composition

One important property of the mucus is the capacity to maintain a layer of mucus adjacent to the epithelium despite vigorous movements as swallowing, coughing, intestinal peristalsis or copulation. Skin mucus can trap and immobilize pathogens before they can contact epithelial surfaces, because it is impermeable to most bacteria and many pathogens (Mayer, 2003; Cone, 2009). Furthermore, the continuously capably of the fishes to replace the mucus prevent the permanent colonization of potential microorganisms (Woof & Mestecky, 2005; Subramanian *et al.,* 2007; Cone, 2009).

Mucus is equal with slime and slime is the material that makes fishes slippery to touch. This property is due to the high-water content (95% approx.) and the presence of high-molecular-weight macromolecules of the mucus (Shephard, 1994). These macromolecules are a kind of glycoproteins called mucins but also contains salts and lipids (e.g. fatty acids, phospholipids or cholesterol). The glycoproteins in fish mucus appear to be similar to mammalian mucins in make-up. Some of them have a defensive role while others may also have a function in the organization of the mucus structure (Thornton, 2004). The proteins and glycoproteins constitute the complement system. This system is synthesized by hepatocytes, but important amounts are produced by other cells as macrophages, blood monocytes or epithelial cells of the genitourinary tract and gastrointestinal tract. Complement proteins have a wide range of functions, including the elimination of invading pathogens, promotion of inflammatory responses, clearance of cell debris, and modulation of adaptive immune responses (Castro & Tafalla, 2015).

The composition and the thickness of the mucus vary throughout the epithelial surface and define the immune functions of the skin mucus. The properties of the mucus gel are dictated in large part by the oligomeric mucins and, over the past decades, we have gained a better understanding of the molecular nature of these complex O-linked glycoproteins in some species as in humans (Thornton, 2004). The proteins that contains are required to maintain their properties under harsh conditions such as high temperatures or hydraulic pressure (Esteban, 2012). Lipids (including fatty acids) contribute to increasing the viscoelasticity of the gel that allows some small fishes to collect nutrients suspended in water (Cone, 2009). Mucus transport needs a well-regulated viscoelasticity which is mostly controlled by hydration. However, many other

mechanisms contribute to control the viscoelasticity, as secreted lipids, trefoil factor, pH, calcium and non-mucin glycoproteins (Thornton, 2004).

Mucus composition varies between the fish species. For example, over the 85% of the dry weight of mucus in Arabian Gulf catfish (*Arius bilineatus*) are protein, 13,4% are lipids and only a small quantity are carbohydrates and nucleic acids (Ali *et al.,* 1989). More studies are needed to characterize all the different fish mucus and to describe their defence mechanisms (Esteban, 2012).

Fish mucus also serves as a repository of a lot of defensive molecules of both the innate and the acquired immune system (Yano, 1996; Subramanian & MacKinnon, 2007; Esteban, 2012; Guardiola *et al.,* 2015a; Cammarata *et al.,* 2016). Some components only have a defensive purpose, whereas others may also act by modifying the organization and properties of the gel. A report of the main mucus components is now numbered. In summary, common features of these MALTs resemble those of mammals and include the following: (i) a copious mucus layer that actively barriers pathogen adherence and agglutinates; (ii) secreted antimicrobial proteins (such as lysozyme, lectins, complement proteins, histones, and defensins), antibodies (igM and igtT/Z isotypes), immune mediators (cytokines and chemokines), and enzymatic disruptors (mainly proteases, peroxidases, and phosphatases); and (iii) interposed myeloid and lymphoid immune cells (including mast cells, dendritic-like cells, macrophages, neutrophils, and B and T lymphocyte families), natural killer cells (NK/NCC-like), epithelial phagocytic cells, and immune-associated cells such as thrombocytes and erythrocytes (Alexander, 1992; Inami *et al.*, 2009; Castro & Tafalla, 2015; Khansari *et al.*, 2018)

2.4.2.1. Mucins

Mucins are glycoproteins with a lot of carbohydrate chains (fig. 6). They are the most abundant components in the mucus layer, responsible for its viscous property due to their high molecular weight. Defence is the main role of the mucins forming an adhesive matrix in which different molecules can be found (McGuckin, 2011).

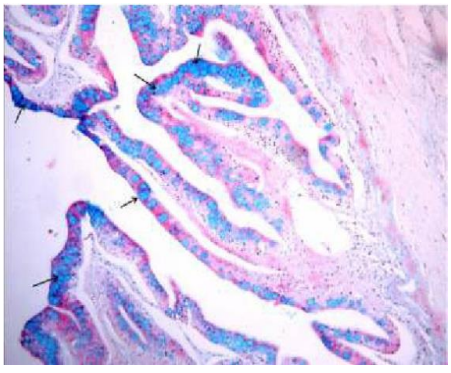


Figure 6. Intestine Nile perch (*Lates niloticus*) showing the mucins in the goblet cells. AB & PAS (x 400). Namulawa *et al.* (2014).

2.4.2.2. Enzymes

Some innate immune components were found in skin mucus. Lysozyme (N-acetylmuramide glucanohydrolase or muramidase) is the most studied enzyme present in fish mucus because is a mucolytic enzyme that acts on the peptidoglycan layer of bacterial cell walls resulting in the lysis of bacteria, catalyzing the hydrolysis of the linkages between the N-acetylmuramic acid and the N-acetyl-D-glucosamine. It is produced mainly by monocytemacrophages and neutrophils and, consequently, is abundant in lymphoid tissues, serum, mucus, and eggs. There are three determined isoforms in skin mucus and are present in some fishes but not in others (as cod or wolfish) (Nigam *et al.,* 2012)**.** The isoforms and their levels depend on the fish species and their environmental conditions. To date, serum lysozyme has been identified in practically all aquacultured species and its modulation, in response to infection and physiological stress, has been demonstrated in the mucus of many species such as European plaice (*Pleuronectes platessa L*.), channel catfish (*Ictalurus punctatus*), yellowtail (*Seriola quinqueradiata*), common carp, ayu (*Plecoglossus altivelis*), rainbow trout, Atlantic salmon, Japanese flounder and coho salmon (Fletcher & White, 1973; Grinde *et al.*, 1989; Lie *et al.,* 1989; Schrock *et al.*, 2001; Fast *et al.,* 2002; Subramanian *et al.*, 2007; Saurabh & Sahoo, 2008).

Another mucus enzyme, alkaline phosphatase (AP) has been demonstrated as a potential stress indicator in the epidermal mucus of Atlantic salmon (Ross *et al.,* 2000). It is also thought to act in a protective role in the initial stage of wound healing in carp (*C. carpio*) and as an antibacterial agent because of its hydrolytic activity (Iger & Abraham, 1990, 1997). Acid and alkalines phosphatases are also found in the skin mucus of some species like in the Asian stinging catfish (*Heteropneustes fossilis*) or in the common carp.

Other studied enzymes are copper and zinc superoxide dismutase (SOD) isolated from European plaice, esterases found in Gilthead seabream and European seabass, trypsine, beta-galactosidase, beta-glucoridase or alfa-fucosidase (Esteban, 2012).

By last, proteases are enzymes classified into serine, cysteine, aspartic and metalloproteases (Hartley, 1960). The ability of these enzymes to lyse formalin killed some bacteria as *V. anguillarum* so it seems that play a role in defence against bacteria (Ellis, 2001). Serine protease compounds more than the 25% of the complement system and it is reported as one of the main mucus proteases in fish skin mucus (Nigam *et al.,* 2012). Cell lysis is accomplished by the complement. The complement and the mucins act in synergy both interact with other components of fish mucus (such a C-reactive protein) to initiate the “complement cascade” (Alexander, 1992). Proteases as Cathepsin

or Metalloproteases also activate the production of others fish mucus components as immunoglobulins or antimicrobial peptides (Cho *et al.,* 2002). Cathepsins have been demonstrated in species as Japanese eel (*Anguilla japonica*) or catfish (Esteban, 2012). Enzymes that inhibits the action of a protease are called antiprotease.

2.4.2.3. Antimicrobial peptides (AMPs)

AMPs are enzymes discovered by Boman's group in 1982 (Rakers *et al.,* 2013). However, the first AMP reports in teleost date back some years after (Thompson *et al.*, 1986). To date, more than one thousands of antimicrobial peptides have been classified (http://www.bbcm.uniV.trieste.it/~tossi/).

AMPs are evolutionarily well preserved and found in higher vertebrate skin (including human epidermis). In fact, several AMPs in fish mucus may act as antibiotic for human diseases. AMPs as piscidin-2 from hybrid bass seem to exhibit a potent antifungal activity and epinecidin-1 also acts as a potential antitumor against fibro-sarcoma cells. Furthermore, each fish secretes their own AMPs with structural differences which may be exploited as antimicrobial agents, vaccine adjuvants or antitumoral agents. For that, fish skin offers the opportunity to study the origins of innate antimicrobial defence systems and might constitute a rich source of antiviral compounds for fighting against human infections (Falco *et al.,* 2009; Esteban, 2012; Rakers *et al.,* 2013).

AMPs are present in exposed tissues as skin and mucosal surfaces (Cho *et al.,* 2002) and have the ability to fight against numerous pathogenic organisms as bacteria (gram-positive and gram-negative), yeasts, fungus, viruses and other parasites (Najafian & Babji, 2012). However, relatively a few species of fish (i.e. zebrafish, pufferfish, rainbow trout, common carp, gilthead seabream, tilapia, winter flounder, American plaice, American halibut, mud dab, Atlantic salmon or Atlantic cod) have been studied to characterize their AMPs (Rakers *et al.,* 2013).

2.4.2.4. Proteins

Lectines

The innate immune system is largely defined by pattern-based recognition of non-self-cells due to the carbohydrates on their surfaces. In animals, the proteins that recognize these groups and also agglutinate cells are soluble molecules called lectines (Ewart, Jonhson, & Ross, 2001).

Lectine is a term used to include an extensive variety of carbohydrate-binding proteins without immune origin. Lectines are widely distributed in lot organisms from prokaryotes

to vertebrates. The first vertebrate lectines were described in European eel (*Anguilla anguilla*). It is known that lectines are involved in an immense range of key biological processes as the immune response (Cammarata *et al.,* 2016). They are found in skin mucus but also in gills and can interact with the pathogenic surface for their subsequent destruction by phagocytic cells and/or activate the complement pathway (Ewart *et al.,* 2001; Esteban, 2012).

Animal lectines are classified in several molecular families differing in carbohydrate recognition domain (CRD) structure and organisation: C-type lectines (CTLs), Galectins or S-type lectines, Rhamnose-binding lectines (RBLs), F-type lectines (FTLs), X-type lectines (XTLs). I-type lectines, P-type lectines and Pentraxins (Cammarata *et al.,* 2016). Selectins and other lectines gens have been reported in the current available fish genomes and CTLs, FTLs, Galectins or pentraxins have been characterized in both cartilaginous and bony fish. In addition, recent studies have identified new lectines families, some of them present in other taxa (Vasta *et al.,* 2011).

Cytokines

Cytokines include a broad category of small proteins that mediate cell signalling within the immune system. Cytokines are released by cells (mainly leucocytes) and regulate immune functions through the interaction with a specific receptor on the surface of other cells (paracrine) or the same cell that produced it (autocrine). Sometimes, systemic effects can also be produced through their release (endocrine). Each cytokine can be produced by different cell types, but in the same way; its receptor can be expressed on the surface of many different leucocyte types. Finally, several cytokines may exert very similar roles and thus there is a high degree of duplication (Castro & Tafalla, 2015). Some important cytokines are the chemokines or the interleukines.

Chemokines are produced by different cells and regulate immune cell migration, maturation, and functionality of the recruited cells in response to inflammation. They are defined by the presence of four conserved cysteine residues and are divided into four subfamilies depending on the arrangement of the first two conserved cysteines in their sequence, into CXC, CC, C, and CX3C classes. Despite the great number of chemokine genes identified in diverse fish species, it is still difficult to understand the mechanism of action of the molecules (Castro & Tafalla, 2015).

Others

Other several proteins have been studied in fish mucus. Some of them are calmodulin, lactoferrin, histones or ribosomal proteins (Esteban, 2012). Calmodulins are ubiquitous calcium-dependent activators of some enzymes with antigenic properties (Flik *et al.*, 1984). Lactoferrin is related to induce systemic immunity and inhibit allergic responses (González-Chávez *et al.,* 2009). In addition, Histone H2B is defined as an endogenous antibiotic due to its property to inhibit important fish bacteria and fungus as *Aeromonas hydrophila* or *Saprolegnia spp* (Bergsson *et al.,*2005).

2.4.2.5. The complement system

The complement system is one of the main mechanisms in the innate response. It is composed of about 30 plasma proteins synthesized as pro-enzymes (Secombes *et al.,* 2012). These proteins are responsible for a variety of functions including the elimination of invasive pathogens, the activation of phagocytosis and inflammatory response, the elimination of apoptotic cells and cellular debris, and modulation of the adaptive immune response (Holland & Lambris, 2002). This system can be activated by three different routes: classic, initiated by immune complexes; alternative, initiated by the union to microbial structures; and lectins as mentioned above, initiated by a bacterial mannose-binding lectin (Nakao *et al.*, 2011). mRNA's encoding complement components have been detected in teleost in a wide variety of tissues such as liver, cephalic kidney, gut or gills (Løvoll *et al.*, 2007).

Complement system includes an important component called pentraxins, an ancient pattern recognition of molecules that are conserved throughout phylogeny from arthropods to mammals. All these proteins play important roles in innate immunity, homeostatic regulation and the acute phase response, involving the immune system as well as other biological and physiological processes (Magnadóttir *et al.,* 2018). In fish, some pentraxins have been detected in several species, such as rainbow trout (Murata *et al.,* 1994, 1995), dogfish (*Mustelus canis*) (Robey *et al.,* 1983), plaice (White *et al.,* 1981), channel catfish. japanese eel, murrel (*Channa punctatus*) or goldfish (*Carassius auratus*) (Nunomura, 1991; Mitra and Bhattacharya, 1992; Kovacevic *et al.,* 2015).

2.4.2.6. Immunoglobulins

The adaptive immune system of the majority of vertebrates is based on key molecules such as immunoglobulins (Ig), T cell receptors (TCR) and major histocompatibility complex (MHC). Secretory immunoglobulins (Ig) are molecules found secreted into serum or mucosal secretions, or molecules adhered to B cells surfaces acting as membrane-bound form (BCR). The immunoglobulins found in fluid liquids are called antibodies (Ab) and constitute the main effectors in the humoral part of the immune system. These molecules are composed by two identical heavy chains (H) and two identical light chains (L) binding by two amino-terminal variable domains as shown in fig. 7 (Parra *et al.,* 2015).

Immunoglobulins are produced by plasmablast and plasma cells and play a key role in the mucosal homeostasis from many animals (Salinas *et al.,* 2011). They are well characterized in higher vertebrates as mammals. However, in lower vertebrates and particularly in teleost fish were firstly reported in the late 1960's by Fletcher and Grant (1969).

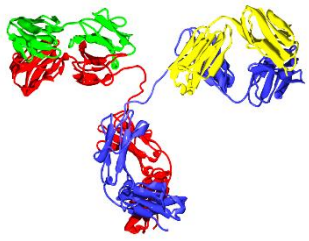


Figure 7. Heavy chains (H) (red and blue) and light chains (L) (green and yellow) from an immunoglobulin.

The kind of immunoglobulins varies in the different taxa. In fish mucosal surfaces, there are defined three types of Ig produced by teleost B cells: IgM, IgD and the recently discovered IgT/IgZ (Hansen *et al.,* 2005; Xu *et al.,* 2016). IgT has been reported to be an immunoglobulin specialized in gut and gill mucosal immunity while IgM is the predominant isotype in plasma, bile and skin mucus (Zhang *et al.,* 2010). Except catfish and medaka, all the other fish species studied present the IgT and constitutes the most ancient mucosal immunoglobulin found in vertebrates (Xu *et al.,* 2016). However, the presence of IgD in mucosal skin secretions in fish has not been reported (Parra *et al.*, 2015).

The concentration of Ig serum varies between species and may change depending on fish size, environmental temperature, water quality, season of the year or stressful conditions (reviewed in (Solem & Stenvik, 2006)). There are several studies that described the immunoglobulins from some bony fishes as giant grouper (*Epinephelus itaira*), margate (*Haemulon album*), sheepshead (*Archosargus probatocephalus*),

common carp, rainbow trout, European perch (*Perca fluviatilis* L.), olive flounder, European seabass or southern Bluefin tuna (*Thunnus maccoyii* Castelnau) (reviewed by Salinas *et al.,* 2011; Guardiola *et al.*, 2015b). However, there is no information about the characterization of the immunoglobulins in Senegalese sole as in the case of the other components (enzymes or antimicrobial peptides).

There is a discussion about the synthesis and transport of immunoglobulins in mucosal sites. It seems that the spleen is a site for B cells activation, plasmablast formation and differentiation into plasma cells. In addition, the spleen is involved in trapping antigens from the bloodstream. After that, plasma cells migrate to the head-kidney and produce immunoglobulins (Solem & Stenvik, 2006; Ye *et al.,* 2011a,b). The authors concluded that Ig must have resulted from local synthesis and transported across the hepatocytes to be secreted in the bile, and not transudation or transport from serum (Lobb & Clem, 1981; Abelli *et al.,* 2005). Finally, Xu *et al.* (2016) provided the first demonstration that dedicated mucosal immunoglobulins are locally induced at the mucosal surface of a non-tetrapod species.

Immunoglobulins are the principal components of the immune response against pathogenic organisms. Immunomodulatory products, including nucleotides, glucans and probiotics, are increasingly used in aquaculture production. The use of these products reduces the need for therapeutic treatments, enhances the effects of vaccines and, in turn, improves the indicators of production (Uribe *et al.,* 2011).

2.5. Skin mucosal immunity response

Mucus composition can be influenced by endogenous and exogenous factors (Esteban, 2012; Parra *et al.,* 2015). Sex or developmental stages are some of the endogenous factors while stress, acidification, hyper osmotic pressure, seasonality or infections are some of the studied exogenous factors (Blackstock, 1982; Wu *et al.,* 2004; Costas *et al.,* 2011; Esteban, 2012). Nevertheless, the presence of invasive pathogens is the main influencer in mucus composition (Esteban & Cerezuela, 2015). The fish innate immune system recognises pathogenic and non-pathogenic microorganisms via germline encoded pathogen pattern recognition receptors (PRRs) that sense particular structures of the microorganisms (pathogen-associated molecular patterns, PAMPs) and initiate a well-orchestrated immune response (Boltaña *et al.* 2011).

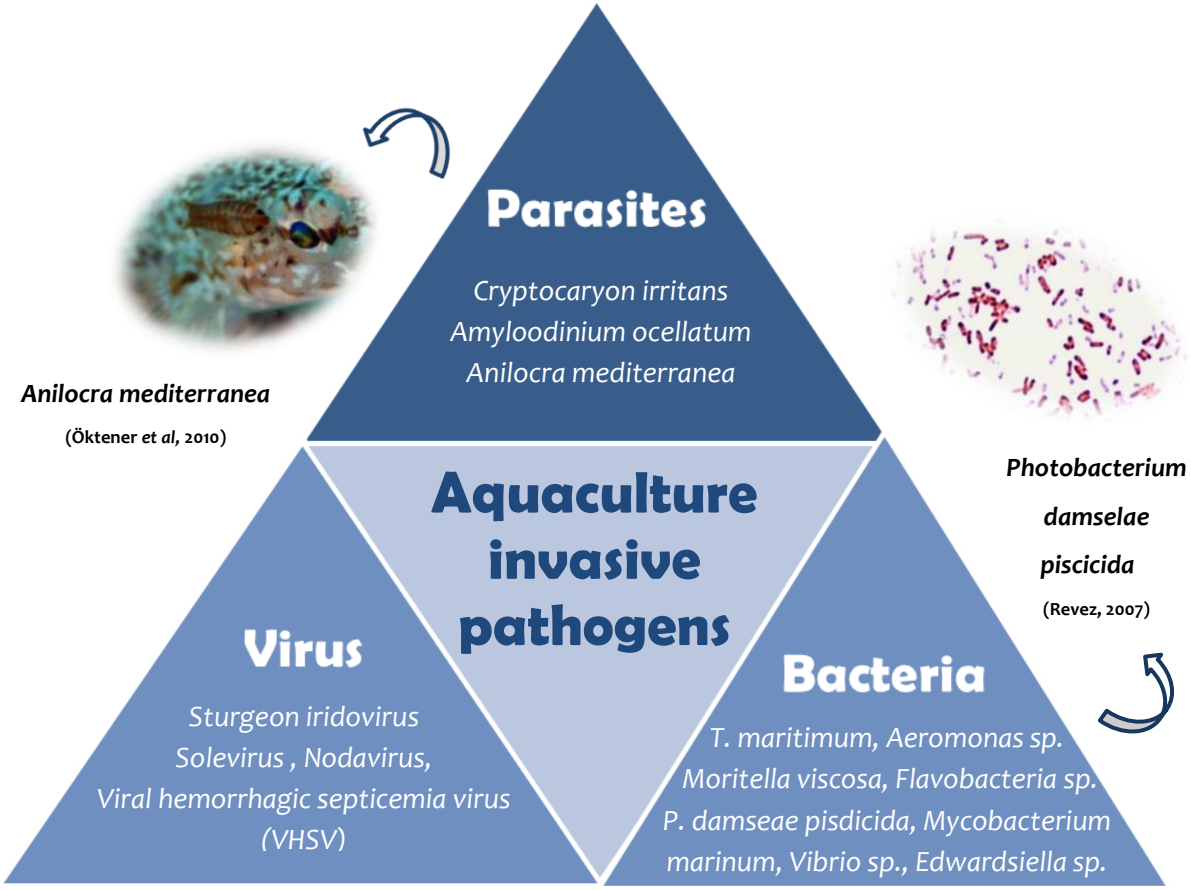


Figure 8. Resume of the most invasive pathogens in marine aquaculture.

As shown in Fig. 8, fish are immersed in a sea of pathogens and the importance of the mucus is now better documented due to their Ig responses (Salinas *et al.,* 2011; Esteban & Cerezuela, 2015). Some bacterium as *Flavobacterium columnare* or protozoan as *Ichthyophthirius multifiliis* cause fatal diseases penetrating by the skin and gills, points of entry for the pathogens to the fish body (Matthews, 2005). By this way, *Moritella viscosa* is considered the hardest pathogen causing ulcer and septicaemia on the fish skin due to its capacity to inhibit the epidermal regeneration abilities of the keratocytes (Karlsen *et al.,* 2012).

In addition, the concept of common mucosal immune system (CMIS) is believed to happen in fish but is not yet demonstrated in all the mucosa systems. CMIS suggests that an initial response in one of the mucosal places could be generate similar response in other mucosal tissues (Salinas *et al.,* 2011). Thus, more studies are needed to show the presence or absence of the CMIS.

2.5.1. Fish responses against bacteria

There is an increasing interest in fish studies after bacterial change but no much is known in this area related to soles. White sturgeon (*Acipenser transmontanus*), striped bass (*Morone saxatilis*), Chinook salmon (*Oncorhynchus tshawytscha*) and channel catfish (*I. punctatus*) were immersion challenged with *Edwardsiella ictaluri* and a Gram-negative septicaemia occurred in infected fishes, suggesting that *E. ictaluri* is a potential pathogen of salmonid fishes (Baxa *et al.,* 1990). After that, some studies in catfish treated with *Flavobacterium columnare* revealed a response in IgM levels from gill, skin, and liver samples (Shoemaker *et al.,* 2005). However, no specific changes in IgM levels were found when catfish were infected with *Edwardsiella tarda*. In addition, immune responses and expression profiles of some immune-related genes from head kidney and serum in Indian major carp (*Labeo rohita*) were studied after *E. tarda* infection (Mohanty & Sahoo, 2007). Nile tilapia (*Oreochromis niloticus*) was challenged with *Aeromona hydrophila* in Tellez-Bañuelos *et al.* (2010) and *M. viscosa* bath challenge was performed to Atlantic salmon, turbot, cod and Atlantic halibut. Samples of serum or head kidney were studied, and only Atlantic halibut was resistant to the pathogen (Björnsdóttir *et al.,* 2004; Løvoll *et al.*, 2009). Furthermore, Gilthead seabream and European seabass were subjected to either experimental infection with *Photobacterium damselae* subsp. *piscicida* in Mauri *et al.* (2011). Oral antigens also were studied in coho salmon skin infected with *V. anguillarum* and in barramundi (*Lates calcarifer*) challenged with *Streptoccocus iniae* (Delamare-Deboutteville *et al.,* 2006). Other studies tested this bacterial influence in

species as European eel by immersion with *Vibrio vulnificus. V. anguillarum* was also studied in eels and in common carp, where an increased goblet cell number was observed (van der Marel *et al.,* 2010; Salinas *et al.,* 2011). In addition, effects after bacterial challenge were described in other marine species as viera (*Pecten maximus*) or corals and demonstrate the strength of this kind of studies in the last years (Genard, *et al.,* 2014; Welsh *et al.*, 2017).

These experiments are also being accomplished on Senegalese sole specifically challenged with *P. damselae piscicida.* Leucocyte responses to inflammation as well as some innate immune parameters of Senegalese sole were determined after challenge with two strains of *P. damselae* subsp. *piscicida* in Costas *et al.* (2013). A challenge experiment using this bacterium was carried out to evaluate the effects of corticosteroids on the susceptibility to this important pathogen (Salas-Leiton *et al.,* 2012). A similar experiment was developed by Barroso *et al.* (2016) to determine the effect of a dietary multi-species probiotic on growth, gut morphology and immune parameters in the same flatfish species. Nowadays, the response of cultured Senegalese sole serum to *P. damselae piscicida* infection had been studied through the determination of transcriptional changes in genes related to iron metabolism, stress response and innate immune system (Núñez-Díaz, 2016). However, none of those studies gathered any data from the skin mucus of the species.

2.5.2. Skin mucus response against bacteria

Despite the important roles of mucus in fish immunity, the knowledge of detailed events happened within it during infection process is still limited. Some studies have focused on characterizing the protein and enzyme activities in the skin mucus following challenge as in Atlantic salmon mucus affected by *Neoparamoeba perurans* (Valdenegro-Vega *et al.,* 2014) or the characterization of the probiotic strain *Vagococcus fluvialis* in European seabass after the experimental challenge against *V. anguillarum* (Sorroza, 2012). Japanise eel was other of the study species in order to check different levels of antibacterial activities in skin mucus against three strains of Gram-negative bacteria: *E. tarda, A. hydrophila, Aeromonas sp.* and one Gram-positive *Bacterium Micrococcus leteus* (Liang *et al.,* 2011). Similar responses were observed from the zebra fish mucosa after colonization with the resident microbial flora (Cheesman & Guillemin, 2007). Other studies have examined the gene expression profiles as in the channel catfish skin mucus following *Flavobacterium columnare* challenge (Ren *et al.*, 2015).

However, there is a great lack of knowledge about the properties of skin mucus in Senegalese sole after bacterial infection. The first report about a bath challenge with *T. maritimum* was reported by Mabrok *et al.* (2016). This study aimed to optimize bacterial yields as well as to establish a challenge model for tenacibaculosis induction and suggested that the body surface can be considered the primary site of *T. maritimum* infection, as was also suggested in Vilar *et al.* (2012). In addition, data regarding mucus and plasma activities against *T. maritimum* suggested both a lack of host innate immune responses against this particular pathogen and evading strategies of *T. maritimum* against Senegalese sole.

3. STATEMENT FOR THE SIGNIFICANCE OF THE STUDY: OBJECTIVES

Considering the information reviewed in the literature, the present work studied for the first time, the *in vivo* mucosal immune response of Senegalese sole after the infection with one of its main threatening pathogens, *T. maritimum*. Therefore, one of the aims of the study was to determine the haematological profile and several humoral parameters in plasma and skin mucus after bacterial bath-challenge with *T. maritimum.* Interestingly, due to their condition of flatfish, it was wanted to differentiate between the skin mucus from ocular and blind sites in order to evaluate if there are differences in the mucosal immune response between both zones. The second aim of the present study was to assess possible resistance to bath challenge with *T. maritimum* through the evaluation of innate immune parameters at systemic (plasma) and local level (skin mucus), as well as, the disease resistance.

For these reasons, the present study was divided into two experiments to evaluate:

1) Haematological profile, several immune-related enzymes, natural haemolytic complement and bactericidal activities in skin mucus (from ocular and blind sides) and plasma after 1, 2 and 3 weeks of bath challenge with *T. maritimum*.

2) Several immune-related enzymes and bactericidal activities in skin mucus (from ocular and blind sides) and plasma after 4 weeks of bath challenge with *T. maritimum*, as well as, the survival rates after a second bath challenge with *T. maritimum* (ten times higher) at 5 weeks of first bath challenge.

4. GRAPHICAL ABSTRACT

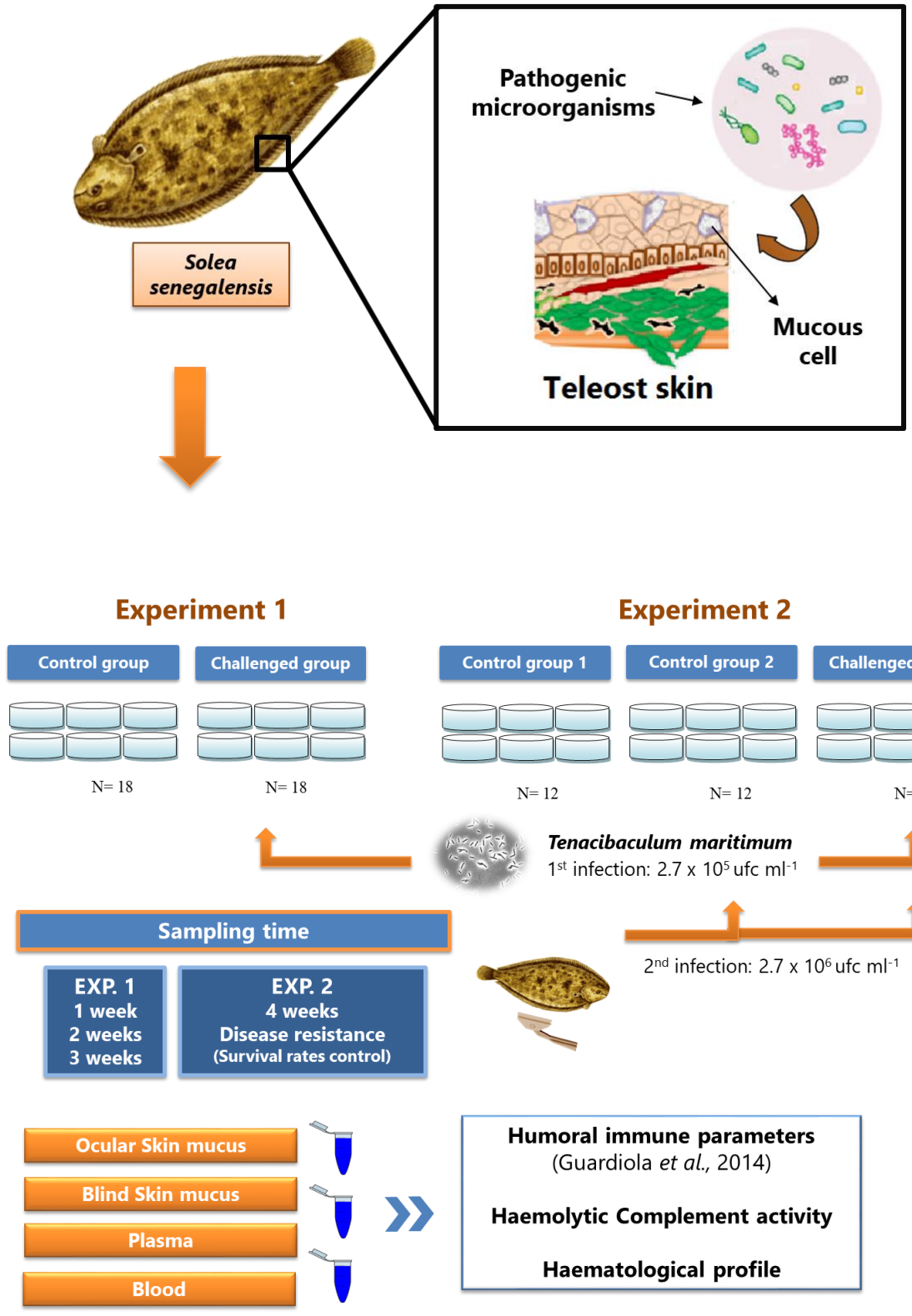


Figure 9. General graphical abstract.

5. MATERIALS AND METHODS

5.1. Fish care and maintenance

Healthy adult specimens of Senegalese sole with no history of tenacibaculosis (164.09 ± 5.08 g mean body weight and 25.28 ± 1.32 cm mean body length) were obtained from a commercial fish farm, located in north-west Portugal, and transported to the CIIMAR's experimental facilities (University of Porto, Portugal). Prior to the trials, fish were maintained in a recirculating aerated seawater (33 ‰ salinity) system with mechanical and biological filtration where dissolved oxygen was maintained around 90%, water temperature at 21 ± 1ºC, and a 12 h light/12 h dark photoperiod was adopted. Fish were fed to apparent satiety with commercial pellets (Skretting LE-2 ELITE, Spain). Ammonia and nitrite levels in the water were measured twice a week using commercial kits and never exceeded 0.025 and 0.3 mg L^{-1}, respectively.

5.2. Bacterial culture condition and inoculum preparation

T. maritimum strain ACC6.1 isolated from the Senegalese sole in a local fish farm was kindly provided by Professor Alicia E. Toranzo (*Departamento de Microbiología y Parasitología, Facultad de Biología*, University of Santiago de Compostela, Spain). Bacteria were kept frozen at -80°C until being used. The recovery of bacteria was achieved using marine agar (CONDA, Spain) at 25 °C for 48 hours. For inoculum preparation, the bacteria were harvested and inoculated into 50 ml of marine broth (MB) for additional 48 hours under the same temperature with continuous shaking (140 rpm). Exponentially growing bacteria were harvested by centrifugation at 4,000 *x g* for 30 min, re-suspended in sterile physiological saline (0.9% NaCl solution) and adjusted to the final concentration (2.7×10^5 CFU ml^{-1}) according to Mabrok *et al.* (2016).

5.3. Experimental design and sampling

This experiment was directed by trained scientists (following FELASA category C recommendations) and conducted according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes. It was divided into two parts: Experiment 1 and 2.

Experiment 1

MUCOSAL IMMUNE RESPONSES IN SENEGALESE SOLE JUVENILES AFTER *TENACIBACULUM MARITIMUM* CHALLENGE: A COMPARATIVE STUDY BETWEEN OCULAR AND BLIND SIDES

Fish were randomly distributed in 2 identical recirculated water systems composed of 6 tanks filled with 8 L of aerated seawater at flow rate 900 L h^{-1} as shown in Fig. 11. Thereafter, fish were left to acclimate for fifteen days prior to bacterial challenge. Subsequently, one of those systems (6 tanks, n=18) were bath challenged with a final bacterial concentration of 2.7 x 10^5 CFU mL^{-1} in 1 L of sea water at 23 ± 1 °C with strong aeration for 24 hours. Afterwards, the rearing water in each tank was changed three times and the recirculation system was re-established. The remaining system (6 tanks, n=18) was challenged under the same conditions with sterile marine broth (MB) instead of bacteria and served as control. One fish was then removed from each tank at the following times after bacterial challenge: 1, 2 and 3 weeks; then, skin mucus, blood and plasma were collected as described below. Fish were fed daily at a ratio of 1% of total fish biomass. In addition, ammonia and nitrite levels were assessed daily and kept below 0.025 and 0.3 mg L^{-1}, respectively.

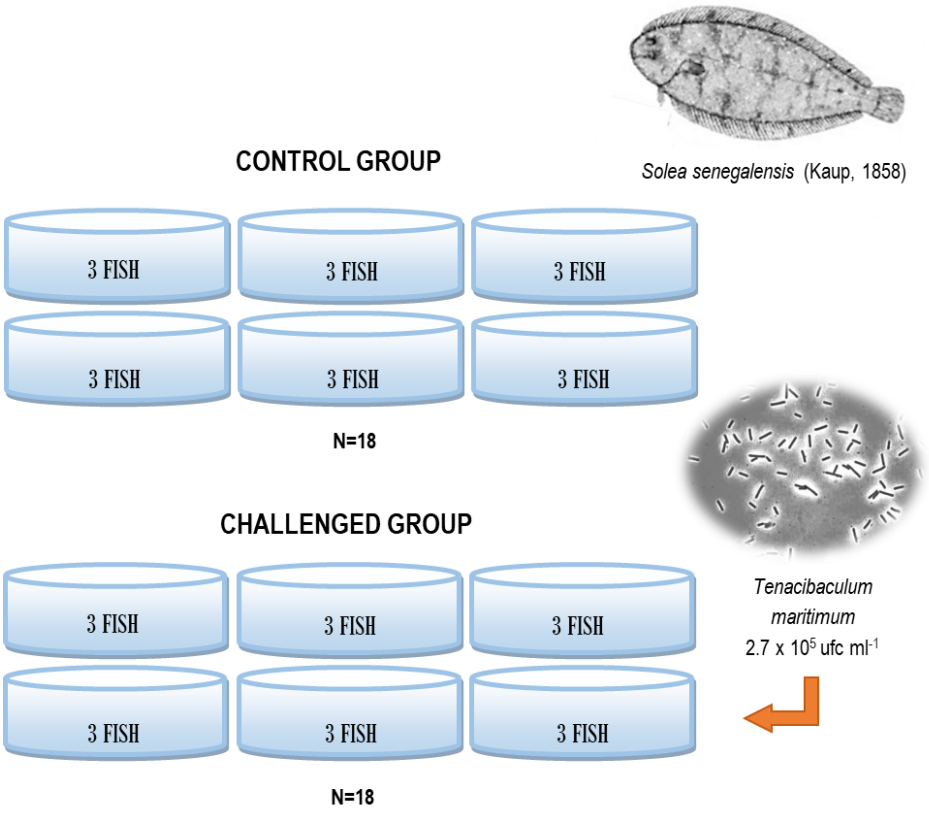


Figure 10. Experiment 1 experimental design.

Experiment 2

DISEASE RESISTANCE AND IMMUNE RESPONSE IN SKIN MUCUS AND PLASMA OF SENEGALESE SOLE AFTER *TENACIBACULUM MARITIMUM* CHALLENGE

Eighteen groups of 2 fishes each one were randomly distributed in 3 identical recirculated water systems composed of 6 tanks each one filled with 8 L of aerated sea water at flow rate 900 L h^{-1}. Two of the systems were selected as control and were defined as "Control A" and "Control B". The other system was considered the experimental group as it is represented in Fig. 12. Thereafter, fish were left to acclimate for 15 days prior to bacterial challenge. Subsequently, the experimental group (6 tanks, n=12) were bath challenged in 1 L of sea water at 23 ± 1°C with strong aeration for 24 hours with the same bacterial concentration than the previous experiment (2.7 x 10^5 CFU mL^{-1} of *T. maritimum*) as described in figure 12.

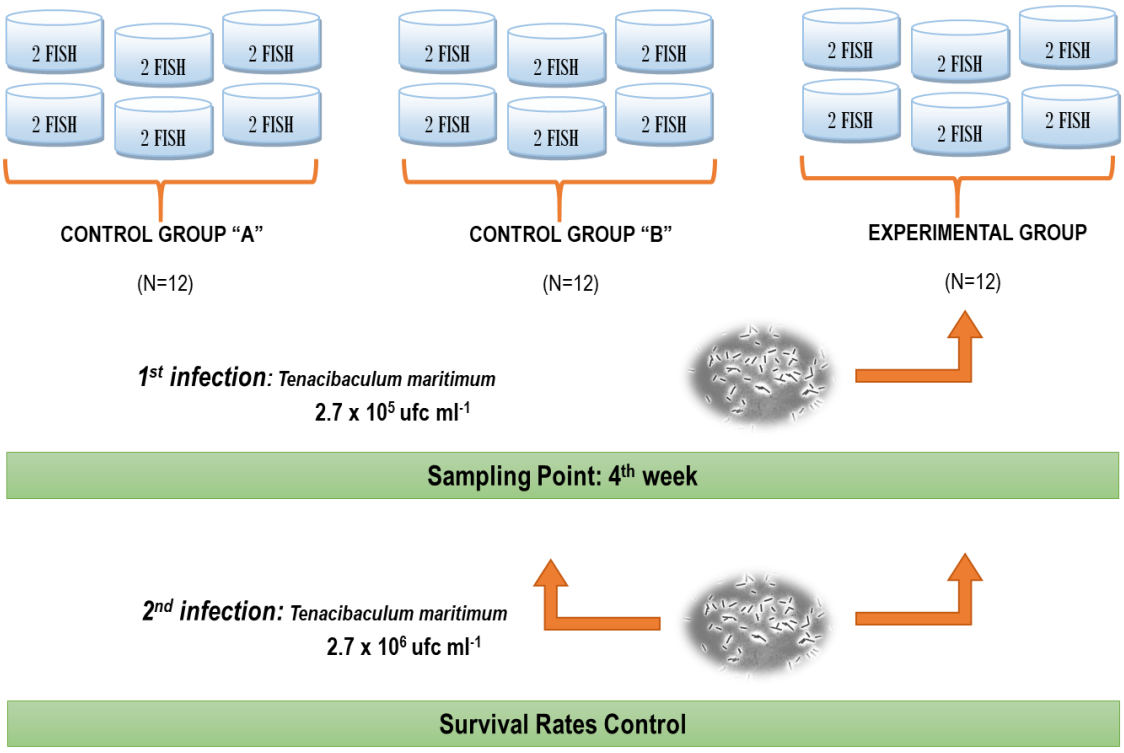


Figure 11. Experiment 2 experimental design.

Afterwards, the rearing water in each tank was changed three times and the recirculation system was restored. The remaining systems "Control A and B" (12 tanks, n=24) were

challenged under the same conditions with sterile marine broth (MB) instead of bacteria and served as controls. All fish were then removed from each tank at 4th week, and skin mucus of both sides (up and down), blood and plasma were collected as described below.

After the sampling point a new infection ten times more concentrated than before of *T. maritimum* (2.7×10^6 CFU mL^{-1} of *T. maritimum*) were applied in the previous challenged groups and in one of the previous control systems (6 tanks, N=12) to become the "2nd challenge-control" as shown in figure 12. After this second infection, the survival rates were determined.

5.4. Samples collection

Figure 12. Blood collection from the caudal vein.

Prior to each sampling point, fish were anesthetized with 2-phenoxyethanol (1 ml L^{-1}; Sigma). Skin mucus was aseptically collected from specimens using the method described by Guardiola *et al.* (2014) with slight modifications. Firstly, the fish were drained and subsequently the ocular and blind sides of both control and challenged fish were gently scraped by using a cell scraper with enough care to avoid the mixture of skin mucus between both sides as well as the contamination with urogenital and/or intestinal excretions. Collected mucus samples were then centrifuged at 2,000 × *g* and 4 ºC for 10 min. The supernatant was then filtrated (0.2 µm pore size; Sarstedt), aliquoted and stored at -80 ºC until further analyses. Following mucus collection, blood samples were withdrawn from the caudal vessel with heparinized syringes as shown in Fig. 13, placed in heparinized Eppendorf tubes and used to determine total erythrocytes and leucocytes counts and to prepare blood smear preparations. The remaining blood was used to collect plasma, following centrifugation (10,000 × *g*, 10 min, at 4 °C). Plasma was then frozen in liquid nitrogen and stored at −80 °C until further analysis.

5.5. Haematological procedures

The haematological profile consisted of total red (RBC) and white (WBC) blood cells counts, haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also calculated as follows:

$$\mathbf{MCH}\ (\mathbf{pg\ cell^{-1}}) = \left(\mathbf{Hb}/_{\mathbf{RBC}}\right) * \mathbf{10}$$

$$\mathbf{MCV}\ (\mu\mathbf{m^3}) = \left(\mathbf{Ht}/_{\mathbf{RBC}}\right) * \mathbf{10}$$

$$\mathbf{MCHC}\ (\mathbf{g\ 100\ ml^{-1}}) = \left(\mathbf{Hb}/_{\mathbf{Ht}}\right) * \mathbf{10}$$

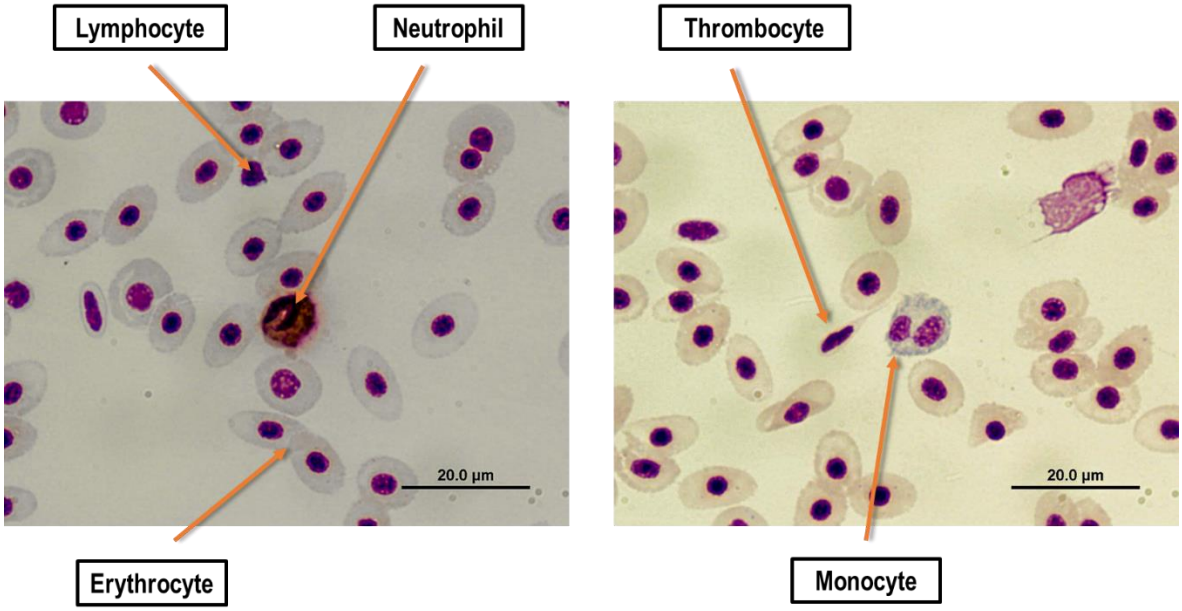


Figure 13. Leucocytes classified as thrombocytes, lymphocytes, monocytes and neutrophils in *Solea senegalensis*. Blood smear, Wright's stain (Haemacolor; Merck), Microscope at 1000x. Photo taken at the CIIMAR facilities.

Immediately after blood collection, blood smears were performed from homogenized blood, air dried, and stained with Wright's stain (Haemacolor; Merck) after fixation with formolethanol (10% of 37% formaldehyde in absolute ethanol). Detection of peroxidase activity to label neutrophils was carried out according to Afonso *et al.* (1998). The slides were examined under oil immersion (1,000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils as shown in Fig. 14. The absolute values ($\times 10^4$ μl^{-1}) of each leucocyte type were calculated.

5.6. Humoral immune parameters

5.6.1. Natural haemolytic complement activity (ACH_{50})

The natural haemolytic complement activity (ACH_{50}) was measured according to Sunyer and Tort (1995) with some modifications. The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Horse red blood cells (HoRBC; Probiologica Lda, Portugal) were used for haemolytic complement activity determination. HoRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5 x 10^8 cells ml^{-1}. Twenty µl of HoRBC suspension was then added to 60 µl of serially diluted plasma and skin mucus in Mg-EGTA-GVB buffer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 40 µl of distilled water or Mg-EGTA-GVB buffer to 20 µl samples of HoRBC, respectively. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged, and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a microplate reader (Synergy HT). The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y\ (1\text{-}Y)^{-1}$ against the volume of plasma and skin mucus homogenates added on a log-log scaled graph. The volume of plasma and skin mucus homogenates producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish. It was not possible to determine the complement in the plasma in experiment 2 due to lack of sample.

5.6.2. Peroxidase activity

Peroxidase activity in plasma and skin mucus was measured using the procedure described by Quade and Roth (1997). Briefly, 15 µl of plasma and 30 µl of skin mucus were diluted with 135 or 120 µl of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} in flat-bottomed 96-well plates. Then, 50 µl of 20 mM 3,3',5,5'-tetrametilbenzidine hydrochloride (TMB; Sigma-Aldrich) and 50 µl of 5 mM H_2O_2 were added. The reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the optical density (OD) was read at 450 nm in a microplate reader (Synergy HT). Wells without plasma and skin mucus were used as blanks. One unit of peroxidase was defined

as the amount producing an absorbance change of 1 and the final results were expressed as units ml^{-1}.

5.6.3. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Swain *et al.* (2007) with some modifications. Briefly, 20 µl of plasma or skin mucus were placed in flat-bottomed 96-well plates. To each well, 180 µl of freeze-dried *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma) in 40 mM sodium phosphate (pH 6.2) was added as lysozyme substrate. As blanks of each sample, 20 µl of plasma or skin mucus were added to 180 µl of sodium phosphate buffer. The absorbance at 450 nm was measured after 20 min at 35 ºC in a microplate reader (Synergy HT). The amounts of lysozyme present in plasma and skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) through serial dilutions in the above buffer. Plasma or skin mucus lysozyme values are expressed as µg ml^{-1} equivalent of HEWL activity.

5.6.4. Protease

Protease activity was quantified in plasma and skin mucus samples using the azocasein hydrolysis assay according to Guardiola *et al.* (2016) with some modifications. Briefly, 100 µl of skin mucus was incubated with 100 µl of 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) for 24 h at 30ºC. In the case of plasma, 10 µl of plasma was diluted in 100 µl of ammonium bicarbonate (100 mM) and incubated with equal volume of 100 mM ammonium bicarbonate containing 2% azocasein (Sigma) at the same conditions (24 h at 30ºC). The reaction was stopped by adding 4.6% (skin mucus) or 10% (plasma) trichloroacetic acid (TCA) and the mixture centrifuged (6000 *x g*, 5 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5 N NaOH in the case of skin mucus samples and 1 N in the plasma ones. The OD read at 450 nm using a microplate reader (Synergy HT). Skin mucus and plasma were replaced by trypsin solution (5 mg ml^{-1}, Sigma), as the positive control (100% of protease activity), or by buffer, as the negative control (0% activity). The percentage of trypsin activity compared to the positive control was calculated.

$$\%\ \textbf{non inhibited trypsin} = \frac{\textbf{Sample Abs. x 100}}{\textbf{Abs. of the reference sample}}$$

5.6.5. Antiprotease activity

Total antiprotease activity was determined by the ability of skin mucus or plasma to inhibit trypsin activity with some modifications (Machado *et al.,* 2015). Briefly, 50 µl of skin mucus or 10 µl of plasma were incubated for 10 min at 22ºC with 10 µl of standard trypsin solution (5 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) for 10 min at 22 ºC in polystyrene microtubes (Fig. 14). To the incubation mixture, 60 µl or 100 µl of 115 mM PBS (NaH_2PO_4, 13.9 mg ml^{-1}, pH 7.0) for skin mucus and plasma samples, respectively, and 125 µl of 2% azocasein (in 60 mM sodium bicarbonate, pH 8.3) was added and the samples incubated for 60 min at 22ºC. Finally, 250 µl of 10% of TCA (trichloroacetic acid) was added a new incubation for 30 min at 22ºC was done. The mixture was then centrifuged (10,000 *x g*, 5 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 1 N NaOH, and the OD read at 450 nm using a microplate reader (Synergy HT). PBS in place of skin mucus, plasma and trypsin served as blank whereas the reference sample was PBS in place of skin mucus and plasma. The percentage inhibition of trypsin activity compared to the reference sample was calculated.

Figure 14. Material used for antiprotease activity assay. Photo taken at the CIIMAR facilities.

$$\%\ \boldsymbol{non\ inhibited\ trypsin} = \frac{\boldsymbol{Sample\ Abs.\ x\ 100}}{\boldsymbol{Abs.\ of\ the\ reference\ sample}}$$

$$\%\ \boldsymbol{inhibited\ trypsin} = \mathbf{100}\ -\ \%\ \boldsymbol{non\ inhibited\ trypsin}$$

5.7. Bactericidal activity

Bactericidal activity present in skin mucus and plasma samples was determined using two marine pathogenic bacteria: *P. damselae piscicida* and *V. anguillarum* (strains PP3 and PC696.1 respectively). Bacteria were grown in agar plates at 25ºC in tryptic soy media (TSB, Sigma) for *V. anguillarum* and *P. damselae piscicida* for 24 h. Afterwards, fresh single colonies of 1-2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 24 h at 25ºC on an orbital incubator (250 rpm) until exponential growing, at which point bacteria were resuspended in sterile HBSS and adjusted to 1×10^6 and 1×10^8 colony-forming unit (cfu) ml^{-1} for *P. damselae piscicida* and *V. anguillarum*, respectively.

Skin mucus and plasma bactericidal activity were then determined by evaluating their effects on the bacterial growth curves according to Machado *et al.,* (2015) with some modifications. Briefly, 20 µl of skin mucus or plasma were added to duplicate wells of a U-shaped 96-well plate as it was shown in fig.15. Hank's balanced salt solution was added to some wells instead of sample and served as positive control. To each well, 20 µl of each bacteria solution were added and plates were incubated for 2.5 h at 25ºC. To each well, 25 µl of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg ml^{-1}; Sigma-Aldrich) was added and incubated for 10 min to allow the formation of formazan. Plates were then centrifuged at 2,000 *x g* for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich). The absorbance of the dissolved formazan was measured at 560 nm in a microplate reader (Synergy HT). Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

$$\%\ \boldsymbol{viable\ bacteria} = \frac{\boldsymbol{Sample\ Abs.\ x\ 100}}{\boldsymbol{Abs.\ of\ the\ reference\ sample}}$$

$$\%\ \boldsymbol{no\ viable\ bacteria}\ (\boldsymbol{bactericidal\ activity}) = \mathbf{100} - \%\ \boldsymbol{viable\ bacteria}$$

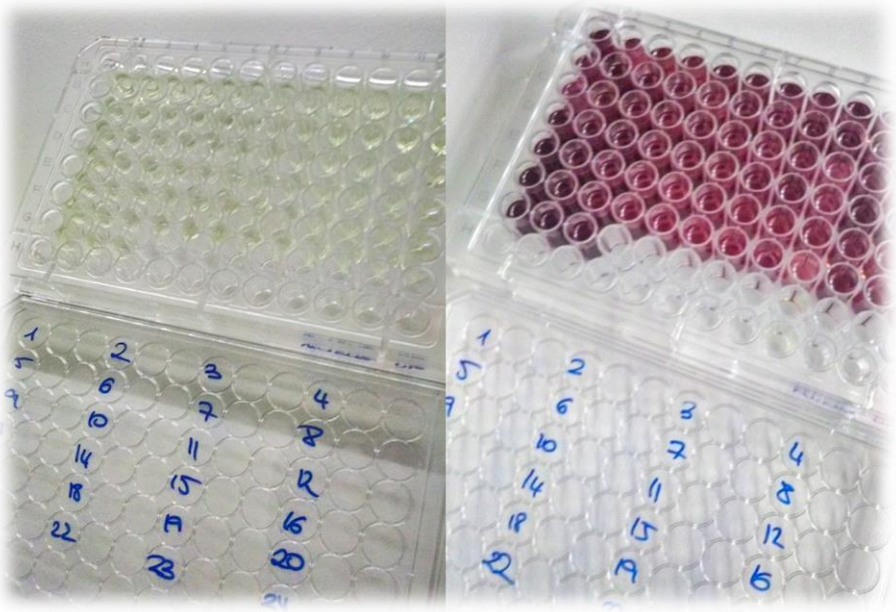


Figure 15. U-shaped 96-well microplates used for bactericidal activity assay.

5.8. Statistical analysis

All analyses were conducted in triplicates and the results are expressed as means ± standard error of the mean (SEM). Data were analysed by Two-way analysis of variance (ANOVA) (haemotological profile and plasma immune-related analysis) or Multifactorial ANOVA (mucosal immune-related analysis) with experimental group, sampling time and origin (ocular or blind sides) as variables. Both procedures were followed by Tukey post hoc test (sampling time) or t-test student (experimental group and skin mucus origin) to identify differences in the experimental groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test and, when necessary, outliers were removed using the SPSS tool for outliers and extremes removal. All statistical analyses were conducted using SPSS 19.0 and differences were considered statistically significant when $p < 0.05$.

6. Results

6.1. Experiment 1

6.1.1. Haematological profile and differential cell counts

Ht, Hb, MCV, MCH, MCHC, RBC and WBC of unchallenged and challenged with *T. maritimum* Senegalese soles are shown in Table 1 whilst the relative proportion and absolute values of peripheral blood leucocytes are represented in Table 2. Overall, bacterial bath challenge did not affect the haematological parameters, as well as the relative proportion and absolute numbers of the different leucocyte types. Only a decreased in the absolute value of monocytes was observed at 2 weeks in challenged fish compared to control group (unchallenged). Comparing each experimental group with the time, no variations were observed in any parameters (data not shown).

Table 1. Haematocrit (Ht), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC) and white blood cells (WBC) in Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. Values are expressed as means ± SE (n=6).

Parameters	Experimental time (weeks)					
	1		2		3	
	Control	Challenged	Control	Challenged	Control	Challenged
Ht (%)	13 ± 1.38	13 ± 1.41	13.17 ± 3.02	13.60 ± 4.03	14.83 ± 3.02	17.00 ± 2.97
Hb (g dl^{-1})	0.38 ± 0.14	0.55 ± 0.22	0.62 ± 0.24	0.81 ± 0.30	0.60 ± 0.28	0.86 ± 0.09
MCV (μm^3)	124.1 ± 19.2	119.3 ± 4.3	123.6 ± 19.2	133.5 ± 37.7	123.3 ± 20.6	135.3 ± 37.6
MCH (pg $cell^{-1}$)	3.59 ± 1.58	5.05 ± 2.26	6.18 ± 2.99	7.85 ± 2.75	4.85 ± 1.77	6.76 ± 0.47
MCHC (g 100 ml^{-1})	2.80 ± 0.87	4.21 ± 1.85	5.22 ± 2.74	6.40 ± 2.86	3.88 ± 1.02	5.19 ± 0.86
RBC ($\times 10^6$ μl^{-1})	1.08 ± 0.21	1.09 ± 0.12	1.06 ± 0.15	1.04 ± 0.18	1.22 ± 0.22	1.29 ± 0.16
WBC ($\times 10^4$ μl^{-1})	9.78 ± 3.83	8.98 ± 1.56	7.95 ± 2.04	7.08 ± 1.38	7.02 ± 3.56	8.96 ± 1.75

Table 2. Relative proportion and absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. Values are expressed as means ± SE (n=6). Asterisk denotes significant differences between treatment groups ($p<0.05$).

Parameters		Experimental time (weeks)					
		1		2		3	
		Control	Challenged	Control	Challenged	Control	Challenged
Thrombocytes	(% WBC)	34.15 ± 5.42	32.47 ± 3.60	38.68 ± 14.7	46.64 ± 9.46	41.80 ± 5.95	47.6 ± 5.54
	($\times 10^4$ μl^{-1})	3.84 ± 1.22	3.49 ± 0.78	3.41 ± 1.33	3.75 ± 1.31	3.72 ± 1.41	5.92 ± 1.32
Lymphocytes	(% WBC)	58.36 ± 4.06	59.83 ± 5.63	51.54 ± 14.9	43.67 ± 11.4	49.79 ± 7.64	46.00 ± 5.68
	($\times 10^4$ μl^{-1})	6.68 ± 2.32	6.38 ± 1.05	4.66 ± 2.17	3.34 ± 0.70	4.62 ± 2.11	5.68 ± 0.92
Monocytes	(% WBC)	1.81 ± 0.38	1.81 ± 0.37	2.41 ± 0.60	1.20 ± 0.51*	1.50 ± 0.57	1.1 ± 0.37
	($\times 10^4$ μl^{-1})	0.20 ± 0.07	0.19 ± 0.05	0.22 ± 0.08	0.09 ± 0.03	0.13 ± 0.08	0.14 ± 0.06
Neutrophils	(% WBC)	5.68 ± 2.32	5.90 ± 4.29	7.40 ± 2.49	8.49 ± 2.33	6.91 ± 2.54	5.3 ± 1.29
	($\times 10^4$ μl^{-1})	0.69 ± 0.44	0.60 ± 0.37	0.65 ± 0.24	0.69 ± 0.29	0.56 ± 0.25	0.64 ± 0.12

6.1.2. Innate immune parameters and bactericidal activity in plasma

Protease (Fig. 16A), antiprotease (Fig. 16B), Natural haemolytic complement (Fig. 17A), peroxidase (Fig. 17B) and lysozyme (Fig. 17C) activities were unaffected by bacterial challenge.

The bactericidal activity against pathogenic bacterium *P. damselae piscicida* (Fig. 18A) was not modified by challenge whilst an increase in bactericidal activity against *V. anguillarum* in plasma from challenged fish compared to the control group was observed at 1 week (Fig. 18B).

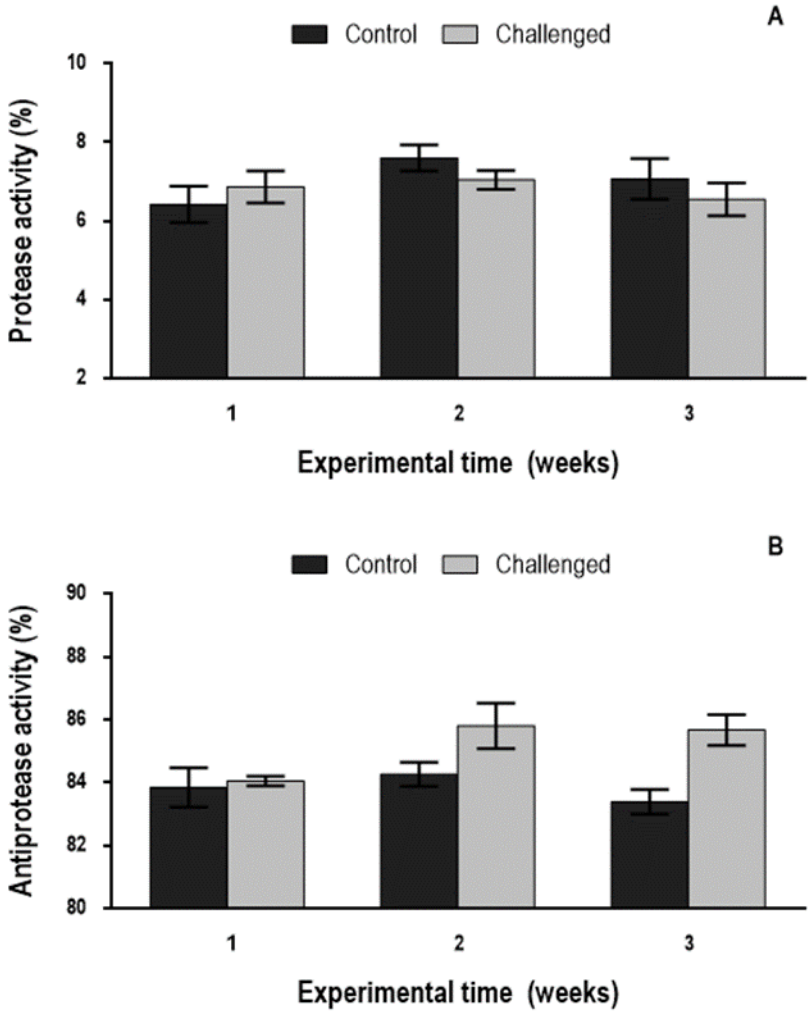


Figure 16. Protease (%) (A) and antiprotease (%) (B) activities in plasma samples of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SE (n=6). Asterisk denotes significant differences between experimental groups (T-test; $p < 0.05$).

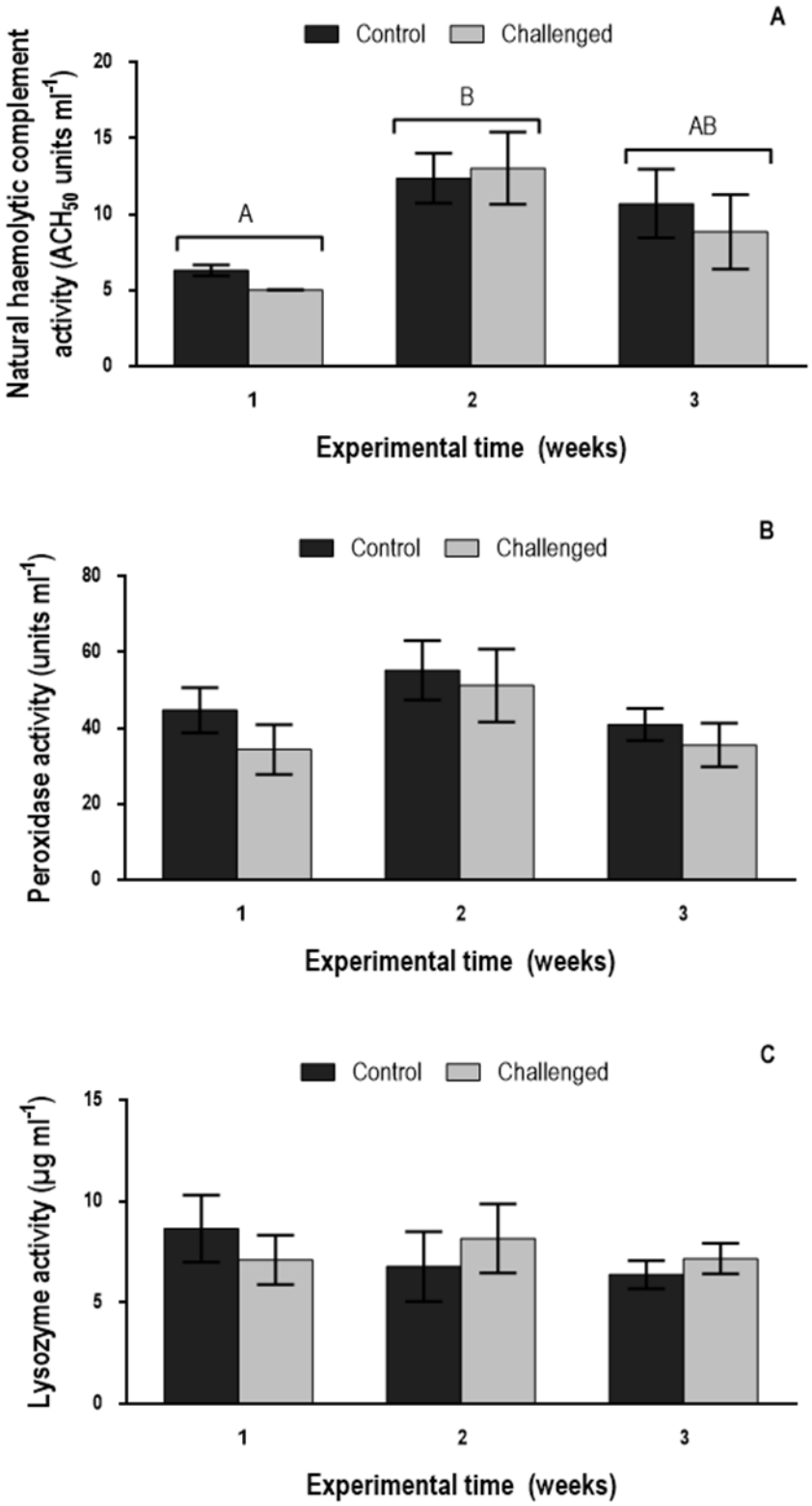


Figure 17. Natural haemolytic complement (ACH_{50} units ml^{-1}) (A), peroxidase (units ml^{-1}) (B) and lysozyme (µg ml^{-1}) (C) activities in plasma samples of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SE (n=6). Different letters denote significant variations among experimental groups regarding to time (Two way ANOVA; $p<0.05$).

Respect to time factor, the increases of bactericidal activity against *P. damselae piscicida* (Fig. 18A) observed in both experimental times at 3 weeks were significant respect to values found at 1 week. In the case of haemolytic complement (Fig. 17A), the activity was increased at 2 weeks compared to values observed at first sampling point (1 week).

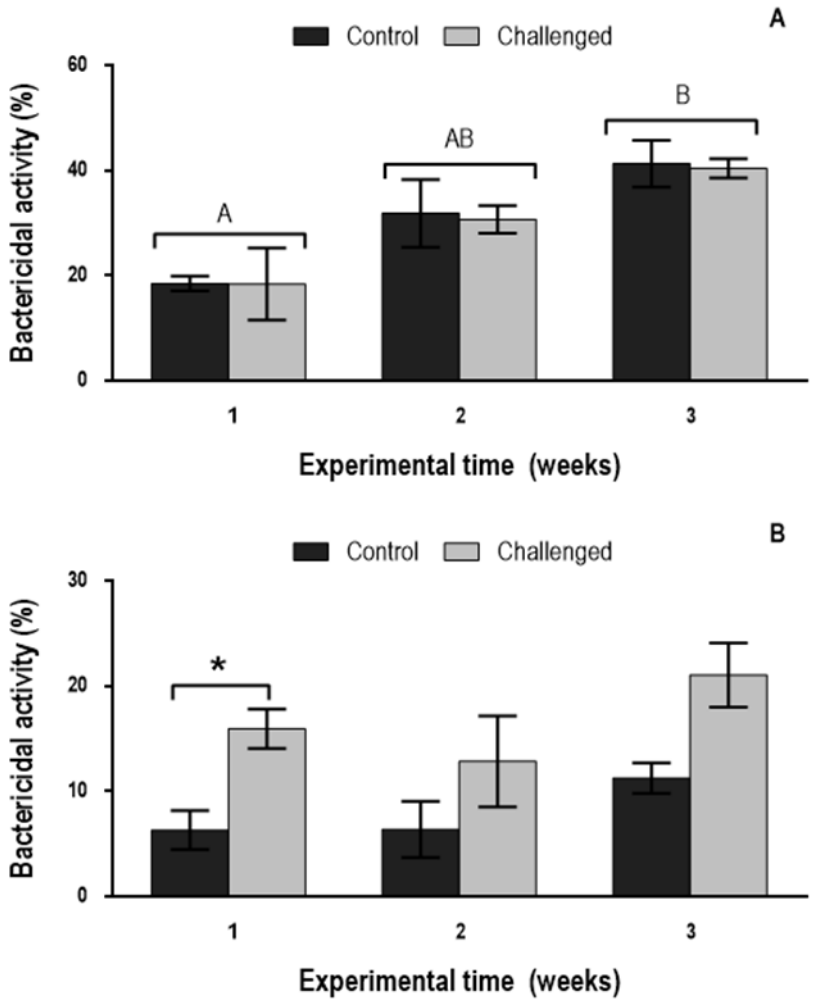


Figure 18. Bactericidal activity (%) against *Photobacterium damselae* subsp. *piscicida* (A) and *Vibrio anguillarum* (B) in plasma samples of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SEM (n=6). Asterisk denotes significant differences between experimental groups (T-test; $p<0.05$) whilst letters denote significant variations among experimental groups regarding to time (Two way ANOVA; $p<0.05$).

6.1.3. Immune parameters and bactericidal activity in skin mucus from ocular and blind sites

Natural haemolytic complement increased in skin mucus from the blind side of challenged fish respect to unchallenged (control) ones at 2 weeks of trial (Fig. 20A). After 2 weeks of the challenge, peroxidase (Fig. 20B) and bactericidal activities against *V. anguillarum* (Fig. 21B) were higher in ocular skin mucus of challenged fish respect to control group. Similarly, bactericidal activity against *P. damselae piscicida* was also incremented in ocular skin mucus of challenged fish compared to unchallenged ones but at the end of the experiment (3 weeks) (Fig. 21A). Contrarily, bactericidal activity against *V. anguillarum* was reduced in blind skin mucus of challenged fish comparatively to control group (Fig. 21B). Finally, no variations were observed in protease (Fig. 19A), antiprotease (Fig. 19B) and lysozyme (Fig. 20C), activities in skin mucus of challenged fish compared to control group from both origins (ocular and blind) at any sampling point.

Comparing each experimental group with both origins (ocular and blind), the activities of haemolytic complement (2 week), lysozyme (1 and 3 weeks), protease (1, 2 and 3 weeks), bactericidal against *P. damselae piscicida* (1 and 2 weeks) and *V. anguillarum* (1 week) were higher in ocular skin mucus of control fish (unchallenged) compared to blind site, except to peroxidase activity at 2 weeks (Fig. 22). Following a similar pattern, the activities of haemolytic complement (1 week), lysozyme (1 and 2 weeks), protease (1, 2 and 3 weeks), bactericidal against *P. damselae piscicida* (2 and 3 weeks) and *V. anguillarum* (2 and 3 weeks) were higher in ocular skin mucus of challenged fish respect to values found in blind skin mucus, except to peroxidase activity at 1 week (Fig. 22).

In respect of time, only the bactericidal activity against *V. anguillarum* showed variations (Table 3). Concretely, a decrease in bactericidal activity in ocular skin mucus of unchallenged and challenged fish was observed at 3 weeks compared to values found at 1 and 2 weeks. Similarly, a bactericidal activity reduction was observed at 2 and 3 weeks in blind skin mucus of control fish respect to first sampling point (1 week).

Regarding to skin mucus origins (ocular and blind) and independently of time and experimental group variables, a higher activity was observed of all immune-related parameters measured in the ocular side compared to blind one, except for peroxidase which showed more activity in skin mucus from blind side.

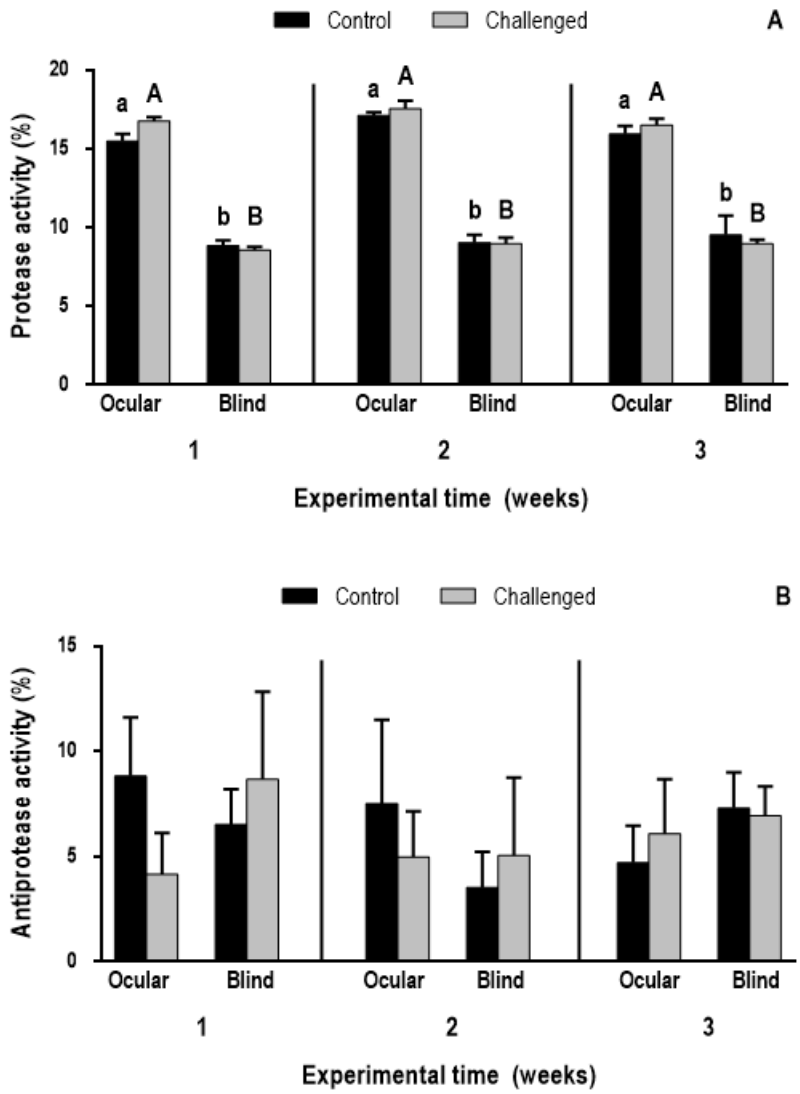


Figure 19. Protease (%) (A) and antiprotease (%) (B) activities in ocular and blind skin mucus samples of Senegalese sole control (unchallenged) and challenged with *Tenacibaculum maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SE (n=6). Asterisk denotes significant differences between experimental groups (T-test; $p<0.05$). Small and capital letters denote significant variations between ocular and blind skin mucus at the same time, respectively (T-test; $p<0.05$).

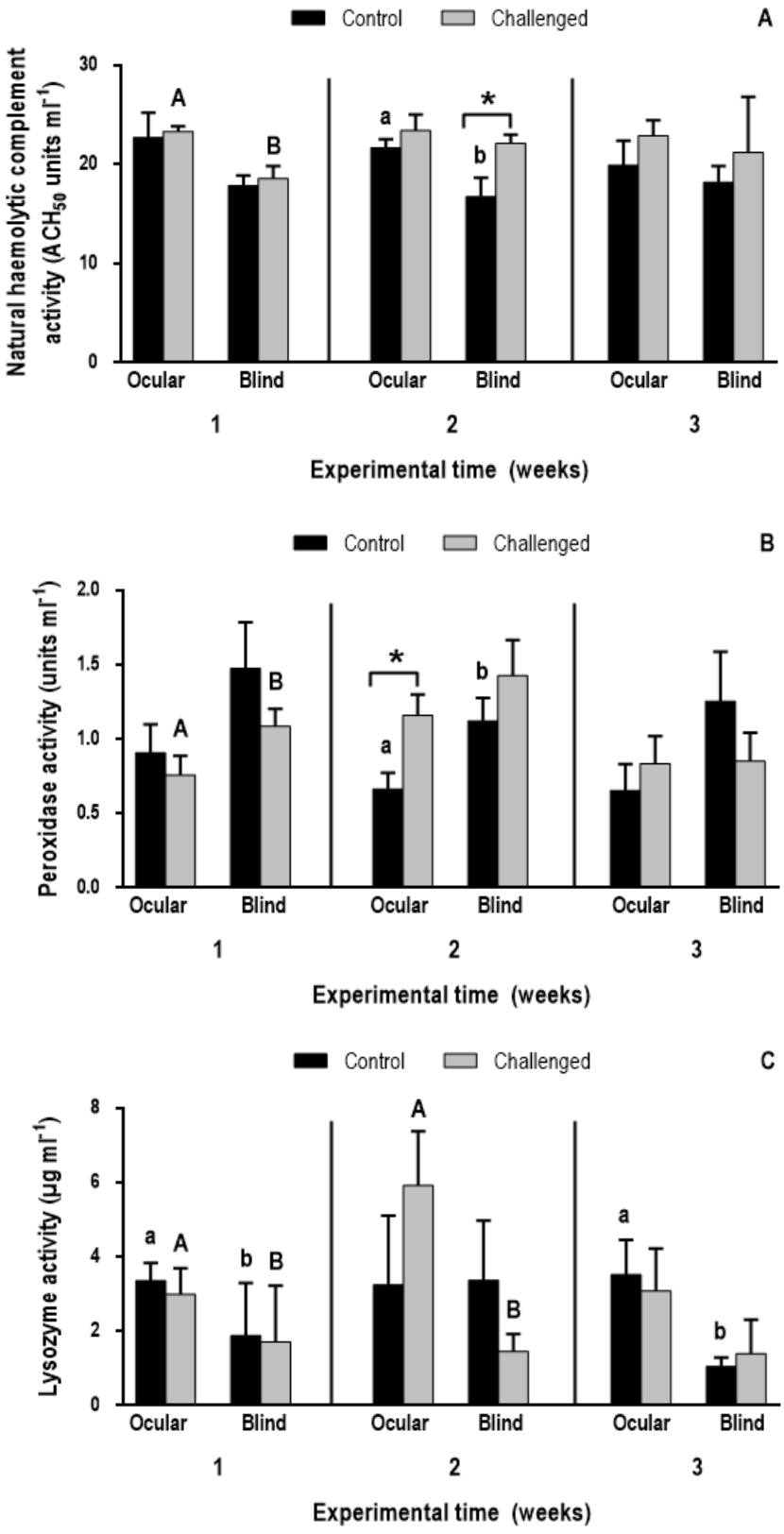
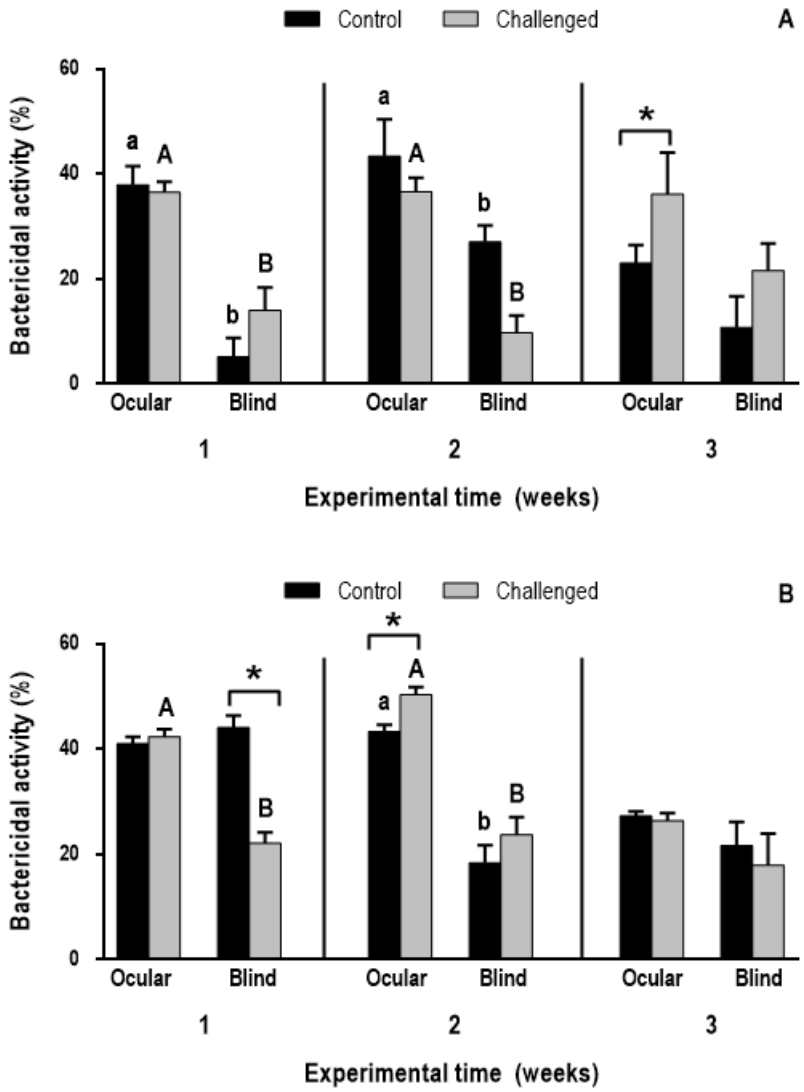
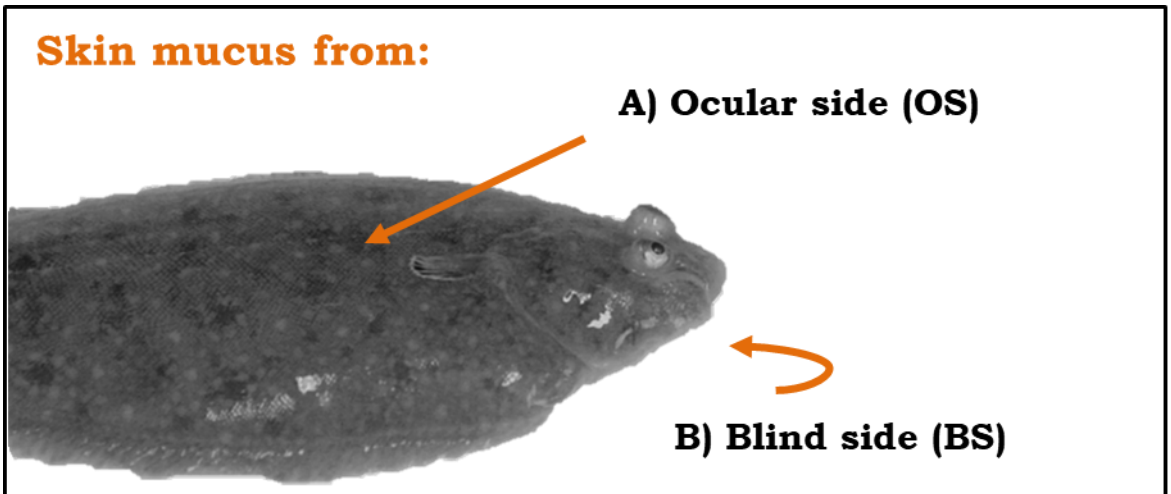


Figure 20. Natural haemolytic complement (ACH_{50} units ml^{-1}) (A), peroxidase (units ml^{-1}) (B) and lysozyme (µg ml^{-1}) (C) activities in ocular and blind skin mucus samples of Senegalese sole control (unchallenged) and challenged with *Tenacibaculum maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SE (n=6). Asterisks denote significant differences between experimental groups whilst small and capital letters denote significant variations between ocular and blind skin mucus at the same time, respectively (T-test; $p<0.05$).



Bactericidal activity (%)	Origin	Experimental times (weeks)			P values
		1	2	3	
A) Pdp	Ocular side	-	-	-	ns
	Blind side	-	-	-	ns
B) V. anguillarum	Ocular side	a	a	b	0.00
	Blind side	a	b	b	0.00

Figure 21. Bactericidal activity (%) against *Photobacterium damselae* subsp. piscicida (Pdp) (A) and *Vibrio anguillarum* (B) in ocular and blind skin mucus samples of Senegalese sole control (unchallenged) and challenged with *Tenacibaculum maritimum* during 24 hours at 1, 2 and 3 weeks. Bars represent the means ± SEM (n=6). Asterisks denote significant differences between experimental groups whilst small and capital letters denote significant variations between ocular and blind skin mucus at the same time, respectively (T-test; $p<0.05$). In the table, different letters denote significant variations between among experimental groups regarding to time (Two way ANOVA; $p<0.05$). Ns: non-significant.



Control	OS *vs* BS		
Activities	Time (weeks)		
	1	2	3
ACH_{50}	>	-	-
PER	<	-	-
LYZ	>	>	-
PRO	>	>	>
ANTI	-	-	-
Pdp	>	>	-
V.ang	>	>	-

Challenge	OS *vs* BS		
Activities	Time (weeks)		
	1	2	3
ACH_{50}	-	>	-
PER	-	<	-
LYZ	>	-	>
PRO	>	>	>
ANTI	-	-	-
Pdp	>	>	-
V.ang	-	>	-

Figure 22. Comparative graphic diagram showing the activity levels (> or <) of the immune-related parameters found in skin mucus from ocular versus (vs) blind sides of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours at 1, 2 and 3 weeks. ACH_{50}: natural haemolytic complement; PER: peroxidase; LYZ lysozyme; PRO: protease; ANTI: antiprotease; Pdp: bactericidal activity against Photobacterium damselae subsp. piscicida; V. ang: bactericidal activity against Vibrio anguillarum.

Table 3. Differences found in the parameters evaluated in ocular and blind skin mucus of Senegalese sole control (unchallenged) and challenged with *T. maritimum* during 24 hours respect to time (1, 2 and 3 weeks) regardless of experimental group (n=6). Different letters denote significant differences between experimental groups regarding to time. One-way ANOVA was performed: ns: non-significant ($p<0.05$).

Parameters evaluated		Origin	Experimental groups	Experimental times (weeks)			P values
				1	2	3	
HUMORAL FACTORS	Haemolytic complement (ACH_{50} units ml^{-1})	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
	Peroxidase (units ml^{-1})	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
	Lysozyme (µg ml^{-1})	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
	Protease (%)	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
	Antiprotease (%)	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
BACTERICIDAL ACTIVITY (%)	*Photobacterium damselae*	Ocular	Control	-	-	-	ns
			Challenged	-	-	-	ns
		Blind	Control	-	-	-	ns
			Challenged	-	-	-	ns
	Vibrio anguillarum	Ocular	Control	a	a	b	0.00
			Challenged	a	b	c	0.00
		Blind	Control	a	b	b	0.00
			Challenged	-	-	-	ns

6.2. Experiment 2

6.2.1. Innate immune parameters and bactericidal activity in plasma

Lysozyme (Fig. 23B), protease (Fig. 24A) and antiprotease (24B) activities were unaffected after bacterial challenge. However, peroxidase activity (Fig. 23A) was incremented in challenged fish compared to values found in both controls after 4 weeks post-challenge. The bactericidal activity against opportunist pathogenic bacterium *P. damselae* (Fig. 25A) was not modified by *T. maritimum* challenge whilst an decrease in this activity against *V. anguillarum* was observed in challenged fish compared to the control groups at 4 weeks of trial (Fig. 24B).

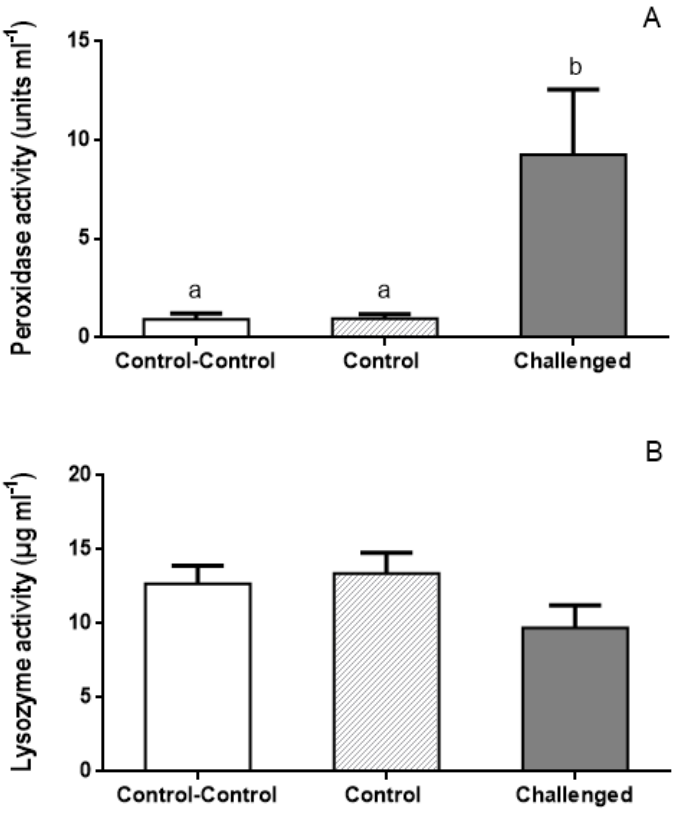


Figure 23. Peroxidase (units ml^{-1}) (A) and lysozyme (µg ml^{-1}) (B) activities in plasma samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12). Different letters denote significant differences between experimental groups (ANOVA; $p<0.05$).

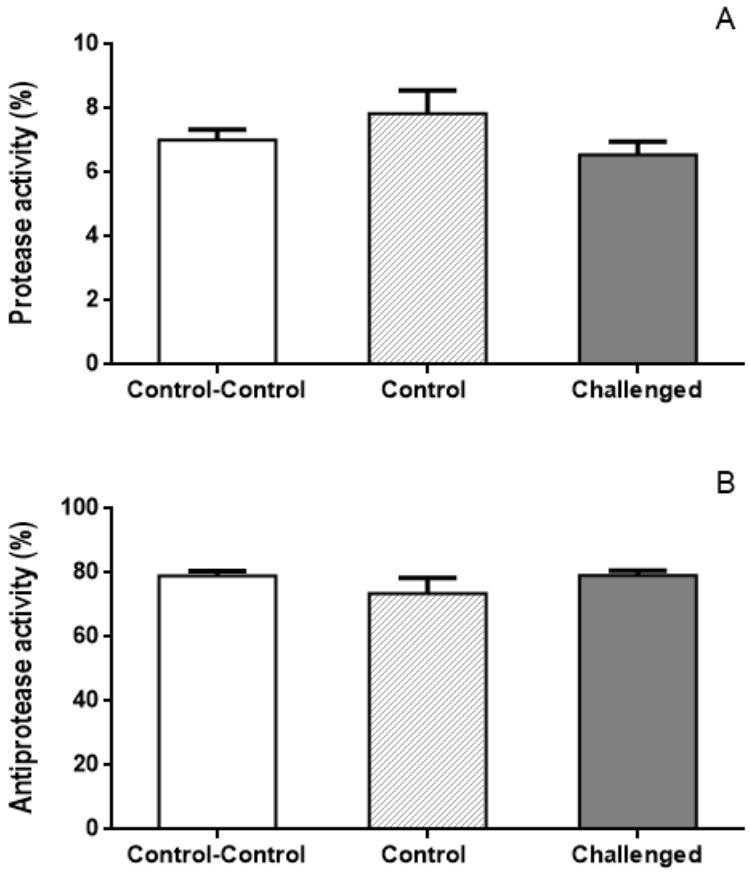


Figure 24. Protease (%) (A) and antiprotease (%) (B) activities in plasma samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12).

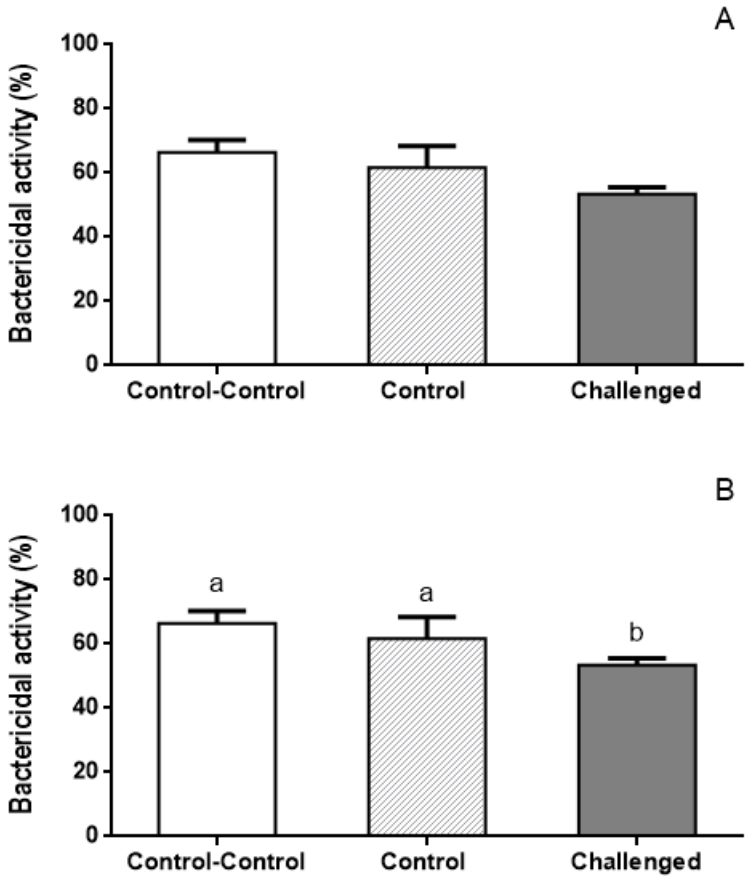


Figure 25. Bactericidal activity (%) against *Photobacterium damselae* subsp. *piscicida* (A) and *Vibrio anguillarum* (B) in plasma samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12). Different letters denote significant differences between experimental groups (ANOVA; $p<0.05$).

6.2.2. Immune parameters and bactericidal activity in skin mucus from ocular and blind sites

No variations were observed in haemolytic complement (Fig. 26A), peroxidase (Fig. 26B), lysozyme (Fig. 26C) and protease (Fig. 27A) activities in ocular skin mucus samples between experimental groups. Contrarily, antiprotease activity was reduced in the ocular skin mucus of challenged fish compared to both control ones (Fig. 27B). A similar pattern was observed in skin mucus from the blind side where no differences were found between experimental groups in none of the activities evaluated (Figs 26 and 27). In addition, no variations were detected in the bactericidal activity against *P. damselae piscicida* (Fig. 28A) and *V. anguillarum* (Fig. 28B) between the experimental groups from ocular and blind skin mucus.

Comparing each experimental group with both origins (ocular and blind), no variations were found in haemolytic complement (Fig. 26A), peroxidase (Fig. 26B) and lysozyme (Fig. 26C) activities in skin mucus from ocular side respect to blind side in none experimental groups. Interestingly, the protease activity was higher in skin mucus from ocular side of all experimental groups compared to blind site one (Fig. 27A), whilst an opposite pattern was observed in the antiprotease activity (Fig. 27B), which was lower in ocular skin mucus respect to values found in skin mucus from blind side of all experimental fish. Regarding bactericidal activity, whilst no variations were observed in bactericidal activity against *P. damselae piscicida*, this activity against *V. anguillarum* was lower in skin mucus from ocular side compared to blind one.

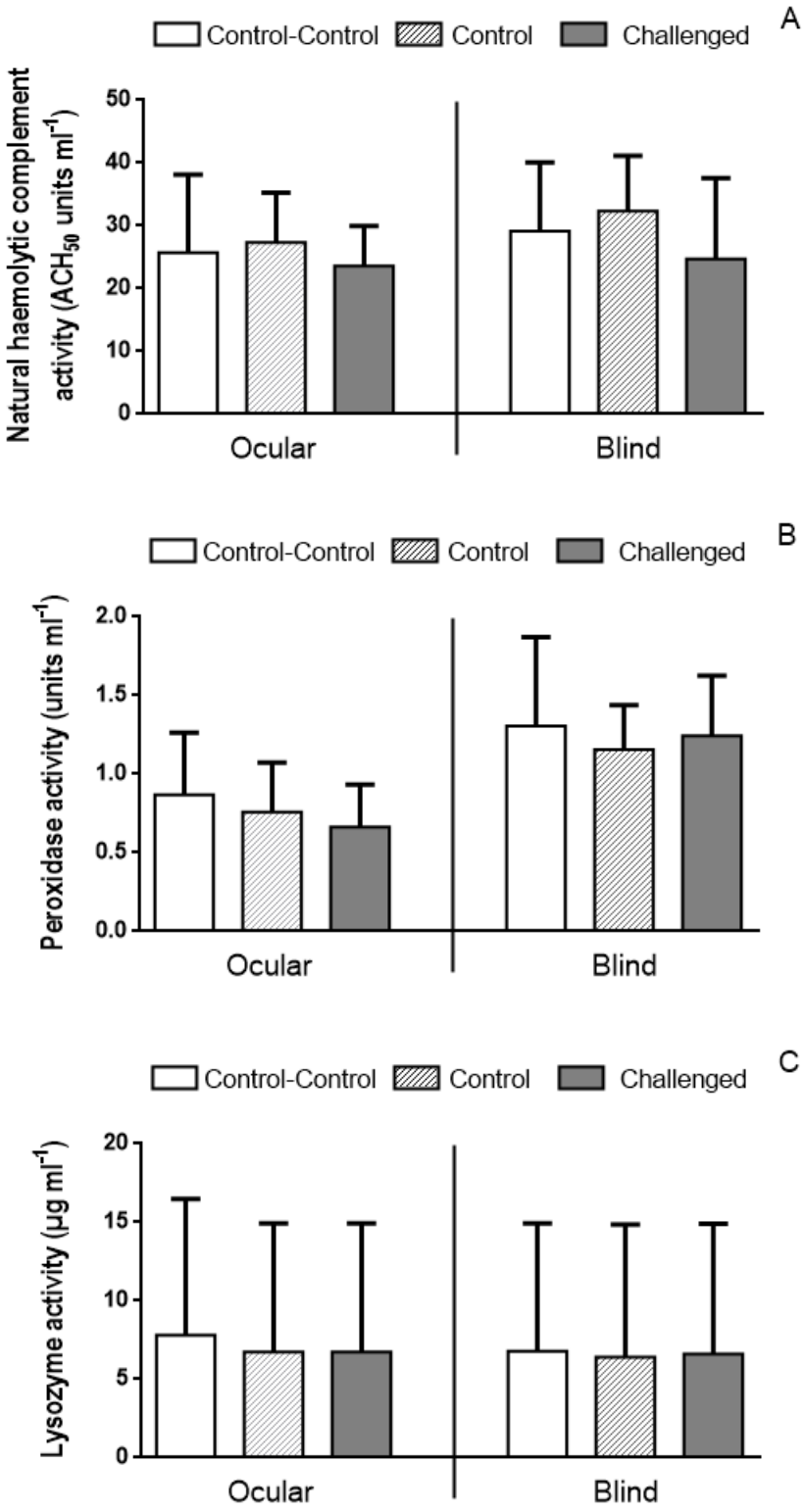


Figure 26. Natural haemolytic complement (ACH_{50} units ml^{-1}) (A), peroxidase (units ml^{-1}) (B) and lysozyme (µg ml^{-1}) (C) activities in ocular and blind skin mucus samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12).

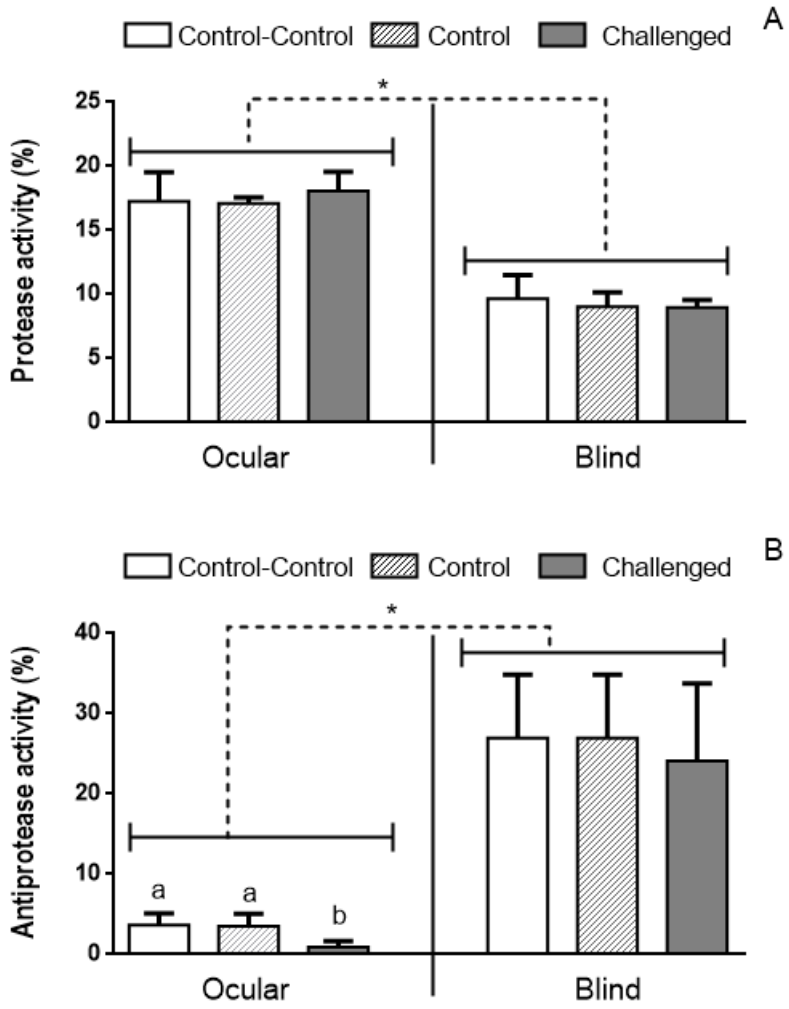


Figure 27. Protease (%) (A) and antiprotease (%) (B) activities in ocular and blind skin mucus samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12). Different letters denote significant variations between experimental groups (ANOVA; $p<0.05$) whilst asterisks denote significant differences between ocular and blind skin mucus of each experimental group (T-test; $p<0.05$).

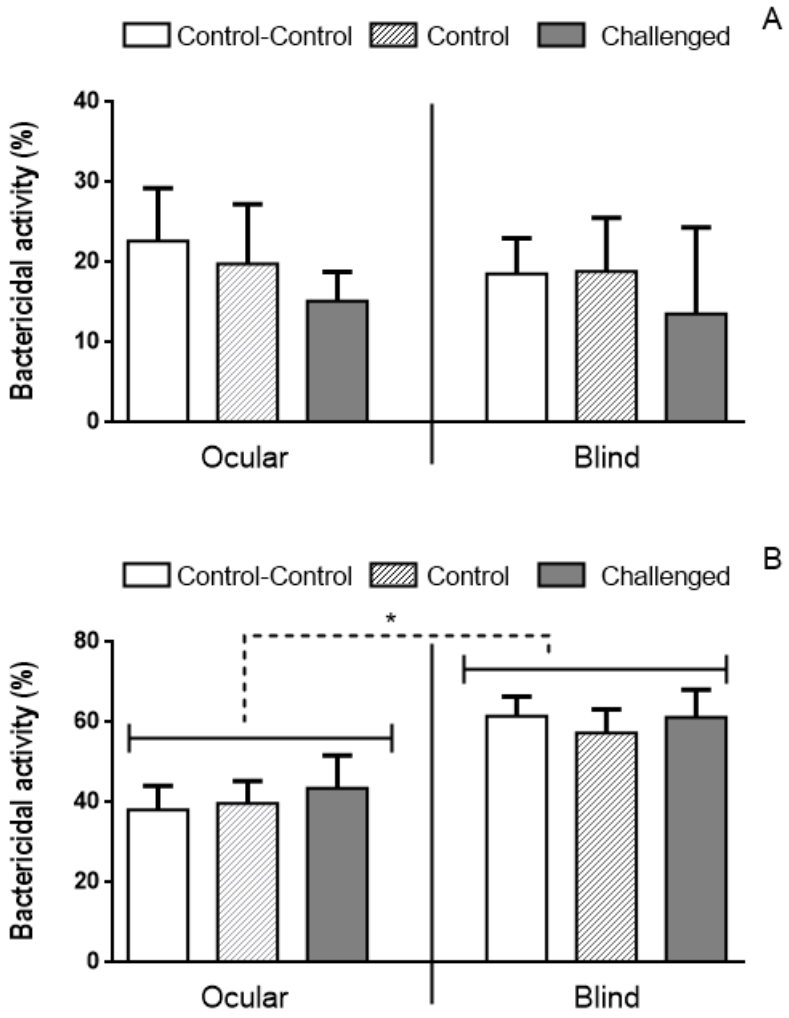


Figure 28. Bactericidal activity (%) against *Photobacterium damselae* subsp. *piscicida* (A) and *Vibrio anguillarum* (B) in ocular and blind skin mucus samples of Senegalese sole control-control, control and challenged with *Tenacibaculum maritimum* during 24 hours at 4 weeks. Bars represent the means ± SE (n=12). Asterisk denotes significant differences between ocular and blind skin mucus of each experimental group (T-test; $p<0.05$).

6.2.3. Disease resistance

After 5 weeks of challenge with *T. maritimum*, a second challenged were carry out on one of control group (control) and the previous challenged group whilst the other control group (control-control) was challenged under the same conditions with sterile MB (instead of bacteria) and served as control of the second challenge.

The mortality of Senegalese soles after the second experimental challenge was assessed for 12 days post-inoculation and survival rates were calculated (Fig. 29). The results showed no mortality in fish from control group. Contrarily, the group challenged for the first time (control-challenged) suffered mortalities after 4 and 7 days post-challenge, reaching a 50 % survival rate at the end of experiment. However, the survival rate was around 80 % in fish from group challenged for the second time, showing mortalities after 10 and 12 days of challenge. Interestingly, significant differences were found between the control and the challenged groups respect to the control-challenged group.

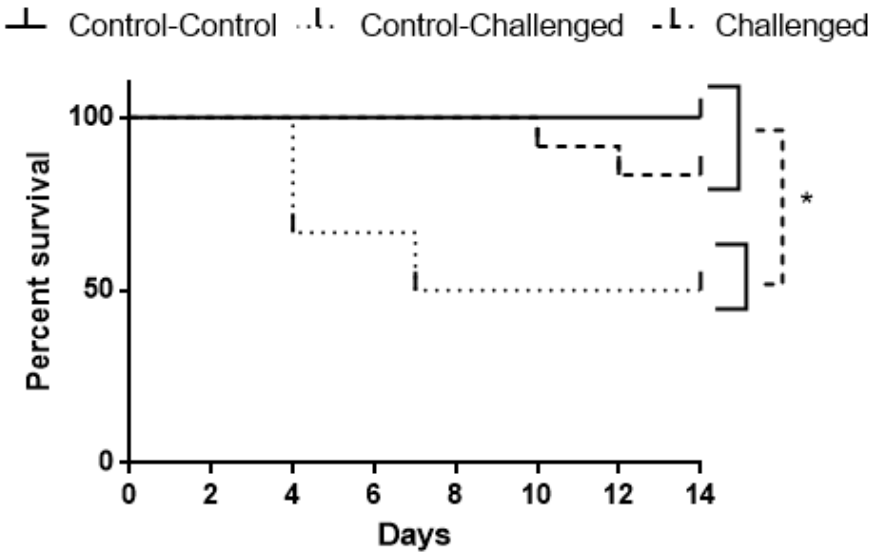


Figure 29. Survival curves of Senegalese sole control-control, control (challenged) and challenged (2nd challenge) with *T. maritimum* (2.7 x 106 CFU ml-1) during 24 hours after 5 weeks from the first infection (2.7 x 105 CFU ml-1). Curves represent the survival percentages during 14 days (n=12). Asterisk denotes significant differences between experimental groups (Log-rank Mantel-Cox test; $p<0.05$).

7. Discussion

Fish skin surfaces are in constant interaction with a wide range of pathogenic microorganisms present in the aquatic environment and consequently, they are provided with a highly effective physical, chemical and biological barrier (Shephard, 1994; Subramanian *et al,* 2007). Due to this localization, skin mucus represents an interface between the environment and the interior milieu, making it of vital importance for aquatic animals (Benhamed *et al.*, 2014). To date, several physico-chemical and biological parameters of skin mucus were already determined in many fish-related aquaculture species, such us gilthead seabream, European seabass, rainbow trout or Atlantic salmon (Fast *et al.,* 2002; Subramanian *et al.,* 2007; 2008; Guardiola *et al.,* 2014). It is likely that the variation in these innate immune factors has different influences on the response of each species to disease processes (Fast *et al.,* 2002). However, few studies have reported data about these parameters in skin mucus of Senegalese sole (Mabrok *et al.,* 2016; Guardiola *et al.,* 2017), thus it is necessary to gather more knowledge about the biology and function of this defensive barrier in Senegalese sole, one of the most valuable flatfish species in Southern Europe.

In the last years, many studies have revealed that the skin mucus of several fish species has strong anti-bacterial and/or bacteriostatic activities against a broad range of microbial pathogens (Kanno *et al.* 1989, Fouz *et al.* 1990, Magariños *et al.* 1995, Fast *et al.* 2002, Guardiola *et al.*, 2015b). However, despite the important role of skin mucus in fish defense, the knowledge of the mucosal immune response during the bacterial infection process or after bacterial challenge remains limited. For instance, the results after the challenge of thirteen marine fish species with ten different bacteria showed that the most active skin mucus against Gram-positive bacteria was extracted from brill (*S. rhombus*) and common sole (*S. solea*) whilst the most active skin mucus against Gram-negative bacteria were Ballan wrasse *(Labrus bergylta)* and common sole, being most of them flatfish species (Hellio *et al.*, 2002). Considering that the first report about bath challenge with *T. maritimum* in Senegalese sole was reported recently by Mabrok *et al.* (2016), there is a great lack of knowledge about the properties of skin mucus in flatfish species, especially in Senegalese sole after bacterial challenge against one of its main pathogens.

Despite the impressive gaps concerning *T. maritimum* route of entry, it is suggested that the primary infection site of the pathogen could be the body surface of fish species (Magariños *et al.,* 1995; Avendaño-Herrera *et al.,* 2006; Mabrok *et al.,* 2016). Interestingly, no comparative studies have been reported between skin mucus from both sides of flatfish after a bacterial challenge. To the best of our knowledge, this is the first study presenting data regarding differences in the skin mucus immune defense between

the ocular and blind sides of the flatfish Senegalese sole against *T. maritimum*. Therefore, the present study intended to bring some insight regarding systemic (plasma) and local (ocular and blind skin mucus) immune responses in Senegalese sole after a bath challenge with *T. maritimum*.

7.1. Experiment 1

In this first study, the haematological profile and several immune-related parameters in plasma and skin mucus of Senegalese sole were determined after bacterial bath-challenge to evaluate the systemic and local immune responses in both fish sides.

7.1.1. Haematological profile after T. maritimum challenge

The involvement of neutrophils and macrophages in phagocytosis, killing and degradation of invading microorganisms, as well as different patterns of localization and mobilization into the infected areas is well documented in fish (Steinhagen & Jendrysek 1994; Afonso *et al.* 1998; do Vale *et al.,* 2002). However, in our study, the dose administered of *T. maritimum* (2.7 x 10^5 CFU ml^{-1}) did not change the haematological profile of Senegalese sole, neither the relative proportion and absolute numbers of the different leucocyte types in any of the experimental times. These data are in line with the findings found in Mabrok *et al.* (2016), where changes at haematological level occurred during the first 7 days of challenge and in our case the first sampling was at 1 week, so probably it was not possible to determine these changes. In addition, in Mabrok's experience, peripheral lymphocyte and circulating thrombocyte number in challenged fish remained similar to control individuals until 14 days of challenge at the same conditions as in our experience. This fact could indicate that the first steps in the defence against infection do not translate into a leukocyte migration from the first week of infection, when our first sampling was made. However, they found a significant lymphocytosis in bath challenged specimens which could be attributed to the migration of lymphocyte to the tissues at the end of the trial (Mabrok*,* 2016).

Costas *et al.,* (2013) determined that plasma lysozyme and peroxidase contents correlated well with the increase in peripheral neutrophils. These phagocytes are thought to be the source of plasma lysozyme (Murray & Fletcher, 1976) and peroxidase (Ellis, 1999), and increases in lysozyme and peroxidase levels have been associated with increases in neutrophil numbers (Muona & Soivio 1992; Cerezuela *et al.* 2016). This

correlation was also found in kelp grouper (*Epinephelus bruneus)* against *Vibrio carchariae,* where the activation of the phagocytic cell population had also produced an increased in the serum lysozyme, bactericidal and haemolytic complement activities (Kim *et al.,* 2011). These results were in line with our findings since no significant differences neither in the number of neutrophils nor in the plasma lysozyme and peroxidase activity between the unchallenged and challenged fish were observed but it could be due to the lack of information at the initial phases of the bath-challenge.

7.1.2. Immune response at systemic level after T. maritimum challenge

In general, the present study showed no differences at the systemic level (blood) between unchallenged and bath challenged Senegalese sole. Indeed, other humoral factors beyond lysozyme and peroxidase activities, such as protease, antiprotease and haemolytic complement activities, followed a similar trend and no changes were observed between control and challenged fish. Interestingly, the haemolytic complement activity in plasma of unchallenged fish presented similar values than to the challenged ones, suggesting that the complement system has no major role against *T. maritimum*, at least through the alternative pathway. As it was described previously in Mabrok *et al.* (2016), the resistance of *T. maritimum* to the defence of the alternative complement system may be attributed to the presence of a lipopolysaccharide compound (LPS O-chain) which enhances biofilm formation and seems to be unique for *T. maritimum* (Vinogradov *et al.* 2003). This fact is due to that contains an unusual linkage ([R]-2-hydroxyglutaric acid residue). In another study, Wiklund and Dalsgaard (2002) reported that both virulent and non-virulent strains of *Flabobacterium psychrophilum* can resist alternative complement activity in serum of rainbow trout. However, *T. maritimum* resistance could also be related to certain evolving strategies to withstand fish innate immunity as recorded in many other Gram-negative pathogens (Rooijakkers and Strijp, 2007). This fact could explain that the parameters studied did not present any variation at systemic level after the *T. maritimum* challenge. However, these studies developed in Mabrok's phD thesis (2016), in vitro plasma was compared with complement activity and without activity (heat-inactivated). Future works with some references as in that work would help to clarify if the systemic response does not exist due to strategies of evasion of the pathogen or other factors interfere as pathogen-host interactions, different dispersion of the bacteria during the bath or interaction of the bacteria with the microbiota of the water system.

At the same time, the present study showed an increase in the bactericidal activity against *V. anguillarum* in plasma of the fish challenged with *T. maritimum*. Contrarily, plasma incubated with *P. damselae piscicida* did not present any reaction after the challenge with *T. maritimum*. These results suggest that the systemic immune response of Senegalese sole after the bath challenge is more effective against *V. anguillarum* than against *P. damselae piscicida*. Similarly, plasma of gilthead seabream presented more bactericidal activity against bacteria of the *Vibrio* genus (*V. harveyi*) than against *P. damselae piscicida* and proposed that the bacteria virulence of *Vibrio* genus depend on the site of the wound whilst *P. damselae piscicida* was not-dependent (Ceballos-Francisco *et al.*, 2018). Other studies also suggested that a possible portal of entry for *T. maritimum* and *Vibrio alginolyticus* into the fish body could be the skin while the pathways of entry of *P. damselae piscicida* may vary depending on the host so that some fish can be infected by ingestion of the pathogen (Benhamed *et al.,* 2014). This could explain why Senegalese sole could present a more effective response against *Vibrio anguillarum*, since its entry is more localized.

7.1.3. Immune response at local level (skin mucus) after T. maritimum challenge

In agreement with the data observed in plasma, the skin mucus of Senegalese sole also showed some significant differences between the unchallenged and the bath challenged fish respect to bactericidal activity against the pathogenic bacteria tested. In fact, the bactericidal activity against *V. anguillarum* also increased in ocular skin mucus of challenged fish after 2 weeks post-challenge whilst the increases in bactericidal activity against *P. damselae piscicida* were only observed at 3 weeks. This different capacity to react against *T. maritimum* could be explained in other studies that observed that *P. damselae piscicida* showed lower adhesive rates to Senegalese sole skin mucus than other bacterial pathogens, such as *Vibrio anguillarum, Flavobacterium psychrophilum* or *Aeromonas salmonicida* (Nikoskelainen *et al.,* 2001; Chabrillón *et al.,* 2005).

The fish skin mucus seems to have an important selective role to discriminate between bacterial strains and it plays a critical role in the defence mechanisms of fish by also acting as a biological barrier (Benhamed *et al.,* 2014). *Vibrio anguillarum* is able to adhere preferentially to fish integument, modifying the thickness, quality, and secretory pattern of skin immune defences (Hickey & Lee, 2017). This fact may influence the mucous adherence and may destabilize the host–microbiota interaction in favour of opportunistic pathogens as *T. maritimum* (Rombout *et al.,* 2011). Therefore, as we

mentioned above, *T. maritimum* and *Vibrio spp.* could present similar ways to enter into the fish body through the skin while the portal of entry of *P. damselae piscicida* may vary depending on the host (Toranzo & Barja, 1993; Magariños *et al.,* 1995; Benhamed *et al.,* 2014; Mabrok *et al.,* 2016).

Recently, Guardiola *et al.* (2017) showed that the bactericidal activity of Senegalese sole skin mucus (from a mix of ocular and blind sides) had a bactericidal capacity around to 25% to kill *P. damselae*, followed by *V. anguillarum* (15%) and finally *V. harveyi* (10%) (In decreasing order of bactericidal capacity). However, our results showed that the bactericidal activity against *V. anguillarum* and *P. damselae* were higher in the ocular side (around 40%) than in the blind side (around 20%) in most of the sampling times, independently of experimental group. The differences found in both studies could be due to the origin of mucus (ocular or blind) since Guardiola *et al.* (2017) evaluated the bactericidal activity in the skin mucus of Senegalese sole from a pool of both sides of this flatfish. This fact increases the importance of measuring all the innate parameters separately to discern if there is a difference due to their location in the fish (from ocular or blind sides).

Regarding the immune-related enzymes tested in skin mucus, it has been seen that only the protease activity presented significant variations (in all experimental times) between the ocular and the blind sides in both experimental groups (unchallenged and challenged ones), being higher in ocular than in the blind side. In addition, the values of other parameters measured as haemolytic complement and lysozyme were also higher in ocular side compared to values observed in the blind side but at specific sampling times. By contrast, only peroxidase activity was higher in the blind side than in the ocular one in some experimental times. Independently of time and experimental group variables, our results revealed more activity in all immune-related parameters measured in the ocular side compared to blind one with the exception of peroxidase activity that showed an opposite pattern. These differences could be due to the fact that the ocular part of the flatfish is more exposed to invasion by pathogens than the blind part and the distribution of the immune cells is different. Nevertheless, further studies are needed for this hypothesis to be clarified.

Comparing the immune parameters evaluated between the unchallenged and the challenged fish (independently of the origin of mucus), our results revealed that most of the parameters that varied significantly did it in an increased way in challenged fish. These results are consistent with other studies as Firth *et al.* (2000) or Rajan *et al.* (2013) where skin mucus showed differential expression of different immune-related

components between the challenged and unchallenged fish. Thus, our findings underline the important role of skin mucus, as a biological barrier and a key component of the innate immune system, in the defence mechanisms of the fishes against bacterial challenge with potential application in aquaculture. These results could be of great relevance for future studies related to bacteria diseases in flatfish and more specifically on Tenacibaculosis, one of the most dangerous bacterial diseases in Senegalese sole culture. In addition, as were reported by Guardiola *et al.* (2018), the method of collecting skin mucus is simple, fast and of low invasiveness than other sample methods. Already in some studies with other flatfish species as in Hellio *et al.* (2002) or Loganathan *et al.* (2011), they tried not collect on the ventral side to avoid intestinal and sperm contamination. For all these reasons, these differences could facilitate more efficient sampling in future studies with this species, could help us to understand how the innate immune system works in Senegalese sole and perhaps this knowledge could be extrapolated to other flatfish species.

7.1.4. Immune response at systemic level (plasma) versus local level (skin mucus) after T. maritimum challenge

Comparing the activity of parameters measured in plasma and skin mucus, more variations were found in skin mucus samples after the bath challenge than in plasma. As in the plasma samples, no variations were found after the challenge in lysozyme and protease activities in skin mucus of Senegalese sole at our sampling times. This fact may mean that these parameters are not activated at the systemic and local level by the dose of *T. maritimum* tested. However, as it was said before, other factors could interfere in these results as pathogen-host interactions, different dispersion of the bacteria during the bath or interaction of *T. maritimum* with the microbiota present in the water. Interestingly, haemolytic complement activities increased after 2 weeks of challenge which could suggest the important role of these in the bacterial fight. Peroxidase is an important microbicidal agent that maintains the redox balance of the immune system and it is tempting to consider that peroxidase in skin mucus is essential for mucosal immunity and skin defence (Guardiola *et al.,* 2014). To our knowledge, there are very few studies in which the activity of peroxidase in fish skin mucus was measured after a bacterial challenge. Contrarily to our results, peroxidase activity in skin mucus of the Atlantic salmon challenged with a pathogenic strain of *Aeromonas salmonicida* significantly decreased at day 4 and 6, indicating the decreased lytic capacity of the challenged fish (Du *et al.,* 2015). In addition, Guardiola *et al.* (2016) showed that peroxidase levels

present in the skin mucus of gilthead seabream were stable during and after exposure to stress conditions while were significantly altered in gilthead seabream serum after some stressors.

These studies and a previous PhD Thesis carried out in our research group (Mabrok, 2016) seem to suggest the importance of time in the mucosal immune response. Mabrok (2016) reported a delay in the mucosal immune response compared to that found at systemic level (i.e. blood and plasma) in Senegalese sole challenged with *T. maritimum*. More concretely, a significant increase in skin mucus lysozyme, complement, protease and antiprotease activities were observed at the end of the experiment (14 days post-challenge) suggesting that late response is mainly due to phagocytes recruitment to mucosal surfaces against this pathogen.

7.2. Experiment 2

In a second study, all the immune-related parameters studied in the previous experiment were also determined in plasma and skin mucus of Senegalese sole after 4 weeks of challenge and before a second bacterial challenge with a lethal dose of *T. maritimum* (ten times higher than in the first challenge) in order to evaluate the disease resistance. All these parameters are present in fish mucus and plasma and have already been used as indicators for disease induction in other studies (Magnadóttir *et al.,* 2006). Our results did not show differences between lysozyme, protease and antiprotease activities in plasma of unchallenged or bath challenged fish. Only, peroxidase activity appeared in higher levels in plasma of fish challenged after 4 weeks of challenge with *T. maritimum* compared to control groups. Contrarily, whilst the bactericidal activity against *V. anguillarum* was reduced in challenged fish, the activity against *P. damselae piscicida* did not suffer variations.

In the same line, no variations were detected in haemolytic complement, peroxidase, lysozyme and protease activities measured in skin mucus (independently of the origin of mucus) between experimental groups. However, the antiprotease activity showed a significant decrease in ocular skin mucus of bath challenged fish respect to the other experimental groups. The decrease of antiprotease activity at 4 weeks of challenge disagreed with the experiment 1, where this activity did not show any variations during three weeks after the bacterial challenge. Interestingly, the results of protease activity in skin mucus are in agreement with those of previous experiment (Experiment 1) where this activity was higher in ocular side compared to values found in blind part. However,

the antiprotease and bactericidal (against *V. anguillarum*) activities showed an opposite pattern being higher in blind side than ocular one, although no variations were found in any of the activities in blind skin mucus between unchallenged and bath challenged fish.

In general, the bacterial challenge revealed that both skin mucus and plasma samples do not seem to present a clear answer against *T. maritimum*, suggesting that Senegalese sole does not contain adequate compounds, either local (i.e. skin mucus) or systemically (i.e. plasma), with potent bactericidal activity to act against the pathogen or could even indicate possible evasion strategies of the bacteria. However, it would be interesting to determine if the plasma and skin mucus have bacteriostatic activity that can inhibit the growth of *T. maritimum*.

These results are in line with those previously reported by Magariños *et al.* (1995), who stated that *T. maritimum* has the ability to strongly attach to the external body surface of turbot, gilthead seabream and European seabass thus overcoming the skin mucus antimicrobial activities. However, when a lethal dose of *T. maritimum* was administered in a second bath challenge, lower mortality rates were observed in this group. In fact, the survival rate in unchallenged and bath challenged fish for a second time was significantly higher compared to fish challenged for the first time. The fish challenged twice with *T. maritimum* had around an 80% of survival rate while the group challenged only one time with the bacteria presented around a 50% of survival. This may suggest that the Senegalese sole could have activated an acquired immune response derived from the first contact with the pathogen turning the fish more resistant than the group that had not come into contact with the bacteria before. Little is known about immunization in flatfish species. However, after a first approach in Senegalese sole, it seems that the fish that survived to the first challenge developed an acquired response with antibodies and competent T cells. By remembering, in the second challenge they survived more. This means that vaccination will be a good prophylactic strategy against tenacibaculosis, but more studies are required to affirm this fact.

Regarding immunization by vaccination, there is nowadays a commercially available bacterin to prevent the disease caused by *T. maritimum* in turbot (Icthiovac TM®), which is applied by bath in fish from 1 to 2 g followed by a booster injection in fish from 20 to 30 g (Avendaño- Herrera *et al.* 2006). Although no licensed commercial vaccines are yet available for sole family, auto-vaccines made using the strains isolated from the farms can also be used in these species. Therefore, more investigations are required to define the role of systemic and mucosal immune response against *T. maritimum* to understand their infection mechanisms and assist future studies to increase the vaccine.

8. Conclusions

In summary, the present study provides detailed information about immune response after the challenge with *T. maritimum* in Senegalese sole, a high-value flatfish that presents a great potential for future farming at commercial scale. Our results revealed that all the studied innate immune-related molecules were constitutively present in both skin mucus sides and seem to play a different role in the mucosal immune response. This could indicate that immune response is always alert and fish resistance is not limited to only one factor. In our study, fish were bath challenged with *T. maritimum* in order to evaluate the skin mucus role from ocular and blind sides due to their condition of flatfish. All the innate immune-related enzymes were constitutively present in both sides but in different levels. Interestingly, the activities measured were higher on the ocular side than on the blind side, possibly due to the high exposure to invasion by pathogens on this side. Nonetheless, further studies should be performed to deepen in the knowledge of the fish skin mucus molecules and their precise role in the mucosal immunity. Our results showed that there is a response to the first contact with *T. maritimum* and that makes the Senegalese sole more resistant to a new contact with the pathogen. The present study opened a new window of research in comparative mucosal immunity and further studies should be performed to deepen our knowledge in fish mucosal immunity. Thus, more studies would be required to improve knowledge of the biology and function of this essential barrier in Senegalese sole and in other flatfish species, against one of their main pathogens (*T. maritimum*), which could have important applications for fish farmers.

9. References

Abelli, L., Coscia, M. R., De Santis, A., Zeni, C. & Oreste, U. (2005). Evidence for hepato-biliary transport of immunoglobulin in the antarctic teleost fish *Trematomus bernacchii*. *Developmental and Comparative Immunology*, Vol. 29, pp. 431-442.

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. *Diseases of Aquatic Organisms*, Vol. 34, pp. 27–37.

Ai-Jun, M., Zhi-hui, H. & Xin-An, W. (2013). Changes in protein composition of epidermal mucus in turbot *Scophthalmus maximus* (L.) under high water temperature. *Fish physiology and biochemistry*, Vol. 39, pp. 1411-1418.

Akimenko, M., Marí-Beffa, M., Becerra, J. & Géraudie, J. (2003). Old questions, new tools, and some answers to the mystery of fin regeneration. *Developmental Dynamics*, Vol. 226, pp. 190-201.

Alexander, J. &. (1992). *Non-cellular non-specific mechanisms in fish. Annual Review of Fish Diseases*, Vol. 2, pp. 249-279.

Al-Hassan, J., Dyson, M., Young, S., Thompson, M. & Criddle, R. (1991). Acceleration of wound healing responses induced by preparations from the epidermal secretions of the Arabian Gulf catfish (*Arius bilineatus*, Valenciennes). *Jornal of Wilderness Medicine*, Vol. 2, pp. 153-163.

Ali, M., Thomson, M. & Al-Hassan, J. E. (1989). Comparative biochemical and pharmacological properties of epidermal secretions from Ariid catfish of the Arabian Gulf. *Comparative biochemistry and physiology*, Vol. 92, nº1, pp. 205-2011.

Alonso, M. C., Cano, I., Garcia-Rosado, E., Castro, D., Lamas, J., Barja, J. L. & Borrego, J. J. (2005). Isolation of lymphocystis disease virus from sole, *Solea senegalensis* Kaup, and blackspot sea bream, *Pagellus bogaraveo* (Brünnich). *Journal of Fish Diseases,* Vol. 28, pp. 221-228.

APROMAR (2017). La Acuicultura en España 2017. *Asociación Empresarial de Productores de Cultivos Marinos de España (APROMAR) y la Asociación Española de Productores de Acuicultura Continental (ESCUA)* (www.apromar.es; www. esacua.com).

Austin, B. (1983). Vaccine for the control of vibriosis in eels. *Veterinary Record*, Vol. 113, pp. 394-395.

Austin, B. (2006). The bacterial microflora of fish. *Scientific World Journal*, Vol. 6, pp. 931-945.

Austin, B., Austin, D. A., Austin, B. & Austin, D. A. (2012). *Bacterial fish pathogens*, pp, 652. Heidelberg, Germany: Springer.

Avendaño-Herrera, R., Toranzo, A. E. & Magariños, B. (2006). Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Diseases of aquatic organisms*, Vol. 71, pp. 255-266.

Bader, J. A. & Shotts Jr, E. B. (1998). Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. *Journal of Aquatic Animal Health*, Vol. 10, pp. 311-319.

Barman, D., Nen, P., Mandal, S.C., Kumar, V. (2013). Immunostimulants for aquaculture health management. *Journal of Marine Science: Research and Development*, Vol. 3, pp. 1–11.

Barroso, C., Ozorio, R. O., Afonso, A., Moraes, J. R. & Costas, B. (2016). Immune responses and gut morphology in Senegalese sole (*Solea senegalensis*) fed dietary probiotic supplementation and following exposure to *Photobacterium damselae* subsp. *piscicida*. *Aquaculture research*, Vol. 47, pp. 951-960.

Barton, B. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology Journal*, Vol. 42, pp. 517-525.

Baxa, D. V., Groff, J. M., Wishkovsky, A. & Hedrick, R. P. (1990). Susceptibility of nonictalurid fishes to experimental infection with *Edwardsiella ictaluri*. *Diseases of Aquatic Organisms*, Vol. 8, pp.113-117.

Baynes, S. M. & Howell, B. R. (1993). Observations on the growth, survival and disease resistance of juvenile common sole, *Solea solea* (L.), fed *Mytilus edulis* L. *Aquaculture Research,* Vol. 24, pp. 95-100.

Benhamed, S., Guardiola, F. A., Mars, M. & Esteban, M. Á. (2014). Pathogen bacteria adhesion to skin mucus of fishes. *Veterinary microbiology*, Vol. 171, pp. 1-12.

Bergsson, G., Agerberth, B., Jörnvall, H. & Gudmundsson, G. (2005). Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). *FEBS Journal*, Vol. 272, pp. 4960-4969.

Bernardet, J. F., Campbell, A. C. & Buswell, J. A. (1990). *Flexibacter maritimus* is the agent of 'black patch necrosis' in Dover sole in Scotland. *Diseases of Aquatic Organisms,* Vol. 8, pp. 233-237.

Bjørndal, T., Guillen, J. & Imsland, A. (2016). The potential of aquaculture sole production in Europe: production costs and markets. *Aquaculture economics & management*, Vol. 20, pp. 109-129.

Björnsdóttir, B., Gudmundsdóttir, S., Bambir, S. H., Magnadóttir, B. & Gudmundsdóttir, B. K. (2004). Experimental infection of turbot, *Scophthalmus maximus* (L.), by *Moritella viscosa*, vaccination effort and vaccine-induced side-effects. *Journal of Fish Diseases,* Vol. 27, pp. 645-655.

Blackstock, N. &. (1982). Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and Fry stages of the brown trout Salmo-Trutta. *Journal of Zoology*, Vol. 197, pp. 463-471.

Boltaña, S., Reyes-Lopez, F., Morera, D., Goetz, F. & MacKenzie, S. A. (2011). Divergent responses to peptidoglycans derived from different *E. coli* serotypes influence inflammatory outcome in trout, *Oncorhynchus mykiss*, macrophages. *BMC genomics*, Vol. 12, pp. 34.

Bricknell, I., Dalmo, R.A. (2005). The use of immunostimulants in fish larval aquaculture. *Fish Shellfish Immunology*. Vol. 19, pp. 457–472.

Bullock, A. & Roberts, R. (1974). The dermatology of marine teleost fish. The normal integument. *Oceanography and Marine Biology*, Vol. 13, pp. 383-411.

Cammarata, M., Parisl, M. & Vasta, G. (2016). Evolution and immune function of fish lectines. In: L. Ballarin, & M. Cammarata, *Lessons in Immunity: From single-cell organism to mammals*, pp. 239-256. Elsevier.

Cano, I., Valverde, E. J., Lopez-Jimena, B., Alonso, M. C., Garcia-Rosado, E., Sarasquete, C. & Castro, D. (2010). A new genotype of Lymphocystivirus isolated from cultured gilthead seabream, *Sparus aurata* L., and Senegalese sole, *Solea senegalensis* (Kaup). *Journal of fish diseases*, Vol. 33, pp. 695-700.

Castro, N., Toranzo, A. E., Devesa, S., González, A., Nuñez, S. & Magariños, B. (2012). First description of *Edwardsiella tarda* in Senegalese sole, *Solea senegalensis* (Kaup). *Journal of fish diseases*, Vol. 35, pp. 79-82.

Castro, R. & Tafalla, C. (2015). Overview of fish immunity. In &. Beck, E.B. *Mucosal Health in Aquaculture,* pp. 3-34. Elsevier.

Ceballos-Francisco, D., Guardiola, F. A., Cordero, H., Cuesta, A. & Esteban, M. Á. (2018). Humoral immune parameters in serum of gilthead seabream (*Sparus aurata* L.) after induced skin injury. *Fish & shellfish immunology*, Vol. 75, pp. 291-294.

Cepeda, C. & Santos, Y. (2002). First isolation of *Flexibacter maritimus* from farmed Senegalese sole (*Solea senegalensis*, Kaup) in Spain. *Bulletin-european association of fish pathologists*, Vol. 22, pp. 388-392.

Cerezuela, R., Guardiola, F. A., Cuesta, A. & Esteban, M. Á. (2016). Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: effects on skin mucosal immunity. *Fish & shellfish immunology*, Vol. 9, pp. 100-109.

Chabrillón, M., Rico, R., Balebona, M. & Moriñigo, M. (2005a). Adhesion to sole, *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damselae* subsp. piscicida. *Jornal of Fish Diseases*, Vol. 28, pp. 229-237.

Chabrillón, M., Rico, R., Arijo, S., Días-Rosales, P., Balebona, M. & Moriñigo, M. (2005b). Interactions of microorganisms isolated from gilthead sea bream, *Sparus aurata* L., on *Vibrio harveyi*, a pathogen of farmed Senegalese sole, *Solea senegalensis* (Kaup). *Journal of Fish Diseases*, Vol. 28, pp. 531-537.

Cheesman, S. & Guillemin, K. (2007). We know you are in there: conversing with the indigenous gut microbiota. *Research in Microbiology*, Vol. 158, pp. 2-9.

Cheesman, S. E. & Guillemin, K. (2007). We know you are in there: conversing with the indigenous gut microbiota. *Research in microbiology*, Vol.158, pp. 2-9.

Cho, H., Park, I., Kim, H., Lee, W., Kim, M. & Kim, S. (2002). Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *The FASEB Journal*, Vol. 16, pp. 429-431.

Cone, R. (2009). *Barrier propierties of mucus.* Advanced Drug Delivery Reviers, vol. 61, pp. 75-85.

Constenla, M. & Padrós, F. (2010). Histopathological and ultrastructural studies on a novel pathological condition in *Solea senegalensis. Diseases of aquatic organisms*, Vol. 90, pp. 191-196.

Constenla, M., Padrós, F. & Palenzuela, O. (2014). *Endolimax piscium* sp. nov.(*Amoebozoa*), causative agent of systemic granulomatous disease of cultured sole, *Solea senegalensis* Kaup. *Journal of fish diseases,* Vol. 37, pp. 229-240.

Cordero, H., Brinchmann, M. F., Cuesta, A., Meseguer, J. & Esteban, M. A. (2015). Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics*, Vol. 15, pp. 4007-4020.

Costas, B., Conceição, L. E., Dias, J., Novoa, B., Figueras, A. & Afonso, A. (2011). Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). *Fish & shellfish immunology*, Vol. 31, pp. 838-847.

Costas, B., Rêgo, P. C. N. P., 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*, Vol. 36, pp. 543-553.

Cutrin, J. M., Dopazo, C. P., Thiery, R., Leao, P., Olveira, J. G., Barja, J. L. & Bandin, I. (2007). Emergence of pathogenic betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian Peninsula. *Journal of fish diseases,* Vol. 30, pp. 225-232.

Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbomel, P. & Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity*, Vol. 17, pp. 693-702.

Delamare-Deboutteville, J., Wood, D. & Barnes, A. (2006). Response and function of cutaneous mucosal and serum antibodies in barramund (*Lates calcarifer*) acclimated in seawater and freshwater. *Fish and Shellfish Immunology*, Vol. 21, pp. 92-101.

Dinis, M., Ribeiro, L., Soares, F. & Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture*, Vol. 176, pp. 27–38.

do Vale A., Afonso A. & Silva M.T. (2002) The professional phagocytes of sea bass (*Dicentrarchus labrax* L.): cytochemical characterisation of neutrophils and macrophages in the normal and inflamed peritoneal cavity. *Fish and Shellfish Immunology*, Vol. 13, pp. 183–198.

Drennan, J., LaPatra, S., Swan, C., Ireland, S. & Cain, K. (2007). Characterization of serum and mucosal antobody responses in white sturgeon (*Acipenser transmontanus* Richardson) following immunization with WSIV and a protein hapten antigen. *Fish and Shellfish Immunology*, Vol. 23, pp.657-669.

Du, Y., Yi, M., Xiao, P., Meng, L., Li, X., Sun, G. & Liu, Y. (2015). The impact of *Aeromonas salmonicida* infection on innate immune parameters of Atlantic salmon (*Salmo salar* L). *Fish & shellfish immunology*, Vol. 44, pp. 307-315.

Elliott, D. (2000). Integumentary system. In: G. OStrander, *The laboratory fish*. Pp. 271-306. California, USA.: Academic press.

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

Esteban, M. (2012). *An overview of the inmunological defenses in fish skin.* ISRB Immunology. Article ID 853470, 2, pp. 29.

Esteban, M. Á. & Cerezuela, R. (2015). Fish mucosal immunity: skin. In *Mucosal Health in Aquaculture*, pp. 67-92.

Ewart, K., Jonhson, S. & Ross, N. (2001). Lectines of the immune system and their relevance to fish health. *Journal of marine science*, Vol. 58, pp. 380-385.

Fagan, M. S., O'Byrne-Ring, N., Ryan, R., Cotter, D., Whelan, K. & Mac Evilly, U. (2003). A biochemical study of mucus lysozyme, proteins and plasma thyroxine of Atlantic salmon (*Salmo salar*) during smoltification. *Aquaculture*, Vol. 222, pp. 287-300.

Falco, A., Ortega-Villaizan, M., Chico, V., Brocal, I., Perez, L., Coll, J. M. & Estepa, A. (2009). Antimicrobial peptides as model molecules for the development of novel antiviral agents in aquaculture. *Mini reviews in medicinal chemistry*, Vol. 9, pp. 1159-1164.

FAO (2018). Cultured Aquatic Species Information Programme: *Solea* spp. (*S. solea, S. senegalensis*). In FAO, *Fisheries and Aquaculture Department [Online].*

FAO. (2016). Aquaculture topics and activities. Aquaculture. In FAO, *Fisheries and Aquaculture Department [Online].*

Fast, M., Sims, D. & Burka, J. M. (2002). Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. *Comparative Biochemistry and Physiology*, Vol 132, pp. 645-657.

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B. & Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of fish diseases*, Vol. 34, pp. 643-661.

FEAP (2014). European Aquaculture Production Report 2005–2014. *Federation of European Aquaculture Producers*, Liege, Belgium.

Firth, K. J., Johnson, S. C. & Ross, N. W. (2000). Characterization of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis)* and in whole-body louse homogenate*. Journal of Parasitology,* Vol. 86, pp. 1199-1205.

Fletcher, T. & Grant, P. (1969). Immunoglobulins in the serum and mucus of the plaice (*Pleuronectes platessa*). *Biochemical Journal*, Vol. 115, pp. 65.

Fletcher, T. & White, A. (1973). Lysozyme activity in the plaice (*Pleuronectes plaessa* L.). *Experientia*, Vol. 29, pp. 1283-1285.

Flik, G., Van Rijs, J. H. & Wendelaar Bonga, S. (1984). Evidence for the presence of calmodulin in fish mucus. *European Journal of Biochemistry*, Vol. 138, pp. 651-654.

Fouz B., Devesa S., Graviningen K., Bajar J. L. & Toranzo A. E. (1990). Antibacterial action of the mucus of turbot. *European Association of Fish Pathologists*, Vol. 10.

Genard, B., Larouche, O., Nicolas, J. L., Miner, P., Beaudin, M. L. & Tremblay, R. (2014). Effect of the probiotic strain *Phaeobacter gallaeciensis* after bacterial challenge on the complete larval development of *Pecten maximus*. *Aquatic Living Resources*, Vol. 27, pp. 27-34.

Genten, F. & Terwinghe, E. &. (2009). *Atlas of fish histology.* Science Publishers, pp. 57-74.

Gomez, D., Sunyer, J.O. & Salinas, I. (2013). The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish & Shellfish Immunology.* Vol. 35, pp. 1729–1739.

Gonzalez, S. F., Chatziandreou, N., Nielsen, M. E., Li, W., Rogers, J., Taylor, R. & Cossins, A. (2007). Cutaneous immune responses in the common carp detected using transcript analysis. *Molecular Immunology*, Vol. 44, pp. 1664-1674.

González-Chávez, S., Arévalo-Gallegos, S. & Rascón-Cruz, Q. (2009). Lactoferrin: structure, function and applications. *International Journal of Antimicrobial Agents*, Vol. 33, pp. 301.e1-301.e8.

Grabowski, L., LaPatra, S. & Cain, K. (2004). Systemic and mucosal antibody responses in tilapia, *Oreochromis* niloticus (L.) followin immunization with *Flavobacterium columnare*. *Journal of Fish Diseases*, Vol. 27, pp. 573-581.

Grinde, B., Jolles, J. & Jolles, P. (1988). Purification and characterization of two lysozymes from rainbow trout (*Salmo gairdneri*). *European Journal of Biochemistry*, Vol. 173, pp. 269-273.

Guardiola, F., Cuesta, A., Arizcun, M., Meseguer, J. & Esteban, M. (2014). Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*). *Fish & Shellfish Immunology* , Vol.36, pp. 545-551.

Guardiola, F., Cuartero, M., Collado-González, M., Arizcún, M., Guillermo Díaz Baños, F., Meseguer, J., Cuesta, A. &. Esteban, M.A. (2015a). Description and comparative study of physico-chemical parameters of the teleost fish skin mucus. *Biorheology* , Vol. 52, pp. 247–256.

Guardiola, F., Cuartero, M., Collado-González, M. M., Díaz-Baños, F., Cuesta, A., Moriñigo, M. A. & Esteban, M. Á. (2015b). Terminal carbohydrate composition, IgM level and enzymatic and bacteriostatic activity of European sea bass (*Dicentrarchus labrax*) skin epidermis extracts. *Fish & Shellfish Immunology*, Vol. 47, 352-359.

Guardiola, F. A., Cuesta, A. & Esteban, M. Á. (2016). Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.*). Fish & shellfish immunology*, Vol. 59, pp. 323-330.

Guardiola, F. A., Cuartero, M., del Mar Collado-González, M., Baños, F. G. D., Cuesta, A., Moriñigo, M. Á. & Esteban, M. Á. (2017). Terminal carbohydrates abundance, immune related enzymes, bactericidal activity and physico-chemical parameters

of the Senegalese sole (*Solea senegalensis*, Kaup) skin mucus. *Fish & shellfish immunology*, Vol. 60, pp. 483-491.

Guardiola, F. A., Saraiva-Fraga, M., Cuesta, A. & Esteban, M. A. (2018). Changes in natural haemolytic complement activity induced by stress in gilthead seabream (*Sparus aurata* L.). *Fish & shellfish immunology,* Vol. 78, pp. 317-321.

Hadden, J. W. & Smith, D. L. (1992). Immunopharmacology: immunomodulation and immunotherapy. *JAMA,* Vol. 268, pp. 2964-2969.

Hansen, J. D., Landis, E. D. & Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences,* Vol. 102, pp. 6919-6924.

Hartley, B. (1960). Proteolytic enzymes. *Annual review of biochemistry*, Vol. 29, pp. 45-72.

Hawkes, J. (1974). The structure of fish skin: General organitation. *Cell and Tissue Research*, Vol. 149, pp. 147-157.

Hellio, C., Pons, A. M., Beaupoil, C., Bourgougnon, N. & Le Gal, Y. (2002). Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. *International Journal of Antimicrobial Agents,* pp. Vol. 20, 214-219.

Hickey, M. E. & Lee, J. L. (2017). A comprehensive review of *Vibrio (Listonella) anguillarum*: ecology, pathology and prevention. *Reviews in Aquaculture.* Vol. 10, pp. 585-610.

Hills, B. (1988). The biology of surfactants. *Cambridge: Cambridge Univ. Press*. pp 408.

Hodneland, K., Garcia, R., Balbuena, J. A., Zarza, C. & Fouz, B. (2011). Real-time RT-PCR detection of betanodavirus in naturally and experimentally infected fish from Spain. *Journal of fish diseases*, Vol. 34, pp. 189-202.

Holland, M. C. H. & Lambris, J. D. (2002). The complement system in teleosts. *Fish & shellfish immunology,* Vol.12, pp. 399-420.

Holmes, B. (1992). Synonymy of *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986 and Cytophaga marina Reichenbach 1989. *International Journal of Systematic and Evolutionary Microbiology*, Vol. 42, pp. 185-185.

Iger, Y. & Abraham, M. (1990). The process of skin healing in experimentally wounded carp. *Journal of Fish Biology*, Vol. 36, pp. 421-437.

Iger, Y. & Abraham, M. (1997). Rodlet cells in the epidermis of fish exposed to stressors. *Tissue and Cell*, Vol. 29, pp. 431-438.

Imsland A.K., Foss A., Conceic¸a˜o L.E.C., Dinis M.T., Delbare D., Schram E., Kamstra A., Rema P. & White P. (2003) A review of the culture potential of *Solea solea* and *Solea senegalensis*. *Reviews in Fish Biology and Fisheries, Vol.* 13, pp. 379–407.

Inami, M., Taverne-Thiele, A. J., Schrøder, M. B., Kiron, V. & Rombout, J. H. (2009). Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.). *Fish & shellfish immunology*, Vol. 26, 751-759.

John D. Hansen, J., Landis, E. & Phillips, R. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences*, Vol. 102, pp. 6919–6924.

Jurado, J., Fuentes-Almagro, C., Francisco Antonio Guardiola, F., Alberto Cuesta, A., Esteban, M. & Prieto-Álamo, M. (2015). Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*). *Journal of Proteomics*, Vol. 120, pp. 21-34.

Kanno T., Nakai T. & Muroga K. (1989). Mode of transmition of vibriosis among ayu *Plecoglossus altivelis*. *Journal of Aquatic Animal Health* Vol.1, pp.2-6.

Karlsen, C., Sorum, H., Willasen, N. & Sbakk, K. (2012). *Moritella viscosa* bypasses Atlantic salmon ephidermal keratocyte clearing activity and might use skin surfaces as port of infection. *Veterinary Microbiology*, Vol. 154, pp. 353-362.

Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Parra, D., Reyes-López, F. E. & Tort, L. (2018). Comparative Immune-and Stress-Related Transcript Response Induced by Air Exposure and *Vibrio anguillarum* Bacterin in Rainbow Trout *(Oncorhynchus mykiss*) and Gilthead Seabream (*Sparus aurata*) mucosal Surfaces. *Frontiers in immunology*, Vol. 9.

Kim, J. S., Harikrishnan, R., Kim, M. C., Jang, I. S., Kim, D. H., Hong, S. H. & Heo, M. S. (2011). Enhancement of Eriobotrya japonica extracts on non-specific immune response and disease resistance in kelp grouper *Epinephelus bruneus* against *Vibrio carchariae*. *Fish & shellfish immunology*, Vol. 31, pp. 1193-1200.

Kovacevic, N., Hagen, M. O., Xie, J. & Belosevic, M. (2015). The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the

goldfish (*Carassius auratus* L.). *Developmental & Comparative Immunology*, Vol. 53, pp. 112-122.

LaPatra, S., Kao, S., Erhardt, E. & Salinas, I. (2015). Evaluation of dual nasal delivery of infectious hematopoietic necrosis virus and enteric red mouth vaccines in rainbow trout (*Oncorhynchus mykiss*). *Vaccine*, Vol. 33, pp. 771-776.

Lazado, C. & Caipang, C. (2014). Mucosal immunity and probiotics in fish. *Fish and Shellfish Immunology*, Vol. 39, pp. 78-89.

Liang, Y., Guan, R., Huang, W. & Xu, T. (2011). Isolation and identification of a novel inducible antibacterial peptide from the skin mucus of Japanese eel, *Anguilla japonica*. *The protein journal*, Vol. 30, pp. 413.

Lie, O., Evensen, O., Sorensen, A., Froysadal, E. (1989). Study on lysozyme activity in some fish species. *Diseases of aquatic organisms*. Vol. 6, pp. 1–5.

Litman, G. W., Cannon, J. P. & Dishaw, L. J. (2005). Reconstructing immune phylogeny: new perspectives. *Nature Reviews Immunology*, Vol. 5, pp. 866.

Lobb, C. & Clem, L. (1981). The metabolic relationships of the immunoglobulins in fish serumn cutaneous mucus, and bile. *Journal of Immunolgy*, Vol. 127, pp. 1525-1529.

Løvoll, M., Dalmo, R. A. & Bøgwald, J. (2007). Extrahepatic synthesis of complement components in the rainbow trout (*Oncorhynchus mykiss*). *Fish & shellfish immunology*, Vol. 23, pp. 721-731.

Løvoll, M., Wiik-Nielsen, C. R., Tunsjø, H. S., Colquhoun, D., Lunder, T., Sørum, H. & Grove, S. (2009). Atlantic salmon bath challenged with *Moritella viscosa*–pathogen invasion and host response. *Fish & shellfish immunology*, Vol.26, pp. 877-884.

Loganathan, K., Muniyan, M., Prakash, A. A., Raja, P. S. & Prakash, M. (2011). Studies on the role of mucus from *Clarias batrachus* (Linn) against selected microbes. *International journal of pharmaceutical Applications*, Vol. 2, pp. 202-206.

Luo, X., Xie, M., Zhu, W. & Li, A. (2007). Protective immunity in grouper (*Epinephelus coioides*) following exposure to or injection with *Cryptocaryon irritans*. *Fish & Shellfish Immunology*, Vol. 22, pp. 427-432.

Mabrok, M. (2016). The host/pathogen interaction during experimental infection of Senegalese sole (*Solea senegalensis*) by *Tenacibaculum maritimum* (Doctoral

dissertation). Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Mabrok, M., Machado, M., Serra, C. R., Afonso, A., Valente, L. M. P. & Costas, B. (2016). Tenacibaculosis induction in the Senegalese sole (*Solea senegalensis*) and studies of *Tenacibaculum maritimum* survival against host mucus and plasma. *Journal of fish diseases*, Vol. 39, pp. 1445-1455.

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*, Vol. 42, pp. 353-362.

Machado, M., Malheiro, D., Couto, A., Wilson, J. M., Guerreiro, M., Azeredo, R., Svendsena, J. C., Afonsoa, A., Serradeiro, R. & Costas, B. (2018). Acute hyperoxia induces systemic responses with no major changes in peripheral tissues in the Senegalese sole (*Solea senegalensis* Kaup, 1858). *Fish & shellfish immunology,* Vol. 74, pp. 260-267.

Magariños B, Pazos F, Santos Y, Romalde JL &. Toranzo AE (1995). Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish. *Diseases of Aquatic Organisms* Vol. 19, pp. 103-108.

Magariños, B., Devesa, S., González, A., Castro, N. & Toranzo, A. E. (2011). Furunculosis in Senegalese sole (*Solea senegalensis*) cultured in a recirculation system. *Veterinary Record*, Vol.168, pp. 431-431.

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

Magnadóttir, B., Hayes, P., Gísladóttir, B., Bragason, B. Þ., Hristova, M., Nicholas, A. P., Guðmundsdóttir, S. & Lange, S. (2018). Pentraxins CRP-I and CRP-II are post-translationally deiminated and differ in tissue specificity in cod (*Gadus morhua* L.) ontogeny. *Developmental & Comparative Immunology*, Vol. 87, pp. 1-11.

Magnadóttir, B., Jonsdóttir, H., Helgason, S., Björnsson, B., Jorgensen, T. & Pilström, L. (1999). Humoral immune parameters in Atlantic cod (*Gadus morhua* L.) I. The effects of environmental temperature. *Comparative Biochemistry and Physiology*, Vol. 122, pp. 173-180.

Magnadottir B. (2010) Immunological control of fish diseases. *Marine Biotechnolog*, Vol. 12, pp. 361–379.

Makesh, M., Ponnerassery, S. & Cain, K. (2015). Systemic and mucosal immune response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes. *Fish and Shellfish immunology*, Vol. 44, pp. 156-163.

Masumura, K. & Wakabayashi, H. (1977). An outbreak of gliding bacterial disease in hatchery-born red seabream (*Pagrus major*) and gilthead (*Acanthopagrus schlegeli*) fry in Hiroshima. *Fish Pathology*, Vol. 12, pp. 171-177.

Matthews, R. (2005*). Ichthyophthirius multifiliis* Fouquet in fresh water teleosts. *Advances in parasitology*, Vol. 59, pp. 159-241.

Mauri, I., Romero, A., Acerete, L., MacKenzie, S., Roher, N., Callol, A. & Tort, L. (2011). Changes in complement responses in Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) under crowding stress, plus viral and bacterial challenges. *Fish & shellfish immunology*, Vol. 30, pp. 182-188.

Mayer, L. (2003). Mucosal immunity. *Pediatrics,* Vol. 111(Supplement 3), pp. 1595-1600.

McGuckin, M. A., Lindén, S. K., Sutton, P. & Florin, T. H. (2011). Mucin dynamics and enteric pathogens. *Nature Reviews Microbiology*, Vol. 9, pp. 265.

McVicar, A. H. & White, P. G. (1979). Fin and skin necrosis of cultivated Dover sole *Solea solea* (L.). *Journal of Fish Diseases,* Vol. 2, pp. 557-562.

Medina, A., Mancera, J.M., Martínez-Manzanares, E., Moriñigo, M.A. & Arijo, S. (2015). Identification of *Vibrio harveyi* proteins involved in the specific immune response of Senegalese sole (*Solea senegalensis*, Kaup). *Fish & Shellfish Immunology*, Vol. 47, pp. 377-380.

Mitra, S. & Bhattacharya, S. (1992). Purification of C-reactive protein from *Channa punctatus* (Bloch). *Indian journal of biochemistry & biophysics*, Vol. 29, pp. 508-511.

Mohanty, B. R. & Sahoo, P. K. (2007). Edwardsiellosis in fish: a brief review. *Journal of biosciences*, Vol. 32, pp. 1331-1344.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M. & Dinis, M. T. (2016). New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture*, Vol. 8, pp. 227-263.

Muona, M., Soivio, A. (1992). Changes in plasma lysozyme and blood leucocyte levels of hatchery-reared Atlantic salmon (*Salmo salar* L.) and sea trout (*Salmo trutta* L.) during parr-smolt transformation. *Aquaculture*, Vol. 106, pp. 75–87.

Murata, M., Onuma, M. & Kodama, H. (1994). Isolation and characterization of rainbow trout (*Oncorhynchus mykiss*) serum amyloid P component (SAP). *Journal of Veterinary Medical Science*, Vol. 56, pp. 661-665.

Murata, M., Kodama, H. & Onuma, M. (1995). Characterization of rainbow trout C-polysaccharide binding proteins. *Journal of Veterinary Medical Science*, Vol. 57, pp. 419-425.

Murray C.K. & Fletcher T.C. (1976). The immunohistochemical localization oflysozyme in plaice (*Pleuronectes platessa* L.) tissues. *Journal of Fish Biology*, Vol. 9,pp. 329–334.

Najafian, A. & Babji, L. (2012). A review of fish-derived antioxidant and antimicrobial peptides: their production, assessment, and applications. *Peptides*, Vol. 33, pp.178– 185.

Nakao, M., Tsujikura, M., Ichiki, S., Vo, T. K. & Somamoto, T. (2011). The complement system in teleost fish: progress of post-homolog-hunting researches. *Developmental & Comparative Immunology*, Vol. 35, pp. 1296-1308.

Narvaez, E., Berendsen, J., Guzman, F. & Gallardo, J. A. (2010). An immunological method for quantifying antibacterial activity in *Salmo salar* (Linnaeus, 1758) skin mucus. *Fish & Shellfish Immunology* , Vol. 28, pp. 235-239.

Nigam, A. K., Kumari, U., Mittal, S. & Mittal, A. K. (2012). Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. *Fish physiology and biochemistry*, Vol. 38, pp. 1245-1256.

Nikoskelainen, S., Ouwehand, A., Salminen, S. & Bylund, G. (2001). Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis *by Lactobacillus rhamnosus. Aquaculture*, Vol. 198, pp. 229-236.

Nunomura, W. (1991). C-reactive protein in eel: purification and agglutinating activity. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* Vol. 1076, pp.191-196.

Núñez-Díaz, J. A., Fumanal, M., Viguera, E., Moriñigo, M. A. & Balebona, M. C. (2017). Use of in vivo induced technology to identify antigens expressed by

Photobacterium damselae subsp. *piscicida* during infection of Senegalese sole *(Solea senegalensis). Fish & shellfish immunology,* Vol. 64, pp. 446-456.

Öktener, A., Torcu-Koç, H., Erdoğan, Z. & Trilles, J. P. (2010). Scuba diving photography: A useful method for taxonomic and ecologic studies on fish parasites (*Cymothoidae*). *Journal of Marine Animals and Their Ecology*, Vol. 3, pp. 3-9.

Olveira, J. G., Souto, S., Dopazo, C. P., Thiéry, R., Barja, J. L. & Bandin, I. (2009). Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. *Journal of General Virology*, Vol. 90, pp. 2940-2951.

Ottesen, O. & Olafsen, J. (1997). Ontogenic development and composition of the mucus cells and the ocurrence of saccular cells in the epidermis of Atlantic halibut. *Jornal of Fish Biology*, Vol. 50, pp. 620-633.

Palaksha, K. J., Shin, G. W., Kim, Y. R. & Jung, T. S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). *Fish & shellfish immunology,* Vol. 24, pp. 479-488.

Padrós, F., Palenzuela, O., Hispano, C., Tosas, O., Zarza, C., Crespo, S. & Alvarez-Pellitero, P. (2001). *Myxidium leei (Myxozoa*) infections in aquarium-reared Mediterranean fish species. *Diseases of aquatic organisms*, Vol. 47, pp. 57-62.

Padrós, F., Zarza, C., Estévez, A., Crespo, S. & Furones, M. D. (2003). Patología como factor limitante para el desarrollo del cultivo del lenguado. In *IX Congreso Nacional de Acuicultura.* Junta de Andalucía. Cádiz, Spain, May. Pp. 343-345.

Parra, D., Reyes-López, F. & Tort, L. (2015). Mucosal immunity and B cells in teleost: effect of vaccination and stress. *Frontiers in Immunology*, Vol. 6, pp. 354.

Peatman, E. & Beck, B. (2015). Why mucosal health? In: B. Beck, & E. Peatman, *Mucosal Health in Aquaculture*, pp. 1-2. Elsevier.

Peleteiro, M. & Richards, R. (1988). Immunocytochemical studies on immunoglobulin-containing cells in the epidermis of rainbo trout *Salmo gairdneri* Richardson: influence of bath vaccination. *Journal of Fish Biology*, Vol. 32, pp. 845-858.

Peres, H., Costas, B., Perez-Jimenez, A., Guerreiro, I. & Oliva-Teles, A. (2015). Reference values for selected hematological and serum biochemical parameters of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles under intensive aquaculture conditions. *Journal of Applied Ichthyology*,Vol. 31, pp. 65-71.

Perez-Prieto, S., Garcia-Rosado, E., Rodriguez, S., Castro, D. & Borrego, J. J. (2001). Antigenic properties and experimental transmission to several fish species of a marine birnavirus isolated from sole (*Solea senegalensis). Veterinary microbiology,* Vol. 82, pp. 11-25.

Plant, k. & LaPatra, S. (2011). Advances in fish vaccine delivery. *Developmental and Comparative Immunology*, Vol. 35, pp. 1256-1262.

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

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., Delforges, C., Costes, B., Farnir, F., Leroy, B., Wattiez, R., Melard, C., Mast, J., Lieffrig, F. & Vanderplasschen, A. (2011). Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary research*, Vol. 42, pp. 92.

Rajan, B., Lokesh, J., Kiron, V. & Brinchmann, M. F. (2013). Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. *BMC veterinary research*, Vol. 9, pp. 103.

Rakers, S., Niklasson, L., Steinhagen, D., Kruse, C. & Schauber, J. &. (2013). Antimicrobial Peptides (AMPs) from Fish Epidermis: Perspectives for Investigative Dermatology. *Journal of Investigative Dermatology* , Vol. 133, pp. 1140–1149.

Rauta, P. R., Nayak, B. & Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunology letters*, Vol.148, pp. 23-33.

Ren, Y., Zhao, H., Su, B., Peatman, E. & Li, C. (2015). Expression profiling analysis of immune-related genes in channel catfish *(Ictalurus punctatus*) skin mucus following *Flavobacterium columnare* challenge. *Fish & shellfish immunology*, Vol. 46, pp. 537-542.

Revez, J. M. C. (2007). Immunological and histological changes of gilthead sea bream (*Sparus Aurata*) to Alum, when used as an adjuvnat in *Photobacterium damselae* ssp *piscicida* vaccine (Doctoral dissertation).

Robey, F. A., Jones, K. D., Tanaka, T. & Liu, T. Y. (1984). Binding of C-reactive protein to chromatin and nucleosome core particles. A possible physiological role of C-reactive protein. *Journal of Biological Chemistry*, Vol. 259, pp. 7311-7316.

Rodríguez, R. B. (1984). Biología y Cultivo de *Solea senegalensis* Kaup, 1858 en el Golfo de Cádiz. Doctoral dissertation: *Universidad de Sevilla*.

Rodríguez, S., Vilas, M. P., Gutierrez, M. C., Pérez-Prieto, S. I., Sarasquete, M. C. & Rodríguez, R. B. (1997). Isolation and preliminary characterization of a birnavirus from the sole *Solea senegalensis* in southwest Spain. *Journal of Aquatic Animal Health*, Vol. 9, pp. 295-300.

Rojo-Cebreros, A. H., Ibarra-Castro, L. & Martínez-Brown, J. M. (2018). Immunostimulation and trained immunity in marine fish larvae. *Fish & shellfish immunology.* Vol. 80, pp. 15-21.

Roman-Padilla, J., Rodriguez-Rua, A., Claros, M. G., Hachero-Cruzado, I. & Manchado, M. (2016). Genomic characterization and expression analysis of four apolipoprotein A-IV paralogs in Senegalese sole (*Solea senegalensis* Kaup). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, Vol. 191, pp. 84-98.

Rombout, J. H., Abelli, L., Picchietti, S., Scapigliati, G. & Kiron, V. (2011). Teleost intestinal immunology. *Fish & shellfish immunology*, Vol. 31, pp. 616-626.

Rombout, J., Yang, G. & Kirom, V. (2014). Adaptative immune responses at mucosal surfaces of teleost fish. *Fish and Shellfish Immunology*, Vol. 40, pp. 634-643.

Rooijakkers, S.H.M. & Strijp, J.A.G. (2007). Bacterial complement evasion. *Molecular Immunology,* Vol. 44, pp. 23–32.

Ross, N. W., Firth, K. J., Wang, A., Burka, J. F. & Johnson, S. C. (2000). Changes in hydrolytic enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. *Diseases of aquatic organisms*, Vol. 41, pp. 43-51.

Salas-Leiton, E., Coste, O., Asensio, E., Infante, C., Cañavate, J. P. & Manchado, M. (2012). Dexamethasone modulates expression of genes involved in the innate immune system, growth and stress and increases susceptibility to bacterial disease in Senegalese sole (*Solea senegalensis* Kaup, 1858). *Fish & shellfish immunology*, Vol. 32, pp. 769-778.

Salinas, I. (2015). The mucosal immune system of teleost fish. *Biology*, Vol. 4, pp. 525-539.

Salinas, I., Zhang, Y. & Sunyer, J. (2011). Mucosal immuneglobulins and B cells of teleost fishes. *Developmental and Comparative Immunology*, Vol. 35, pp. 1346-1365.

Sanderson, S. L., Cech, J. J. & Patterson, M. R. (1991). Fluid dynamics in suspension-feeding blackfish. *Science*, Vol. 25, pp. 1346-1348.

Santos Y., Pazos F. & Barja J.L. (1999) *Flexibacter maritimus*, causal agent of flexibacteriosis in marine fish. In: Oliver G. (ed) ICES *identification leaflets for diseases and parasites of fish and shellfish*, No. 55. ICES, Copenhagen, pp. 1–6.

Sarasquete, C. (1998). Histochemical study of skin and gills of Senegal sole, *Solea senegalensis* larvae and adults. *Histology and Histopathology*, Vol. 13, pp. 727-735.

Starkey, W. G., Ireland, J. H., Muir, K. F., Jenkins, M. E., Roy, W. J., Richards, R. H. & Ferguson, H. W. (2001). Nodavirus infection in Atlantic cod and Dover sole in the UK. *Veterinary Records.* Vol, 146, pp. 179-181

Saurabh, S. & Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture research*, Vol. 39, pp. 223-239.

Schrock, R., Smith, S., Maule, A. G., Doulos, S. & Rockoswski, J. (2001). Mucous lysozyme levels in hatchery coho salmon (*Oncorhynchus kisutch*) and spring Chinook salmon (*O. tshawytscha*) early in the parr-smolt transformation. *Aqualculture*, Vol. 198, pp. 169-177.

Secombes, C. J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K. J., Cunningham, C. & Zou, J. (2001). Cytokines and innate immunity of fish. *Developmental & Comparative Immunology,* Vol. 25, pp. 713-723.

Shephard, K. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries*, Vol 4, pp. 401-429.

Shoemaker, C., Xu, D., Shelby, R. & Klesius, P. (2005). Detection of cutaneous antobodies against *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque). *Aquaculture Research*, Vol. 36, pp. 813-818.

Solem, S. T. & Stenvik, J. (2006). Antibody repertorie development in teleosts - a review with emphasis on salmonids and *Gadus morhua* L. *Developmental and Comparative Immunology*, Vol. 30, pp. 57-79.

Sorroza, L., Padilla, D., Acosta, F., Román, L., Grasso, V., Vega, J. & Real, F. (2012). Characterization of the probiotic strain Vagococcus fluvialis in the protection of European sea bass (*Dicentrarchus labrax*) against vibriosis by *Vibrio anguillarum*. *Veterinary microbiology*, Vol.155, pp. 369-373.

Souza-Bastos, L. R. D., Freire, C. A. & Fernandes-de-Castilho, M. (2014). Skin extract from *Rhamdia quelen* (Siluriformes: *Heptapteridae*) does not promote stress in conspecifics. *Neotropical Ichthyology*, Vol.12, pp. 125-132.

Steinhagen D. & Jendrysek S. (1994). Phagocytosis by carp granulocytes; in vivo and in vitro observations. *Fish and Shellfish Immunology*, Vol. 4, pp. 521–525.

Subramanian, N. & MacKinnon, S. &. (2007). A comparative study of the innate immune parameters in the epidermal mucus of various fish species. *Comparative Biochemistry and Physiology B*, Vol. 148, pp. 256-263.

Subramanian, S., Ross, N. W. & MacKinnon, S. L. (2008). Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, Vol. 150, pp. 85-92.

Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *International journal of systematic and evolutionary microbiology*, Vol. 51, pp. 1639-1652.

Swain, M. R., Naskar, S. K. & Ray, R. C. (2007). Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisetts by isolated from culturable cowdung microflora. *Polish Journal of Microbiology*, Vol. 56, pp. 103.

Tacchi, L., Musharrafied, R., Larragoite, E., Crossey, K., Erhardt, E. & Martin, S. e. (2014). Nasal immunity is an ancient arm of the mucosal immune system of vertebrates. *Natural commun*, Vol. 5, pp. 5205.

Tellez-Bañuelos, M. C., Santerre, A., Casas-Solis, J. & Zaitseva, G. (2010). Endosulfan increases seric interleukin-2 like (IL-2L) factor and immunoglobulin M (IgM) of Nile tilapia (*Oreochromis niloticus*) challenged with *Aeromona hydrophila. Fish & shellfish immunology*, Vol. 28, pp. 401-405.

Thiery, R., Cozien, J., de Boisseson, C., Kerbart-Boscher, S. & Nevarez, L. (2004). Genomic classification of new betanodavirus isolates by phylogenetic analysis of

the coat protein gene suggests a low host-fish species specificity. *Journal of general virology*, Vol. 85, pp. 3079-3087.

Thompson, S., Tachibana, K. & al., N. K. (1986). Melittin-like peptides from the sharkrepelling defense secretion of the sole pardachirus. *Science*, Vol. 233, pp. 341–343.

Thornton, D. &. (2004). *From mucins to mucus.* Proccedings of America Thoracis Society, Vol. 1, pp. 56-61.

Toranzo, A. E. & Barja, J. L. (1993). Virulence factors of bacteria pathogenic for coldwater fish. *Annual Review of Fish Diseases*, Vol. 3, pp. 5-36.

Toranzo, A. E., Abendaño, R., López-Vázquez, C., Magariños, B., Dopazo, C. P., Romalde, J. L. & Barja, J. L. (2003). Principales patologías bacterianas y víricas en lenguado cultivado: caracterización y agentes etiológicos. In IX Congreso Nacional de Acuicultura (Cádiz, mayo 2003). La acuicultura como actividad económica en las zonas costeras: Libro de Resúmenes (pp. 12-16).

Toranzo A. E., Romalde J. L., Dopazo C. P., Magariños B. & Barja J.L. (2004) Disease trends in the primary marine fish species cultured in Spain: a 20-year study. *World Aquaculture*, Vol. 35, pp. 35–38

Toranzo, A. E; Magarinños, B. & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, Vol. 246, pp. 37–61.

Toyama, T., Kita-Tsukamoto, K. & Wakabayashi, H. (1996). Identification of *Flexibacter maritimus, Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S ribosomal DNA. *Fish Pathology*, Vol. 31, pp. 25-31.

Uribe, C., Folch, H. & Enriquez, R. &. (2011). Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina*, Vol. 56, pp. 486–503.

Vadstein, O. (1993). A strategy to obtain microbial control during larval development of marine fish. *Fish Farmig Technology*, pp. 69-75.

Valdenegro-Vega, V. A., Crosbie, P., Bridle, A., Leef, M., Wilson, R. & Nowak, B. F. (2014). Differentially expressed proteins in gill and skin mucus of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. *Fish & shellfish immunology*, Vol. 40, pp. 69-77.

Van der Marel, M., Caspari, N., Neuhaus, H., Meyer, W., Enss, M. & Steinhagen, D. (2010). Changes in skin mucus of common carp, *Cyprinus carpio* L., after

exposure to water with a high bacterial load. *Journal of Fish Diseases*, Vol. 33, pp. 431–439.

Vasta, G. R., Nita-Lazar, M., Giomarelli, B., Ahmed, H., Du, S., Cammarata, M., Nicolò Parrinello, N., Bianchet, M. A. & Amzel, L. M. (2011). Structural and functional diversity of the lectin repertoire in teleost fish: relevance to innate and adaptive immunity. *Developmental & Comparative Immunology,* Vol. 35, pp.1388-1399.

Vatsos, I., Kotzamanis, Y., Henry, M., Angelidis, P. & Alexis, M. (2010). Monitoring stress in fish by applying image analysis to their skin mucous cells. *European Journal of Histochemistry*, Vol. 54, pp. 22.

Vilar, P., Faílde, L. D., Bermúdez, R., Vigliano, F., Riaza, A., Silva, R., Santos, Y. & Quiroga, M. I. (2012). Morphopathological features of a severe ulcerative disease outbreak associated with *Tenacibaculum maritimum* in cultivated sole, *Solea senegalensis* (L.). *Journal of fish diseases*, Vol. 35, pp. 437-445.

Vinogradov E., MacLean L.L., Crump E.M., Perry M.B. & Kay W.W. (2003) Structure of the polysaccharide chain of the lipopolysaccharide from *Flexibacter maritimus*. *European Journal of Biochemistry*, Vol. 270, pp. 1810–1815.

Wakabayashi, H., Hikida, M. & Masumura, K. (1986). *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *International Journal of Systematic and Evolutionary Microbiology,* Vol. 36, pp. 396–398.

Walker, D. P. & Hill, B. J. (1980). Studies on the culture, assay of infectivity and some in vitro properties of lymphocystis virus. *Journal of General Virology*, Vol. 51, pp. 385-395.

Warsen, A. E., Krug, M. J., LaFrentz, S., Stanek, D. R., Loge, F. J. & Call, D. R. (2004). Simultaneous discrimination between 15 fish pathogens by using 16S ribosomal DNA PCR and DNA microarrays. *Applied and environmental microbiology*, Vol. 70, pp. 4216-4221.

Welsh, R. M., Rosales, S. M., Zaneveld, J. R., Payet, J. P., McMinds, R., Hubbs, S. L. & Thurber, R. L. V. (2017). Alien vs. predator: bacterial challenge alters coral microbiomes unless controlled by *Halobacteriovorax* predators. *PeerJ*, Vol. 5, pp. 3315.

White, A., Fletcher, T. C., Pepys, M. B. & Baldo, B. A. (1981). The effect of inflammatory agents on C-reactive protein and serum amyloid P-component levels in plaice

(*Pleuronectes platessa* L.) serum. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, Vol. 69, pp. 325-329.

Whyte, S. K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish & shellfish immunology*, Vol. 23, pp. 1127-1151.

Wiklund, T. & Dalsgaard, I. (2002). Survival of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) serum in vitro. *Fish & shellfish immunology,* Vol. 12, pp. 141-153.

Wilson, T. & Carson, J. (2003). Development of sensitive, high-throughput one-tube RT PCR enzyme hybridisation assay to detect selected bacterial fish pathogens. *Diseases of aquatic organisms*, Vol. 54, pp. 127-134.

Woof, J. & Mestecky, J. (2005). Mucosal immunoglobulins. *Immunological Reviews*, Vol. 206, pp. 64-82.

Wu, M. H., Dimopoulos, G., Mantalaris, A. & Varley, J. (2004). The effect of hyperosmotic pressure on antibody production and gene expression in the GS-NS0 cell line. *Biotechnology and Applied Biochemistry*, Vol. 40, pp. 41-46.

Xu, Z., Takizawa, F., Parra, D., Gómez, D., Jorgense, L., LaPatra, S. E. & Sunyer, O. (2016). Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. *Nature Communications*, pp. 1-14.

Yang, M., Wei, H., Zhao, T., Wang, X., Zhang, A. & Zhou, H. (2012). Characterization of Foxp3 gene from grass carp (*Ctenopharyngodon idellus*): A rapamycin-inducible transcription factor in teleost immune system. *Developmental and Comparative Immunology*, Vol. 38, pp. 98-107.

Yano, T. (1996). The non specific immune system: humoral defense. In G. Iwama, & T. Nakanishi, *The fish immune system: Organism, Pathogen and Environment*, pp. 105-153. Academic Press, San Diego, USA.

Ye, J., Kaattari, I. & Kaattari, S. (2011a). Plasmablasts and plasma cells: reconsidering teleost immune system organization. *Developmental & Comparative Immunology*, Vol. 35, pp. 1273-1281.

Ye, J., Kaattari, I. & Kaattari, S. (2011b). The differential dynamics of antibody subpopulation expression during affinity maduration in teleost. *Fish and shellfish immunology*, Vol. 30, pp. 372-377.

Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A. (2006). Ontogeny of the immune system of fish. *Fish Shellfish Immunology*, Vol. 20, pp. 126-136.

Zhang, Y., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., Sunyer, J. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Natural immunology*, Vol. 11, pp. 827-835.

Zhao, X., Findly, R. & Dickerson, H. (2008). Cutaneous antobody-secreting cells and B cells in a teleost fish. *Developmental and Comparative Immunology*, Vol. 32, pp. 500-508.

Zorrilla I., Balebona M.C., Moriñigo M.A., Sarasquete C. & Borrego J.J. (1999). Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damselae* ssp. *piscicida*, from sole, *Solea senegalensis* (Kaup). *Journal ofFish Disease*s, Vol. 22, pp.167-172.

Zorrilla, I., Arijo S., Chabrillón, M., Díaz P., Martínez-Manzanares, E., Balebona, M.C, Moriñigo, M.A. (2003). *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup), and evaluation of the potential virulence role of their extracellular products. *Journal of Fish Diseases,* Vol. 26, pp. 103–108.