Mahmoud Abd El Aziz Mabrok

The host/pathogen interaction during experimental infection of Senegalese sole (*Solea senegalensis*) by *Tenacibaculum maritimum*

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Orientador - Doutor Benjamín Costas Refojos
Categoria - Post-doctoral researcher
Afiliação- Centro Interdisciplinar de Investigacão Marinha e Ambiental da Universidade do Porto.

Co-orientador - Professor António Manuel dos Santos Afonso
Categoria – Professor Associado
Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador - Professor Luísa Maria Pinheiro Valente
Categoria - Professor Associado
Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Summary

Global aquaculture is responsible for fish supply as food in a significant percentage, and its importance has been increasing during recent years. Portugal in particular is referenced as offering high potential for this activity and Senegalese sole (*Solea senegalensis*) presents itself as a new opportunity for Portuguese aquaculture. Senegalese sole is highly susceptible to a pathogen that causes serious losses in global aquaculture, *Tenacibaculum maritimum*, a Gram-negative bacterium that is the etiological agent of tenacibaculosis. To allow a better understanding of the immune mechanisms and disease resistance in Senegalese sole challenged with *Tenacibaculum maritimum*, the present PhD thesis aimed to: i) develop an effective and reproducible infection model of *T. maritimum* against sole; ii) evaluate cell host responses against this pathogen as *in vitro* approach; iii) study systemic and mucosal innate immune responses against infection with *T. maritimum* including cell migration dynamics. The present Thesis is composed of six chapters, including a general introduction and a final discussion.

1. Since no immersion challenge with *T. maritimum*, and no evidence for an effective infection model has been successfully performed in Senegalese sole, partially as a result of *T. maritimum* culture constrains, the first part of this thesis **(Chapter 2)** aimed to optimise bacterial yields as well as to establish an optimal and reproducible challenge model for tenacibaculosis induction. Several approaches were performed to optimise bacterial culture conditions, including treatment with non-ionic surfactants, detergents, celullase hydrolysis and strong aeration. A prolonged bath challenge was performed for 24 h at two different temperatures, 16 and 23 ºC. Moreover, mucus and plasma bactericidal activities against *T. maritimum* were also assessed. Culturing bacteria with aeration and continuous shaking provided suitable culture conditions to obtain higher bacterial yields without aggregation and fluctuation, contrarily to most other treatments that showed a huge amount of bacterial aggregates. A prolonged bath method for 24 h, without skin or gill scarification, was considered suitable for disease induction with high mortality rates. Moreover, data regarding mucus and plasma bactericidal activities suggested that there is a lack of host innate immune response against *T. maritimum* or that this particular pathogen presents evading strategies against Senegalese sole.

2. There is a considerable lack of knowledge regarding the mechanisms of infection and the interactions between this particular pathogen and the host. Currently, little is known about *T. maritimum* evading strategies and many aspects regarding the host-pathogen interaction are still not fully elucidated. Hence, in **Chapter 3** it was aimed to assess

Senegalese sole cellular immune responses following stimulation with either live or UV killed *T. maritimum* through both functional (e.g. superoxide anion and nitric oxide production, leucocytes killing capacity) and gene expression approaches. Senegalese sole head-kidney leucocytes were isolated and exposed to several live or inactivated *T. maritimum* strains during 4 h, 12 h, 24 h and 48 h. Results from the present study did not reveal significant changes in superoxide anion and nitric oxide production in leucocytes exposed to different bacterial strains. UV killed *T. maritimum* strains induced higher nitric oxide production by leucocytes in contrast to the lower superoxide anion release induced by live strains. Moreover, lactate dehydrogenase activity was assessed and results suggested some evidence for necrotic cell death induction mainly during the first 4 h following bacterial inoculation. Regarding gene expression, stimulation with live strains induced an increase in interleukin 1β (IL1β), hepcidin antimicrobial peptide (HAMP1), cyclooxygenase 2 (COX2) and g-type lysozyme (gLYS) transcripts at 4 h, which decreased similarly until 48h. Although interleukin 10 (IL10) expression levels presented a similar pattern, an upregulation was observed at 48 h post stimulation. Moreover, the expression levels of IL1β, COX2, HAMP1 and IL10 from host cells stimulated with inactivated bacterial strains increased more than those from leucocytes exposed to live bacteria. Finally, the downregulation of inflammatory and iron regulating genes as well as the extensive destruction of phagocytes were considered important tools in bacterial pathogenesis.

3. In **Chapter 4**, the effects of *T. maritimum* extracellular products (ECPs) and lipopolysaccharides (LPS) against Senegalese sole head-kidney leukocytes (HKL) were assessed through functional approaches (i.e. superoxide anion and nitric oxide production). Furthermore, the cytotoxic activity of such stimuli against HKL was evaluated. Senegalese sole HKL were isolated and exposed to several concentrations of ECPs and LPS from 3 *T. maritimum* strains belonging to the same serotype during 24 h. The current study revealed that *T. maritimum* strains belonging to the same serotype produced similar ECPs profiles at either 24 or 48 h of culture. Senegalese sole HKL produced reactive oxygen species (ROS) while failed to produce nitric oxide (NO) following stimulation with ECPs. The ROS production was found to be concentration dependent, with the highest production observed following stimulation with 10 μg mL^{-1}, whereas the lowest one was recorded following stimulation with 100 μg mL^{-1}. Moreover, HKL did not increase ROS nor NO production following exposure to LPS from *T. maritimum* and *E. coli*, which appear to be related to the low LPS concentration employed. The cytotoxic activity of both ECPs and LPS from *T. maritimum* against HKL was assessed and results showed an increase in host cell death. Moreover, the potent cytotoxicity displayed by *T. maritimum* ECPs against

Senegalese sole HKL may give some insights regarding the mechanism by which this bacterium induces cell death.

4. Regardless of endeavors made in the last decades, there are impressive gaps concerning *T. maritimum* route of entry, the survival strategy of the pathogen and immune responses of the host. Therefore, the final part of our thesis (**chapter 5**) aimed to investigate the skin mucus terminal carbohydrate composition, several immune-related enzymes (i.e. lysozyme, peroxidase, proteases and antiproteases), haemolytic activity of complement and the bactericidal activity in skin mucus and plasma of Senegalese sole in a time-course basis following bath challenge with *T. maritimum*. The haematological profile and the kinetics of cell migration post infection were also considered. Lectin binding results suggest that skin mucus contains in order of abundance, α-D-mannose, α-D-glucose, N-acetylneuraminic acid and N-acetyl-D-galactosamine. Passive changes in the glycosylation pattern following bacterial challenge were observed and may consider an influential process to overcome host mucosal immunity. In general, our results demonstrated a delay in the skin mucus immune response compared to that found in plasma. A significant increase in skin mucus lysozyme, complement, protease and anti-protease activities were observed at the end of the experiment (14 days post challenge). Interestingly, the higher activity of these enzymes was positively related with the skin mucus bactericidal capacity, suggesting that these enzymes play an important role in the defence against Gram-negative bacteria. The haematological profile revealed a significant increase in peripheral lymphocytes in challenged fish after 14 days following infection. Although the present study showed inflammatory processes and immune parameters alteration following challenge with *T. maritimum*, the route of entry and the survival strategy of this particular pathogen still not fully elucidated and required further investigations.

Although, the previous data brought up the conceivable ways for bacterial culture optimization and experimental disease induction, and explained briefly the inflammatory processes and immune parameters alteration following *in vivo* and *in vitro* challenge with *T. maritimum*. The route of entry and the survival strategy of that pathogen still not fully clarified and required further investigations.

Resumo

A aquicultura global é responsável por grande parte do abastecimento total de pescado para alimentação e a sua importância tem vindo a aumentar nos últimos anos. Portugal, em particular, oferece excelentes condições para a prática de aquicultura e o linguado senegalês (*Solea senegalensis*) surge como uma nova oportunidade para a aquicultura Portuguesa. O linguado senegalês é altamente susceptível a um patógeno responsável por grandes perdas, a *Tenacibaculum maritimum (T. maritimum),* uma bactéria Gram-negativa que constitui o agente etiológico da tuberculose. A fim de permitir uma melhor compreensão dos mecanismos de defesa e resistência à doença do linguado infetado com *T. maritimum,* esta tese procurou: i) desenvolver um modelo de infeção eficaz e reproduzível de *T. maritimum* em linguado; ii) avaliar *in vitro* as respostas do hospedeiro contra este patógeno; iii) avaliar a resposta imunitária inata a nível sistémico e da mucosa contra a infeção por *T. maritimum* incluindo a dinâmica de migração celular. A presente Tese é composta por seis capítulos, incluindo uma introdução geral e uma discussão final.

1. Tendo em conta as dificuldades da cultura da *T. maritimum*, a infeção por imersão ou qualquer outro modelo de infeção eficaz nunca foram realizados com sucesso nesta espécie de peixes planos. A primeira parte desta tese (**Capítulo 2**) teve como objetivo otimizar a produção da bactéria, bem como estabelecer um modelo de infeção ótimo e reproduzível para a indução de tuberculose. Várias abordagens foram realizadas para otimizar as condições de cultura bacterianas, incluindo o tratamento com agentes tensioactivos não iónicos, detergentes, hidrólise da celulase e arejamento forte. A infeção por imersão prolongada foi realizada durante 24 h, sob duas temperaturas diferentes, 16 e 23 ºC. Foram também avaliadas as atividades bactericidas do muco e plasma do linguado contra *T. maritimum*. A cultura das bactérias com arejamento, agitação forte e contínua são condições de cultura obrigatórias para se obter rendimentos elevados sem agregação bacteriana ou flutuação, contrariamente à maioria dos outros tratamentos que mostraram ser ineficazes contra a formação de agregados bacterianos. Um método de banho prolongado por 24 h, não invasivo e sem danos para a pele e brânquias, foi considerado adequado para indução da doença resultando numa alta taxa de mortalidade. Além disso, dados sobre as atividade bactericida do muco e do plasma sugerem que há uma falta de resposta imunitária inata contra *T. maritimum* ou que este patógeno em particular apresenta estratégias de invasão contra o linguado senegalês.

2. Há uma considerável falta de conhecimento sobre os mecanismos de infecção e as interações entre este patógeno em particular e o hospedeiro. Do mesmo modo, não são conhecidas, até à data, as estratégias de evasão da *T. maritimum* relativamente às defesas do hospedeiro. Assim, o **Capítulo 3** foca-se na resposta *in vitro* de leucócitos do rim anterior de linguado após estimulação imunitária com *T. maritimum,* viva ou morta por radiação UV, numa perspetiva funcional (ex.: espécies reativas de oxigénio (ROS), óxido nítrico (NO), e medição da viabilidade celular) e de expressão de genes. Os leucócitos do rim anterior foram isolados e expostos às várias estirpes de *T. maritimum* vivas ou mortas por radiação UV durante 4 h, 12 h, 24 h e 48 h. Os resultados obtidos não mostraram qualquer variação significativa na produção de ROS e NO entre as diferentes estirpes. Contudo, independentemente da estirpe, a produção ROS e NO induzidas pela bactéria morta foram mais altas do que os valores induzidos pelas estirpes vivas. Além disso, a actividade da lactato desidrogenase foi avaliada e os resultados indicam uma possível indução de morte celular necrótica, principalmente nas primeiras 4 horas após infeção. Quanto à expressão de genes, a estimulação com estirpes vivas levou a um aumento da expressão da interleucina 1β (IL-1β), hepcidina (HAMP1), ciclooxigenase 2 (COX2) e lisozima g (glys) às 4 horas, decrescendo até às 48 h. Apesar da interleucina 10 (IL-10) apresentar um padrão de expressão semelhante, um aumento de expressão é observada às 48 h após estímulo. Também a IL-1β apresenta um padrão de expressão similar, com um aumento de expressão observado às 48 h após estímulo. Finalmente, a redução da expressão de genes inflamatórios e com papel na regulação do ferro, tal como a extensa destruição dos fagócitos são considerados ferramentas fundamentais na virulência bacteriana e patogenicidade.

3. No **Capítulo 4** e numa abordagem funcional (ex.: ROS e NO) foram avaliados os efeitos dos produtos extracelulares (ECPs) da T. *maritimum* e lipopolissacarídeos (LPS) nos leucócitos do rim anterior (HKL) do linguado senegalês. A atividade citotóxica desses estímulos nos HKL foi também avaliada. Os HKL do linguado senegalês foram isolados e expostos a várias concentrações de ECPs e LPS de 3 estirpes de *T. maritimum* pertencentes ao mesmo serotipo durante 24 h. O presente estudo mostrou que estas estirpes produzem perfis de ECPs similares às 24 h e 48 h de cultura. Apesar de apresentarem produção de ROS, os HKL do linguado senegalês não apresentaram uma produção significativa de NO após estimulação com ECPs. A produção de ROS mostrou ser dependente da concentração, com uma maior produção observada após estímulação com 10 µg mL^{-1}, enquanto a menor produção foi observada com a concentração 100 µg mL^{-1}. Além disso, os HKL não aumentaram a produção de ROS nem de NO após exposição ao LPS de *T. maritimum* e *Escherichia coli (E. coli)*, parecendo estar

relacionado com a baixa concentração de LPS usada. A atividade citotóxica de ambos ECPs e LPS de *T. maritimum* foi avaliada e os resultados aqui apresentados mostram um aumento na morte de células do hospedeiro. Por fim, o potencial citotóxico demonstrado pelos ECPs da *T. maritimum* face aos HKL do linguado senegalês pode dar indicações relativas aos mecanismos pelos quais esta bactéria induz morte cellular.

4. Vários estudos acerca da *T. maritimum* têm sido desenvolvidos nas últimas décadas. Contudo, existe ainda uma grande falta de conhecimento quanto à sua via de infeção, estratégias de sobrevivência, bem como acerca da resposta imunitária do hospedeiro. Assim, o capítulo final desta tese (**Capítulo 5**) foca-se na composição do muco da pele e do plasma do linguado senegalês em termos de hidratos de carbono e enzimas (lisozima, peroxidase, protéases e anti-protéases) além das atividades hemolítica do complemento e bactericida. A avaliação destes parâmetros foi desenvolvida ao longo do tempo após infeção por imersão com *T. maritimum*. O perfil hematológico e a cinética de migração celular no hospedeiro foram igualmente avaliados após infeção. As observações a nível de lectinas presentes no muco da pele apontam para a presença, segundo maior abundância, de α-D-manose, α-D-glucose, ácido N-acetilneuramínico e N-acetil-D-galactosamina. Algumas alterações a nível do padrão de glicosilação foram observadas após o desafio imunitário apontando assim para a possível existência de uma estratégia de evasão às defesas imunitárias da mucosa do linguado. Em geral, os resultados obtidos sugerem um ligeiro atraso da resposta do muco, relativamente à resposta no plasma. Ao fim de 14 dias após infeção, verificou-se um aumento significativo das atividades da lisozima, complemento, protéases e anti-protéases do muco da pele. Esta variação foi proporcional à atividade bactericida, evidenciando assim a grande importância destas enzimas durante a resposta do hospedeiro a bactérias Gram-negativas. Por fim, relativamente ao perfil hematológico, e ao fim de 14 dias de infeção, o número de linfócitos do sangue dos peixes infetados aumentou significativamente.
Os dados aqui apresentados sugerem soluções para a otimização da cultura bacteriana de *T. maritimum* e indução experimental da doença. Além disso, também são demonstrados os processos inflamatórios e as alterações a nível de parâmetros imunitários que ocorrem após infeção *in vivo* e *in vitro* com *T. maritimum*. Contudo, é ainda necessário aprofundar o estudo deste agente patogénico, particularmente a sua via de infeção e estratégias de sobrevivência.

Chapter 1

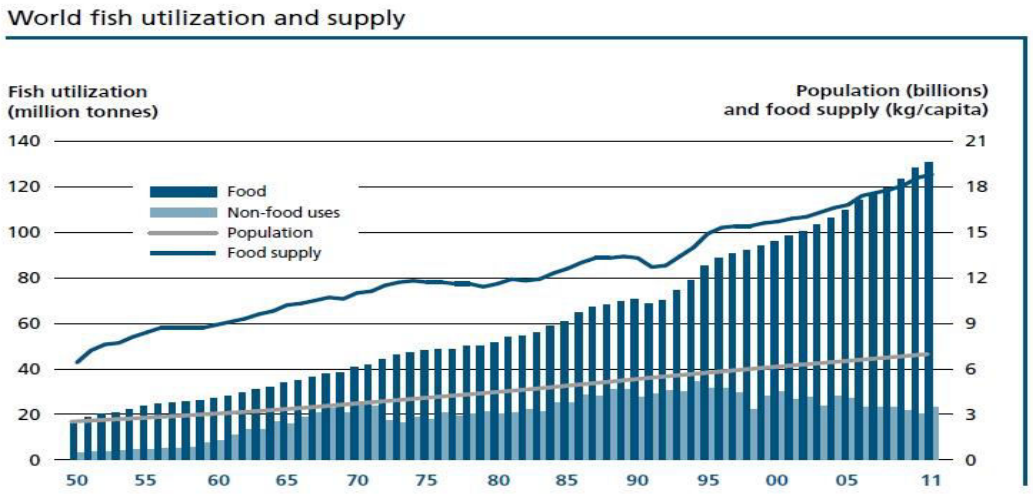
General Introduction

M. Mabrok

1. World Aquaculture

Aquaculture is considered one of the most important animal food-producing sectors that can globally supply world population with protein sources and cover the gap of food shortage due to over population. Fish are not just a source of health but also of wealth. Currently, around 16 % of animal protein consumed worldwide is derived from fish, and over one billion people depend on fish as the main source of animal protein (Buentello *et al*. 2007). Fish consumption (kg per capita) increased from an average of 9.9 kg in 1960 to 19.2 kg according to FAO 2014 (Fig.1). This rise was mainly attributed to an increase in population, associated with increased income and urbanization, and developing facilities for aquaculture rearing and expansion. Moreover, there is a general increasing attitude towards increased fish consumption due to its health benefits. The apparent fish consumption in developing regions raised from 5.2 kg in 1961 to 17.8 kg per capita in 2010, and in low-income food deficit countries (LIFDCs) (from 4.9 to 10.9 kg per capita) according to FAO, 2014 annual report. This increasing fish demand has to be supported by a concomitant increase in aquaculture production due to fisheries stagnation in the last years (FAO 2014). Overall, fish production in 2013 reached 160 million tonnes with 90 million tonnes coming from capture fisheries and 70 million tonnes from aquaculture (FAO 2014). But FAO estimates that by 2020 it will be required a further production of approximately 25 million tonnes worldwide that can only be supplied by aquaculture. Nevertheless, in the European Union (EU 28) aquaculture production only represents 3.2% of the world aquaculture production in volume and 8.2% in value, reaching 1.28 million tonnes and 3.5 billion Euros (FAO 2012).

Fig. 1: World fish utilization and supply according to FAO, 2014:



2. Senegalese sole, *Solea senegalensis* (Kaup) nutritional value and economic importance

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a high-value flatfish with a great potential for future farming in European countries (Morais *et al*. 2015). Due to over saturation of markets with other Mediterranean species such as European seabass (*Dicentrachus labrax*) and gilthead seabream (*Sparus aurata*), scientific and technical interests have focused on high nutritive value species whose biological cycle could be reproduced using available breeding techniques (Imsland *et al*. 2003). This was the case of Senegalese sole. The current market price in the EU is around 8.5-16.5 €/kg depending on size and season for *S. solea* and around 8-14 €/kg for *S. senegalensis* which explains the great importance of this species. However, there are still many obstacles hampering the successful industrial development of sole farming, such as reproduction and disease control Disease outbreaks appear to be the most significant limitation, with bacterial infections often diagnosed (Morais *et al*. 2015, Howell 1997, Dinis *et al*. 1999, Imsland *et al*. 2003).

3. Senegalese sole: general aspects of its biology and feeding habitat

Senegalese sole is a flatfish with an oval and asymmetric body (eyes on the right side). The interradial membrane on the pectoral fin on the eye side is of black colour. Sole are a benthic type marine species living at a depth 100 m, in sandy or muddy bottoms of the continental shelf from the Gulf of Biscay to the coasts of Senegal (Whitehead *et al*. 1986). Like other members of the Pleuronectidae family, Senegalese sole is partially euryhaline in the juvenile phase, being able to adapt to a wide range of salinities (5-55 g L^{-1}) in a short time period (Evans 1984). Due to the ability of *S. solea* to live in deep, warmer and low salinity areas, it is considered a famous type of fish in many areas, were more abundant feed is available (Cabral & Costa 1999). In wild, sole feeds basically on benthonic invertebrates, such as larvae from polychaetes, bivalve, molluscs and small crustacean. Contrarily to other flatfish species, such as turbot and halibut (De-Groot 1971), sole has a poorly developed stomach and consequently needs to feed frequently (Navarro *et al*. 2009). In the Portuguese coast, *S. solea* and *S. senegalensis* reach sexual maturity at 3 or 4 years (Cabral *et al*. 2007). The coastal spawning grounds of *S. solea* in Northern European waters are generally located at depths between 40m and 100m (Koutsikopoulos *et al*. 1991, Wegner *et al*. 2003). Artificial spawning of this species has also been obtained under various conditions. The time of year may differ, even at the same location, depending on the water temperature. Sole are batch spawners and the spawning season occurs mostly between the months of March and June with each female ovulating and releasing batches of eggs every few days for several weeks (Imsland *et al*.

2003). In culture conditions, eggs are normally obtained from wild brood stocks kept in captivity (Dinis *et al*. 1999). Intensive sole farming mainly depends on the rate of production, which is a function of both the mean growth rate of the fish and the stocking density. The effects of density on growth are variable among different species and usually reveal inverse relationships in several teleost species such as Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*), and Dover sole (*Solea solea*) (Schram *et al*. 2006, Sanchez *et al*. 2010). In contrast, Senegalese sole juveniles stocked in the range of 30-45 kg m^2 showed similar growth performance (Salas-Leiton *et al*. 2008, 2010). However, in Senegalese sole it was also shown that increasing stocking density may down regulate the expression of some genes (e.g. g-type lysozyme and hepcidin antimicrobial peptide) making fish more susceptible to opportunistic pathogens (Salas-Leiton *et al.* 2010).

4. General feature about Gram-negative bacteria

Aquatic diseases are considered one of the most important problems facing fish industries during fish rearing and husbandry. Diseases are not a solitary event but a final consequence of collaborative reactions between several events including pathogens, fish, and environmental conditions (Wedemeyer 1996). The presence of an infective agent in a big population may greatly affect susceptible fish particularly in adverse environmental conditions. Bacterial diseases were manifested in various ways following impairment of host physiological function (Bassey 2011). Like other animals, marine fish are exposed to numerous diseases, especially induced by the presence of bacteria. It has been estimated that 10% of fish losses in aquaculture are due to diseases; and more than 50% of these losses are due to bacterial pathogens (Freund *et al*. 1990). The normal bacterial flora of fish is a direct reflection of the bacterial population in the water where they swim (Toranzo *et al*. 2005). A relatively small number of pathogenic bacteria are responsible for the most important economic losses in cultured fish. A disease outbreak is a primary constraint in aquaculture and can seriously affect the economic situation of a fish farm. Diseases that affect domestic fish populations may additionally threaten wild fish populations (Thoesen 1994). Marine fish are always exposed to a variable number of environmental stressors, including chemical stressors, pollution and natural and biological invaders. Such stressors are considered a predisposing factor for chronic immune suppression and subsequently for disease progression. As an ultimate fate for the staggering immune-suppression, the bacterial invasion will be the most probable event. There are basically two types of bacteria producing disease: obligate and facultative pathogens. The facultative ones can indefinitely survive in water and when environmental conditions get worse, the infection may spread. Most potentially pathogenic bacteria normally exist in communal association within the host or live free in the environment. Both types of bacteria become pathogenic

when the fish immune system becomes compromised by one of the mentioned stressors (Kirjusina *et al*. 2007).

5. Tenacibaculosis as an important disease affecting marine fishes

Nowadays, tenacibaculosis is considered one of the most threatening bacterial infections limiting the culture of many fish species of commercial value in distinct geographical areas of the world (Toranzo *et al*. 2005). Once the infection happens, different pathopnominic lesions appear. Different bacterial taxonomies have been developed depending on the host species and the lesion developmental patterns. Therefore, many previous studies described the ulcerative lesions as salt water columnaris disease, gliding bacterial disease of sea fish, bacterial stomatitis, eroded mouth syndrome and black patch necrosis (Bernardet 1998, Santos *et al*. 1999). A recent review has been done to avoid further confusions (Avendaño-Herrera *et al*. 2006).

5.1. Taxonomy

The taxonomy of *T. maritimum* raised some confusion for decades but it has been recently classified by Suzuki *et al*. (2001). Firstly, it was described as a gliding bacterial infection affecting intensive system culture of seabream in Japan (Masumura & Wakabayashi 1977). These strains were recognized by Hikida *et al*. (1979) who made a separate formal proposal of the name *Flexibacter marinus*. The adjective *marinus* had previously been used to name the species *Vibrio marinus* therefore; the eventual species formal proposal was *Flexibacter maritimus* (Wakabayashi *et al*. 1986, Holmes 1992). The pathogen was later known as *Cytophaga marina* (Reichenbach 1989), but the priority of the taxon *Flexibacter maritimus* was later recognized (Holmes 1992). These confusing taxonomic denominations were later confirmed using DNA hybridization methods by Bernardet & Grimont (1989). Depending on the nucleotide sequence of the gyrB which is the most recent advanced technique, it was demonstrated that *Flexibacter maritimus* should be transferred to the new genus *Tenacibaculum*, in which seven members are currently included (Suzuki *et al*. 2001).

5.2. Species susceptibility

Tenacibaculum maritimum affects a wide range of marine fish including Japanese flounder, *Paralichthus olivaceus*, yellowtail *Seriola quinqueradiata*, turbot *Psetta maxima*, sole *Solea senegalensis*, white seabass, Pacific sardine *Sardinops sagax*, northen anchovy *Engraulis mordax,* Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus kisutch*), seabass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*) (Baxa *et al*. 1986, Wakabayashi *et al*. 1986, Devesa *et al*. 1989, Alsina & Blanch 1993, Bernardet

et al. 1994, Chen *et al*. 1995, Ostland *et al*. 1999). In Europe, the presence of this pathogen was firstly demonstrated in Scotland for Dover sole *Solea solea* suffering from 'black patch necrosis (Bernardet *et al*. 1990). *T. maritimum* was also isolated from outbreaks in sole farms in Portugal and south of Spain in the last two years. These new *T. maritimum* isolates could not be assigned within the two major serotypes (O1 and O2) (Avendaño- Herrera *et al*. 2005).

5.3. Clinical features

Moribund fish show haemorrhagic or pale liver, petechial haemorrhage on the jaw, fins, ventral aspect of the body with anaemia and erratic swimming (López *et al*. 2010). The main characteristic gross lesions are ulcers, necrosis, eroded mouth, frayed fins, tail rots, and sometimes necrosis on the gills and eyes (Devesa *et al*. 1989, Alsina & Blanch 1993, Chen *et al*. 1995, Hand-linger *et al*. 1997, Cepeda & Santos 2002). Recently, Vilar *et al*. (2012) described morpho-pathological changes in naturally infected farmed *Senegalese sole* affected by *T. maritimum* using visual observation, in addition to light and scanning electron microscopes. They showed that the main characteristic lesions are associated with a complete loss of epidermis and dermis, as well as an extensive necrosis of muscle layers. Mild to moderate inflammatory response with the presence of macrophages was also noted around hyaline degenerated muscle cells. Gram-negative filamentous bacteria could be detected only at the dermis. Scanning under the electron microscope revealed the presence of filamentous bacteria over the scales without evading the epithelium.

In fish, *T. maritimum* primarily attacks the skin, mouth, fins and tail causing bacterial nets that strongly attach to necrotic surfaces, producing powerful toxins (exotoxins) that tend to attenuate the host defence mechanisms (Baxa *et al*. 1988). This pathogen forms a perfect biofilm to which other microorganisms or organic matter are attached, and once it occurs in gills, it impairs fish respiration (Kitatsuji *et al*. 1996). Internally, paleness of the organs is most frequently diagnosed (Toranzo *et al*. 2005). Lesions occurrence and distribution vary depending on the host fish species (Abd El-Galil & Hashem 2012). These authors observed that affected picasso trigger fish had haemorrhagic ulcers, eroded, ulcerated mouth and tail rots while, whereas diseased black damsel showed ulcerated skin lesions surrounded by a white batch of necrotizing tissues, corneal opacity and fin rot. Similarly, broom tail wrasse showed rounded white patches of necrosis all over the body associated with tail and fin rot. The histological features confirmed the presence of adhered bacteria in the eroded surface, which may extend deeply into the connective tissue. Moreover, it revealed small areas of vascular congestions, petechial and haemorrhagic necrosis of the affected tissue, but without internal pathological changes (Abd El-Galil & Hashem 2012).

5.4. Morphological and biochemical characterization of *T. maritimum*

T. maritimum has been identified and recognized by several authors (Hikida *et al*. 1979, Baxa *et al*. 1986, Wakabayashi *et al*. 1986, Bernardet *et al*. 1990, 1994, Alsina & Blanch 1993, Pazos *et al*. 1993, Soltani & Burke 1994, Chen *et al*. 1995, Ostland *et al*. 1999, Suzuki *et al*. 2001, Avendaño-Herrera *et al*. 2004). It is described as a filamentous bacterium with about 0.5 µm wide and - 30 µm long; cells up to 100 µm in length can occasionally be observed. Prolonged incubation of *T. maritimum* results in some morphological changes as some bacteria became smaller, whereas other form spores (Chen *et al*. 1995, Mouriño *et al*. 2008). The gliding motility on wet preparations is a characteristic feature of all isolates. The bacterium is mesophilic and grows well at temperatures ranging from 15 to 34°C (Avendaño-Herrera *et al*. 2006). The optimal growth temperature under normal culture conditions is 30 °C, while in media containing 50-100% sea water it was changed to 22-25 °C. On solid media, the colonies absorb Congo red, however, it does not contain cell wall-associated flexirubin-type pigment, and this characteristic feature can differentiate it from other phenotypic bacteria of the same family (Avendaño-Herrera *et al*. 2006). The bacteria have different types of colonies with variable rhizoid edges and adherence capacity (Sorongon *et al*. 1991). A single culture isolated in Spain from Senegalese sole (*Senegalese sole*) has been introduced as a new species (*Tenacibaculum soleae*.) It is characterized by yellow-pigmented colonies without pigment production (flexirubin), strictly eoropic and with rod shape bacilli that never grow in the presence of sodium chloride. It is catalase and oxidase positive, but H_2S and Indole negative, and can be diagnosed by using API ZYM (Piñeiro- Vidal *et al*. 2008).

5.5. Diagnostic tools

Isolation of *T. maritimum* using traditional and classical methods showed great constraints, as it is a fastidious process. This species is characterized by a slow growth rate giving an opportunity for other saprophytic and pathogenic bacteria to be present within the lesions, resulting in a false recognition of the colonies among mixed population (Pazos *et al*. 1996, Avendaño-Herrera *et al*. 2004). The most penetrating microorganisms are *Vibrio* spp. (Kimura & Kusuda 1983), particularly the species *V. splendidus* (Handlinger *et al*. 1997) and some ciliated protozoa (Mcvicar & White 1979, Devesa *et al*. 1989). Differentiating *T. maritimum* from other phenotypically and phylogenetically related species, particularly those of the genera *Flavobacterium* and *Cytophaga* showed great difficulties (Suzuki *et al*. 2001, Bader & Starliper 2002). Clinical signs are usefulness diagnostic tools as they can be produced by different pathogens (Mcvicar & White 1979), and there is also a great variability among infected fish species (Chen *et al*. 1995). The difficulty of observing this bacterium in a wet preparation during the early stage of infection

hinders its earli diagnosis (Toranzo *et al*. 2005). *T. maritimum* is a ubiquitous and opportunistic pathogen (Avendaño-Herrera *et al*. 2006) able to withstand the bactericidal action of the skin mucus, and can be detected in fish skin even without any evidence of tenacibaculosis (Avendaño-Herrera 2005, Piñeiro-Vidal 2008). Therefore, all traditional methods required from several days to weeks till accurate diagnosis is achieved, resulting in high fish mortalities with severe economic losses. Hence, the presumptive diagnosis of tenacibaculosis might be based on the clinical signs of the affected fish, but particularly using gross and microscopic examination, and more recently relying on molecular diagnosis tools such as PCR and advanced reverse transcription-chain reaction polymerase (RT-PCR). Currently, these specific molecular based methods (Avendaño-Herrera *et al*. 2004) were considered the best alternative for *T. maritimum* diagnosis. PCR technique can provide a powerful tool for accurate identification of the pathogen from plate cultures, as well as from fish tissues (Cunningham 2002, Osorio & Toranzo 2002). Complete sequencing of deoxyribonucleic acid (DNA) coding region for *T. maritimum* 16S rRNA has been reported by Woese *et al*. (1990). These results helped clarifying the phylogenetic position of the pathogen as well as forming the basis for the design of different DNA-based methods. Based on DNA sequences Toyama *et al*. (1996) developed two set of specific primers that amplifies a fragment size of 1088 base pairs (bp), which can differentiate *T. maritimum* bacterial species from other phonotypical related species such as (*Flavobacterium columnare* and *F. branchiophilum*). Bader & Shotts (1998) established a new pair of primers that amplifies a specific chromosomal fragment of 400 bp. There is some evidence that the conventional type PCR methods have low sensitivity in diagnosis. Therefore, Nested-PCR protocols have been established by Toyama *et al*. (1996) who suggested that a not lethal, highly sensitive, specific and easily applied Nested-PCR could be used for the rapid detection of *T. maritimum* in both infected and asymptomatic carrier fish. More advanced RT-PCR with enzyme hybridization assays have been designed by Wilson *et al*. (2002) and Wilson & Carson (2003). Recently, a diagnostic method using oligonucleotide probes specific for *T. maritimum* allow the detection of PCR product (Warsen *et al*. 2004).

For an accurate diagnosis, it is advisable to isolate *T. maritimum* on specific media. The first specific medium used was Anacker and Ordal agar (AOA) (Anacker & Ordal 1959). Several modifications have been further applied to this medium like its preparation in 70% seawater for the isolation of the slow-growing *T. maritimum* from infected fish (Hikida *et al*. 1979, Wakabayashi *et al*. 1984, 1986, Handlinger *et al*. 1997). Furthermore, marine agar medium (Campbell & Buswell 1982, Alsina & Blanch 1993, Mouriño *et al*. 2008) and Hsu-Shotts media supplemented with antibiotics (Chen *et al*. 1995) have also been devised for the recovery and isolation of the pathogen. Other basal media like tryptone casamino acid

yeast extract (Wakabayashi *et al*. 1984, 1986, Ostland *et al*. 1999), tryptone yeast extract salts (Toyama *et al*. 1996), tryptone yeast (Bader & Shotts 1998) and 1/5 LBM (Marine Luria Broth) (Suzuki *et al*. 2001) have been mentioned for lab culturing, but not recommended for isolation. In fact, although all these media support the growth of *T. maritimum* strains, another medium named *Flexibacter maritimus* medium (FMM) has been proposed as the most important and specific medium for the successful isolation of this bacterium (Pazos *et al*. 1996). The pathogen seems to grow better on this medium than other halophilic bacteria such as *Vibrio*, *Pseudomonas* and *Alteromonas* spp., which are usually present in skin samples. The incubation for all media is usually carried out at 20 to 25°C for 48 to72 h. It is important to note that only on FMM and AOA mediums the colonies show their typical flat, pale-yellow irregular edges features that strongly adhereto the medium; on marine agar, colonies are round and show an yellow pigment (Pazos *et al*. 1996).

5.6. *T. martimum* pathogenicity

Knowledge of the pathogenicity, the pattern of lesions and the portal of entry of an organism are essential for understanding the disease induced by the *T. martimum*. Although previous works have examined the different physical characteristics and molecular heterogeneity of various isolates (Avendaño-Herrera *et al*. 2004), it is still necessary to determine this species effects on the host. Pathogenicity can be defined as the inherent or the genetic capacity of a microorganism to induce disease, mediated by specific virulence factors. It also refers to the degree of bacterial virulence and their damage effect to the host tissues (Alcamo 1883). The pathogenicity is not only confined to the virulence of the causative agent but also related to the host susceptibility (Madigan & Martinko 2003).

5.6.1. Natural reservoirs

The *T. maritimum* natural reservoir(s) and mode of transmission have not been recognized yet, due to the availability of few data about the ecology of this microorganism. Certain bacteria take part of the natural flora of marine organisms and their ecosystem as a reservoir host (Yasuda & Kitao 1983). *T. maritimum* was isolated from sediment, surface of tanks and from water that were exposed to infected fish stocks (Santos *et al*. 1999). Some authors reported that natural outbreaks of tenacibaculosis occurred within few weeks after transferring fish from hatchery tanks to net cages (Mcvicar & White 1979, Wakabayashi *et al*. 1984), suggesting that the horizontal transmission is the main route of transmission to the host in seawater. Furthermore, Faíled *et al*. (2013) reported the presence of *T. maritimum* in the intestinal tract of apparently healthy fish without

associated histological changes and pathopnominic lesions, suggesting that the intestine may act as a reservoir for *T. maritimum*. The ability of bacteria to withstand the bactericidal activity of the host skin mucus indicates that this tissue may be considered the main reservoir for *T. maritimum* (Magarinos *et al*. 1995, Pazos *et al*. 1997). The outbreaks recorded in common sole fish in Aberdeen (Scotland), without a previous history of infection, were attributed to the arrival of a new fish species and suggested that transferred fish may act as asymptomatic carriers of the disease (Mcvicar & White 1979). Recent studies suggested that water may be considered an important route of *T. maritimum* infection, particularly for Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and "greenback flounder (*Rhombosolea tapirina*) due to the ability of this pathogen to induce disease even for short period of exposure 60-90 mints (Handlinger *et al*. 1997).

5.6.2. Age susceptibility

Several studies have pointed out differences in the susceptibility of some fish species on the basis of its age. Fish of body weight ranging from 2 to 80 g are more susceptible to the severe form of the disease, while fish above 100 g appears to be resistant (Mcvicar & White 1979, Wakabayashi *et al*. 1984, Bernardet *et al*. 1994, Handlinger *et al*. 1997, Avendaño-Herrera 2005). Smaller fish have greater susceptibility to *T. maritimum*, as severe destruction of the affected tissues could progress from early stages to advanced ulcerative lesions within a few days (Bernardet *et al*. 1994). The same results have been confirmed by Toranzo *et al*. (2005) who observed that both adult and young fish are susceptible to this pathogen, but the youngest ones are more seriously affected.

5.6.3. Prevalence

The first major outbreak of *T. maritimum* has been reported in the summer of 1988/89. Fish in the marine cage located in south-eastern Tasmania, which were up to 30 km apart, were simultaneously affected, the morbidity rate was reported as high as 30% with significant losses (Handlinger *et al*. 1997). Moreover, an increase in the prevalence and severity of the disease at higher temperatures (above15°C) and salinities (30 to 35‰) were reported, as well as with low water quality. Many authors proved that tenacibaculosis is often associated with poor environmental conditions (i.e. high density, poor feeding and water temperature above 15°C), fish stress and mechanical damage of the skin (Chen *et al*. 1995, Santos *et al*. 1999, Magariños *et al*. 1995, Handlinger *et al*. 1997).When these adverse conditions occur, the prevalence of the causative agent in different internal organs increase (Alsina & Blanch 1993, Cepeda & Santos 2002, Avendaño-Herrera *et al*. 2004). Although, the increase in seawater temperature around Tasmania resulted in an increase in the bacterial incidence (from minor outbreak to major chronic one) (Van-

Geldern *et al*. 2009), winter outbreaks of tenacibaculosis have also been reported (Wakabayashi *et al*. 1984, Bernardet *et al*. 1994, Soltani *et al*. 1996). Abd El-Galil & Hashem (2012) also reported the highest disease prevalence in black damsel, Picasso trigger, and broom tail wrasse fishes (20, 16.7 and 28.6%) during winter and the lowest during summer (0%). López *et al*. (2010) recorded three outbreaks of tenacibaculosis during March, April-, May and January in wedge sole fish cultured in south-western Spain when water temperature was 20.5 °C. These records were mainly attributed to the water temperature, as during the winter it mainly ranged from 15 to 20 °C, which is considered the ideal growth condition for *T. maritimum*.

5.6.4. Virulence mechanisms

Microorganism virulence can be defined as its ability to overcome the host immune response and undergo replication and multiplication with the subsequent development of the disease. It is presumed that the presence of highly virulent strains of *Tenacibaculum sp*. is usually associated with organic matter contaminant, which considered a predisposing factor for disease induction. This fact is also correlated to *Flavobacterium columnare* (Bernardet & Grimont 1989).

The first step for a successful colonization of bacteria within the host is adherence. There are two adhesion forms, specific and non-specific, that allows bacteria proliferation within the host tissue and facilitates the dispersal of any toxins (Ofek & Doyle 1994). Specific adhesion is induced through specific compounds present on the surface of the bacterium that bind to receptors present on the host tissue, whereas non-specific adhesion depends on hydrophobic or ionic interactions between some structures on the surface of the bacterium and the supporting substrate (Ofek & Doyle 1994). Burchard *et al*. (1990) observed that the adhesion capacity of *T. maritimum* significantly increases on substrates with low-critical energy surfaces (e.g. hydrophobic). This was attributed to the ability of the pathogen to produce extracellular polymers or slime which allow its firmly attachment to hydrophobic surfaces. This characteristic feature provided indications concerning the mechanism and the pathogen capacity to adhere to external body surfaces of many fish species, once the infection was established. This hypothesis was later confirmed by Magariños *et al*. (1995) who stated that *T. maritimum* strongly adhered to turbot (*Scophthalmus maximus*), seabream and seabass skin mucus, and the adherence was not affected by the salt solution in which the mucus was dissolved. Moreover, *in vitro* work established by Van-Gelderen *et al*. (2010) proved the adhesive nature of different *T. maritimum strains.*

Besides stickiness, the hemagglutinating activity of *T. maritimum* may also influence its virulence. Pazos (1997) reported that *T. maritimum* cells agglutinate a broad spectrum of

erythrocytes. Extracellular products (ECP) of this species show some proteolytic activity with the ability to degrade gelatin, amylase, casein and nucleases (Pazos 1997). Bacterial toxins present in ECP may also present a role during infections either by facilitating bacterial colonization and invasion or via alteration and erosion of the host tissue (Baxa *et al*. 1988). In the Atlantic salmon (*Salmo salar*), *T. maritimum* toxins caused cellular necrosis in the gills, heart, and pyloric caeca, as well as autolysis and rapid lysis to the cell, similar to the one observed on the host external surface during infection (Van-Gelderen. *et al.* 2009). Another putative virulence factor observed in *T. maritimum* is the ability of this pathogen to express high affinity iron uptake mechanisms, which compete with the host iron-binding proteins (Avendaño- Herrera *et al*. 2005).

5.7. *T. maritimum* vaccination

Regardless the impact of tenacibaculosis, relatively few endeavours at vaccination has been described till the end of 1990. At this moment, only one bacterin is commercially available to prevent turbot mortalities caused by *T. maritimum* (Avendaño-Herrera *et al*. 2006). As this disease affects both juvenile and adult turbot, the vaccine is applied by bath when the fish are 1 to 2 g, followed by a booster injection when they attain 20 to 30 g. The relative percentage of survival following bath immunization is about 50%, but the protection increases above 85% after intraperitoneal booster injection. The regular use of this vaccine in some turbot farms has reduced the incidence of tenacibaculosis (Avendaño-Herrera *et al*. 2006).Atlantic salmon (*Salmo salar* L.) injected with a vaccine plus adjuvant showed much better survival rate than other groups after a challenge period of 27 days (van Gelderen. *et al*. 2009). This indicates that the vaccine without adjuvant could not provide enough protection against a moderate challenge of *T. maritimum*.

It has been reported that lipopolysaccharides are the main protective antigens of this pathogen (Salati *et al*. 2005). Nevertheless, the serological diversity mentioned by Toranzo *et al*. (2004) indicates that the vaccine developed for turbot is not effective in preventing tenacibaculosis in other fish species. New bacterins specific for cultured sole have been developed and evaluated by many scientists. There are still considerable gaps of knowledge concerning the pathogen route of entry and survival strategies, the host mechanisms against infection, and the establishment of adequate vaccination programmes for economically important marine fish. There is no effective infection model and few data are available regarding interactions between *T. maritimum* and Senegalese sole (Costas *et al*. 2013).

6. Fish immune system

According to FAO, aquaculture is expected to continue increasing and the production will be duplicated in the next 25 years. The development of the aquaculture industry mainly depends on the control of reproduction, the understanding of farmed fishes biology, technological innovation and development of specific feeds. Nowadays, there is a great tendency to adopt intensive culture farming practices that expose fish to stressful conditions; this often results on diseases-related problems that result in severe economic losses. Controlling fish pathogens by drug administration is forbidden and harmful, due to its cumulative effect on the fish tissues. On the other hand it may induce drug resistant mechanisms by many bacterial species.

Fish are free-living organisms from their embryonic stage of life and in continuous contact with their environment that contain high concentrations of bacteria and viruses. Many of these are saprophytic, some are pathogenic and both may cause tissue degradation and degeneration. Under normal satisfactory conditions, fish have the ability to maintain its healthy state against potent invader microorganisms through a highly complicated defence system, named the immune system (Rombout *et al*. 2005, Uribe *et al*. 2011). Fish are considered important models for comparative immunological studies as represent one member of lower vertebrates and hence serve as an essential link to early vertebrate evolution (Zhu *et al*. 2013). Bony fishes are considered one of the earliest divergent vertebrate lineages and exhibit both innate and acquired immune systems (Magor & Magor 2001).

The immune system of the fish not only gives some sort of protection through preventing the attachment of microorganisms to the external body surface, but also avoids microbes' invasion and multiplication inside the host tissues. The immune system also maintains stable condition (homeostasis) following inflammatory reactions and tissue damage that take place during infections (Magnadottir 2010). Fish immune system is unique among vertebrates due to the absence of bone marrow and lymphatic nodules (Cuesta *et al*. 2008). Therefore, the head-kidney is the main lymph-haematopoietic tissue in fish and is the primary site for the development and production of B cells. The thymus is the main tissue responsible for the development and maturation of T cells, whereas the spleen is the main secondary lymphoid tissue in fish. The fish immune system is divided into innate (also called non-specific) and adaptive immune system (or specific), both types presenting cellular and humoral defences. Fish possess an inherited and acquired immunological response, but in the case of teleost, the innate immune response is considered a more important defence mechanism when compared to endotherms since it is relatively temperature independent (Magnadottir 2006). The innate responses have the ability to stop microbial attacks or keep them in check until an efficient adaptive immune response

has been developed. It's importance is attributed to many reasons, one of them is the acquired specific immune system that being temperature dependent takes a considerable time to respond (Magnadottir 2006). In salmonids, antibody production takes at least 4-6 weeks, even at optimum rearing temperature, resulting in limited antibody repertoire (Ellis 2001). Therefore, many pathogens can induce mortalities within a few days of infection without any response. The protection afforded by the specific response is only important in previously immunized and vaccinated fish with a dramatic history concerning molecular recognition. The proliferation of lymphocytes which are the most important cellular compound of immune response occurs very slowly (Alexander & Ingram 1992). Therefore, the signs of inflammation including redness, heat, swelling, and pain are usually initiated by the innate immune system. The pathogen recognition system depends on a set of receptors (PRRs) that are able to distinguish infectious from non-infectious invaders (Aoki *et al*. 2008). All these receptors have specific tasks, among which is included the opsonization, complement activation and phagocytosis (Pasare & Medzhitov 2004). These PRRs activate tissue macrophages to produce cytokines which in turn, activate the hepatocytes to produce acute phase proteins, resulting in complement activation and opsonization. Activated complement cascades have strong chemotactic activities to macrophages and neutrophils that in turn will kill the invading pathogen by phagocytosis, coagulation, encapsulation and production of ROS and NOS during respiratory burst (Akira *et al*. 2006, Alvarez- Pellitero 2008).

Fish immune systems include several response components classified as physical barriers as well as cellular and humoral defences (e.g. growth inhibitors, lytic enzymes, complement system, lectins such as agglutinins and precipitins, natural antibodies, cytokines, chemokines and anti-bacterial peptides). The physical barriers are represented in skin, mucus and gills and considered the first line of defence against microbial invasions (Shephard 1994, Ellis 2001). Skin is the most important external structure that covers all body surfaces of the fish, protecting it, not only from the entry of pathogens, but also preventing the leakage of water, solutes and nutrients (Ellis 2001). The protective role of the skin is influenced by the mucus and epidermis stratified cellular sheets. The mucosal surfaces of the fish including gills, skin and gastrointestinal tract act as the main physical barriers between internal structures and the external environment with highest microbial exposure. All these surfaces are guarded by protective mucus layers which greatly contribute to defence mechanisms (Woof & Mesteckey 2005). Furthermore, skin mucus also serves as a depository of many biologically active substances and numerous defensive molecules from both innate and acquired immune system (Palaksha *et al*. 2008, Tadiso *et al*. 2011).

Certain pathogens can overcome the antimicrobial activity of skin mucus and penetrate host tissues resulting in disease induction. In such cases, fish activate cellular responses including specialized cells that are capable of killing and digest the pathogens once they pass the physical barriers (Aoki *et al*. 2008). The main cellular components involved in immune defence against microbial invasion are granulocytes, monocytes or macrophages, lymphocytes (including both T and B cells), and the non-specific cytotoxic cells (Evans *et al*. 2001, Neumann *et al*. 2001). The cellular factors have well-developed recognition receptors systems for detection and signalling of microbial infection (Medzhitov 2007, Takeda *et al*. 2003). Other soluble factors that play an important role in innate immune response are humoral parameters. These parameters can be activated by certain cellular and molecular receptors soluble in plasma and other body fluids (Magnadottir 2006, Subramanian *et al*. 2007). Although the majority of fish humoral factors show many similarities to those of the mammalian and other vertebrates, certain parameters have great variations. Previously, it was believed that fish only had one immunoglobulin isoform IgM, but this idea was recently completely changed as the presence of other types of immunoglobulin isoforms including (IgD, IgZ, and IgT) was described in several studies (Hsu & Criscitiello 2006, Hikima *et al*. 2010).

7. Innate immune system recognition patterns (PAMPS)

The innate immune system recognizes all multicellular organisms either pathogenic or saprophytic either via germ-line encoded pattern recognition receptors (PRR), or pattern recognition proteins (PRP) (Janawy 1989, Elward & Gasque 2003). These PRRs have the ability to detect and sense particular components and structures of the micro-organisms called pathogen associated molecular patterns (PAMPs). This interaction results in the recruitment of phagocytic cells, activation of some antimicrobial enzymes, and induction of both innate and adaptive immune responses (Medzhitov 2007, Kaway & Akira 2010, Takeuchi & Akira 2010). There are two types of molecular patterns involved in fish immune responses: a PAMP and a tissue damage molecular pattern which results from infections, necrotic changes and natural cell death (Magnadòttir 2006). The typical PAMPs recognized are lipopolysaccharides (LPS), peptidoglycans (PGNs), porins and flagellins that are either present in the bacterial cell wall or released from bacteria just after contact with the target cells, fungal $\beta_{1,3}$ glucans, viral RNA and bacterial DNA (Magnadòttir 2006). These molecular patterns are released during infection, inflammatory processes, normal cell deaths and act as signals for immune response activation (Matzinger 1998). Furthermore, the carbohydrates present in apoptotic cell surfaces could be identified by certain cell receptors after its modification to form sialic acid (Elward & Gasque 2003).

Gram-negative and Gram-positive bacteria actually differ regarding their structure, as Gram-negative bacteria contain an outer membrane (OM) that is absent in G-positive ones (Boltãna *et al*. 2011). The outer membrane is consisting of phospholipids, proteins, lipoproteins and LPS while the inner membrane mainly consists of phospholipid and proteins. The periplasmic space contains some proteins and enzymes that play an important role in nutrient gain. Furthermore, it contains PGNs that are responsible to synthesis and modify toxins such as penicillin (Funahara & Nikaido 1980, Nikaido 1989). The LPS consists of three parts: lipid A, a core oligosaccharide, and O-specific polysaccharide (O-antigen). The active component of LPS (lipid A) shows great chemical structure complexity and antigenic cross-reactivity (Rietschel *et al*. 1998), therefore, immunological identification of organisms was almost based on saccharides bases (core and O-antigen) attached to the lipid A (Boltãna *et al*. 2011). According to the previously mentioned facts, the Gram-negative LPS must be considered a highly heterogeneous group of PAMPs and the presence of non-LPS contaminants should be considered in view of PRR-PAMP interactions.

8. Innate immune system Pathogen recognition receptors (PRR)

The pathogen recognition receptors (PRRs) are soluble components either present in the plasma or expressed as receptors on phagocytes and other humoral cell membrane (anchored TLRs) innate components (Magnadòttir 2006). All PRRs have the ability to recognize different PAMPs. The main function of PRRs is to provide immediate protection against invading pathogens (Akira *et al*. 2006). They also serve as phagocytic transmembrane receptors (e.g. the mannose receptor and dectin-1) or soluble proteins that are involved in complement activation and opsonisation (e.g., mannose-binding lectin) (Fraser *et al*. 1998). For continuous interactions and coordination, multiple PRR-signalling pathways are required to determine the site and place of microbial colonization whenever symbiotic coexistence, asymptomatic infection and virulent disease (Brodsky & Medzhitov 2009). Receptors which have a binding affinity with LPS of bacterial cell wall have been detected in rainbow trout and seabream macrophages (Mulero *et al*. 2001, Kiryu *et al*. 2003). Teleost fish demonstrate a wide range of PRRs which are involved in the immune response against pathogens. Four main types of PRRs have been described to date in fish (Boltãna *et al*. 2011): Toll-like receptors (TLRs), NOD-like receptors (NLR), C-type lectin receptors (CLRs) and PGRPs (peptidoglycan recognition proteins).

The PGRPs recognize peptidoglycans of both from Gram-positive and Gram-negative bacteria. In zebrafish, three PGRPs genes showed amidase activity and were supposed to be secreted as a mammalian contraceptive (Li *et al*. 2007). The putative signal peptide obtained from PGRPs sequencing in both yellow croaker and rockfish, indicates its

fundamental secretion (Kim *et al*. 2010, Mao *et al*. 2010). The CLR family includes proteins which have at least one carbohydrate recognition domain, but do not always bind carbohydrate structures and may exist as soluble or as trans-membrane proteins (Geijtenbeek & Gringhuis 2009). To date, seven transmembrane CLRs have been described in fish. CLRs are mainly expressed in immune tissue including head kidney cells and peripheral blood leukocytes following stimulation with different pathogens (Chen *et al*. 2010, Goetz *et al*. 2004, Soanes *et al*. 2008).

NLRs are cytosolic receptors which are involved in autoimmunity, and have the ability to recognize different pathogens such as bacteria, virus, as well as some reactions resulting from apoptosis (Inohara *et al*. 2005, Ting *et al*. 2006, Martinon & Tschopp 2005). The NLRs sense pathogens have three different structural domains: a central nucleotide binding domain, a C-terminal leucine-rich domain (LRR) and the N-terminal domain that is mostly involved in apoptosis (Takeuchi & Akira 2010). In teleost fish, NLRs form a large family of receptors that have been divided into three subfamilies (A, B and C) (Laing *et al*. 2008). The NLR-A family, composed of five members, contains mammalian NOD 1-5 orthologues. NOD receptor 1 and NOD like receptor 2 share the same function of TLRs in that these receptors activate NF-kB and induce the expression of pro-inflammatory cytokines (Strober *et al*. 2006). NLR-B family, composed of six members, contain NLR apoptosis inhibitory proteins (Naips), NACHT, LRR, and pyrin domain-containing proteins (Nalps). NLR-B family controls the activation of the inflammasome, a protein complex which in turn regulate the activation of IL-1β from immature to mature stage before being released (Mariathasan & Monack 2007). Moreover, Naips and Nalps recognize bacteria and possibly other classes of microbes by specific patterns that resemble those recognized by TLRs.

TLR group which consist of 10-15 members in most mammalian species is a well-characterized family that comprises membrane and endolysosomal receptors (Kaway & Akira 2010). It is considered the main recognition receptor group involved in inflammation. All TLRs contain an extracellular domain with a series of leucine-rich repeats (LRRs), and an intracellular domain with a conserved signalling module called a Toll/IL-1 receptor (TIR) domain (Boltãna *et al*. 2011). Bacterial PAMPs are mainly recognized by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 (Kumar *et al*. 2011). The main TLR legend that has the ability to detect LPS which is the main constituent of the outer membrane of Gram-negative bacteria is TLR4, while peptidoglycan, lipoteichoic acid, and zymosan are recognized by TLR2. Double-stranded RNA, flagellin, single-stranded RNA, and non-methylated CpG motifs in DNA were recognized by TLR3, TLR5, TLR7 and TLR8, and TLR9, respectively (Boltãna *et al*. 2011). Several members of TLRs have been recognized in teleost fish, including TLR1 in pufferfish (*Tetraodon nigroviridis*) (Wu *et al*. 2008), TLR3

in common carp (*Cyprinus carpio*) (Yang & Su 2010), TLR1 and TLR2 in orange-spotted grouper (*Epinephelus coioides*) (Wei *et al*. 2011), TLR5S, TLR20, and TLR21 in catfish (*Ictalurus punctatus*) (Baoprasertkul *et al*. 2007), and TLR22 in large yellow croaker (*Pseudosciaena crocea*) (Xiao *et al*. 2011). However, TLRs in fish have different characteristic features and show a great heterogeneity compared to mammalian orthologues (Meijer *et al*. 2004, Phelan *et al*. 2005). TLR-3 in zebrafish and rainbow trout (Rodriguez *et al*. 2005), and TLR-5 in rainbow trout (Tsujita *et al*. 2004) showed the same functional analogy to mammalian counterparts. Recently, at least 17 TLRs have been found in teleost, among which TLR14, TLR19, TLR20, TLR21, TLR22, and TLR23 are non-mammalian origin, and the TLR 5S is a soluble isoform of TLR 5 that appears to be unique in fish (Palti 2011). Moreover, TLR4 that recognizes lipopolysaccharides (LPS) of Gram-negative bacteria in mammals has no function in LPS recognition in zebrafish and is not present in most of other fish species (Jault *et al*. 2004).

TLRs are expressed by many phagocytic cells including macrophages, neutrophils, and dendritic cells and they recognized certain components of bacteria, viruses, fungi, and protozoa to activate these phagocytic cells (Akira *et al*. 2001, Medzhitov 2001). In mammals, the main role of TLRs is to activate certain inflammatory cascades such as NF-kB, IFN-regulatory factor (IRF) and transcription factors (Kawai & Akira 2007). These factors regulate the releasing of cytokines, and chemokines such as IL-1β, IL-6, TNF-α, CC-1 and MCP-1, which in turn induced the acute inflammatory response against invading pathogens (Taylor *et al*. 2005, Gordon & Taylor 2005). Therefore, production of ROS (reactive oxygen substances), and activation or declination of some pro-inflammatory genes could serve as indicators for TLR activation (Kaway & Akira 2007, 2010).

9. PAMPS - PRR interaction and induction of Immune system

Pathogen express different pathogen associated molecular patterns (PAMPs). The combination of these patterns with pathogen recognition receptors (PRRs) are essential for integration, activation and opsonization of many target genes which in term promote and accelerate pro-inflammatory mediators (Boltaña *et al*. 2011). Each receptor has specific affinity for recognition of a certain molecular pattern of the pathogen and initiates pro-inflammatory immune responses. Several studies in fish immunology have recognized distinct gene expression profiles and specific cassettes of responsive genes that regulate their patterns in many different fish species following challenge with specific pathogens. For instance, C-type lectin 2-1, a gene whose product is involved in the C/EBP-driven inflammatory response has been identified when bacterial preparations have been used to challenge live fish (Ewart *et al*. 2005, Martin *et al*. 2006, Mackenzie *et al*. 2008). Nowadays, a great attention was paid to Gram-negative bacteria recognition in fish, to

understand the structure-function relationships between G-negative bacteria and PAMPs and fish PRRs. Studies applied microarray-based approaches using both *in vivo* infection and *in vitro* PAMP-cell (Boltaña *et al*. 2011). Across all vertebrates, Toll-like receptors (TLRS) are the main classical receptors that recognise patterns usually transduced into pro-inflammatory signals that activate later numerous transcription factors, including kappa B factors and AP1 (Tato & Hunter 2002, Ghosh & Hayden 2008). Many of these signals are transferred through dendritic cells (DCs) to secondary lymphoid organs and T cells for releasing of cytokines and other mediators resulting in final activation of macrophages, neutrophils and mast cells that are drive to the site of infection for pathogen elimination.

The recognition of LPS is usually accompanied by the release of pro-inflammatory mediators, such as TNFα, IL6, and IL1β which act on local sites of infection to enhance inflammation and host immune response (Boltãna *et al*. 2011). Many studies revealed that LPS stimulated cytokine expression in many fish species such as *Paralichthys olivaceus, Danio rerio* and *Cyprinus carpio* leukocytes (Hirono *et al*. 2000, Zou *et al*. 1999) and in *Oncorhynchus mykiss* macrophages along with IL6 and TNFα (Mackenzie *et al*. 2010). Similarly, the expression of cyclooxygenase 2 (COX2),key mediator of prostaglandin mediated inflammatory responses, has been reported after LPS challenge in *Carassius auratus* and *O. mykiss* (Mackenzie *et al*. 2010, Zou *et al*. 1999). Peptidoglycans which represent half of the cell math in Gram-positive bacteria and a thin rim layer in the Gram-negative ones (Ghuysen 1968) have the ability to recognize many pathogens via peptidoglycan recognition proteins (PGRPs) and initiate the inflammatory processes (Li *et al*. 2007). It can also stimulate specific PGN-recognition-pathways in macrophages and enhance phagocytic activities (Boltaña *et al*. 2011). Furthermore, PGNs stimulate pro-inflammatory cytokines such as (IL1α and IL6) and cyclooxygenase 2 expression in macrophages as well as the release of prostaglandin E2 and D2 (Mackenzie *et al*. 2010, Boltaña *et al*. 2011). Porins are components of the outer membrane protein (OMP) of Gram-negative bacteria. In fish a few studies suggest that protective and damaging effects during *in vivo* challenges with bacteria have occurred (Dooley & Trust 1988, Gudmundsdottir & Bjornsdottir 2007, Lutwyche *et al*. 1995). Motile bacteria have complex surface organelles, known as flagella responsible for the motility. The flagellin is a ligand for TLR5 (Akira *et al*. 2006). Little is known about fish immune responses to flagella. In salmonids, flagella induced NF-$_K$B activity via TLR5 in *O. mykiss* (Tsujita *et al*. 2004), and facilitate chemotactic motility during infection (O'Toole *et al*. 1996, 1999). The external membrane of the Gram-negative bacteria contains lipoproteins (LP) which induce inflammatory responses through activation of mammalian monocytes, macrophages, and other lymphocytes. This resulted in the production of pro-inflammatory cytokines such as

(TNFα, IL-1β, and IL6) which in turn initiate immune response via TLR2 (Takeuchi & Akira 2010, Akira 2003).

10. Inflammatory responses and phagocytic activities against bacterial infection

10.1. Initiation of the inflammatory processes

The inflammatory processes that precede bacterial infection are induced after attachment and entry of the pathogen within the host tissue, resulting in the production of potent bacterial inhibitors and phagocytic cells which have strong bactericidal activities. The main bacterial growth inhibitors released during the inflammatory processes are transferrin, anti-proteases enzymes (Takeuchi & Akira 2010). These enzymes play an important role in minimizing and sustaining the bacterial growth. Many studies have been established to investigate the mechanisms of host immune response against bacterial infections. The inflammatory responses of rainbow trout (Afonso *et al*. 2000), and seabass *Dicentrachus labrax* (Salati *et al*. 2005) following intraperitoneal injection with *T. marititum* formalin-killed strain, extracellular product and crude lipopolysaccharide have been evaluated. The results revealed a significant increase in the total numbers of macrophages and neutrophils after few hours post injection comparing to their initial number, attributing that to the bactericidal and the phagocytic nature of both macrophages and neutrophils.

10.2. Control of inflammation

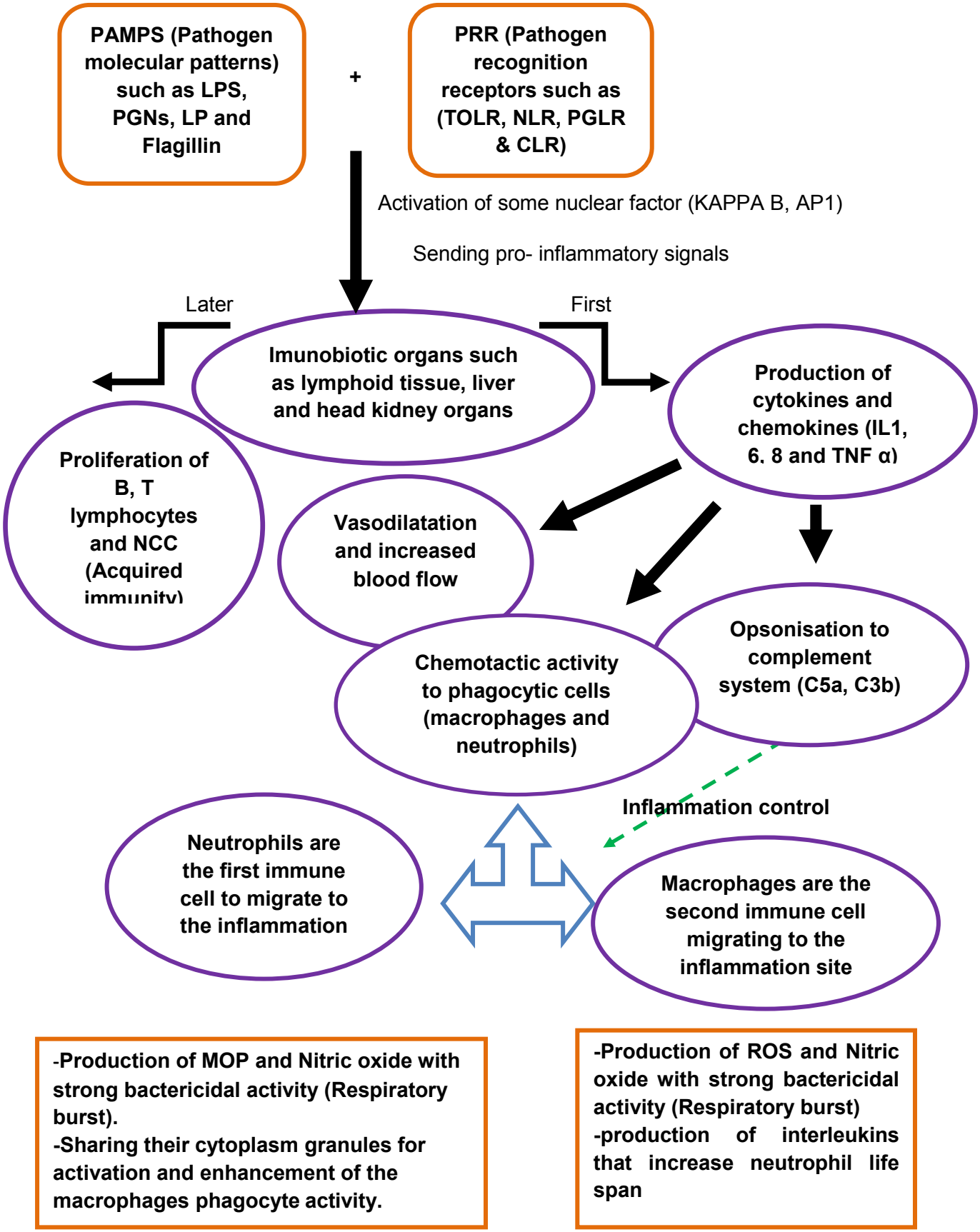
There are several blood enzymes, including clotting enzymes, kinin and complement that play vital roles in the inflammatory control of mammalian tissues. Similar enzymes have been discovered in fish and showed great similarities to their mammalian counterparts (Secombes 1996). Once the bacteria enter the tissue the complement system is activated either directly, through alternative pathway (ALP) or indirectly by lectins or c-reactive proteins. Afterwards, two main complement cascades C5a and C3b are activated. C5a cascade has chemotactic activities for macrophages and neutrophils to the site of inflammation, while C3b remains attached to the bacterial cell wall untill phagocytosis (Yano 1996, Jenkins & Ourth 1993). Furthermore, thrombocytes and eosinophilic granules cells (EGCs) play an equivalent role in the control of inflammation. The degranulation cells enhance the release of histamine like products (5-HT) when induced by bacteria or bacterial products (Matsuyama & Iida 2001). These products have a vasodilating effect resulting in extravasations of neutrophils and monocytes to the site of infection. The cell wall of most Gram-negative bacteria contains lipopolysaccharides LPS which acts as a starter for production of cytokines, mainly IL-1β, after being recognized by PRRs (Scombes *et al*. 1999). Other leukocytes with chemotactic activity have been recorded by

Rowley *et al*. (1995). All these cytokines and chemokines participate in the control process to minimize bacterial invasion and propagation.

10.3. Phagocytic mechanism

Phagocytosis is a process by which the macrophages and neutrophils kill and engulf invading pathogens including bacteria. First of all, the initiation of this mechanism requires the attachment between bacteria (via many PAMPs on it is cell wall, such as LPS, peptidoglycan, flagellin and lipoproteins) and macrophages surface by hydrophobic bond or sugar-lectin interaction (Secombes 1996). The most active promoters for phagocytosis are C5a and C3b complement cascades. After attachment, activated macrophages produce reactive oxygen species (ROS) and nitric oxide (NO) of potent bactericidal activity during respiratory burst (Secombes 1996, Lamas & Ellis 1994). Macrophage bactericidal activities appear to be species specific and strain virulence dependant. Costas *et al*. 2013 showed that Senegalese sole leucocytes responses have significant variations following stimulation with two *T. maritimum* strains (ACC6.1 & ACC13.1), attributing that to the degree of virulence of such strain. Neutrophils which are the main cellular component involved in innate immunity contain large amounts of myeloperoxidase (MPO) in their cytoplasmic granules (Ellis 1999, Afonso *et al*. 1997). In the presence of halide ions and H_2O_2 they can kill bacteria by halogenation of their cell wall and by production of bactericidal halophyte ions (Klebanoff & Clark 1978). Several studies against *T. maritimum* have been conducted in different fish species and revealed that the pathogen presented an increased susceptibility to the phagocyte killing activity (Barnes *et al*. 1999, Costas *et al*. 2013). Not only macrophages and neutrophils work independently but also, synchronization and coordination between them occurs. Studies on the inflammatory response of rainbow trout against *Y. ruckeri* showed that resident macrophages which also phagocytosed the bacteria have been observed to phagocytose neutrophils containing bacteria. Furthermore, neutrophil appears to transfer MPO granules to the macrophages when they closely contact (Afonso *et al*. 1998).

Diagram illustrating the inflammatory process and its control



Objectives

The main goal of this thesis is to allow a better understanding of the immune mechanisms and disease resistance in Senegalese sole challenged with *Tenacibaculum maritimum*. In particular it is intended to:

1. Optimize bacterial culture conditions to avoid bacterial fluctuation and aggregation.

2. Developing of an effective and reproducible infection model against Senegalese sole.

3. Investigate the cellular immune responses following stimulation with whole bacteria, extracellular product (ECP) and lipopolysaccharides (LPS) of different *T. maritimum* strains (*in vitro* study).

4. Assess the mucus and plasma innate immune response and cell migration dynamics following challenge with *T. maritimum* (*in vivo* study).

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Chapter 2

Tenacibaculosis induction in the Senegalese sole (*Solea senegalensis*) and studies of *Tenacibaculum maritimum* survival against host mucus and plasma

Mahmoud Mabrok[1,2,3], Marina Machado[1,2], Cláudia R. Serra[1], António Afonso[1,2], Luísa M.P. Valente[1,2], Benjamín Costas[1,2*]

[1]CIIMAR/CIMAR, Centro Interdisciplinar de Investigacão Marinha e Ambiental, Universidade do Porto, Porto,Portugal.

[2]ICBAS–UP, Instituto de Cîencias Biomedicas Abel Salazar, Universidade do Porto, Porto, Portugal.

[3]Department of Fish Diseases and Management, Faculty of Veterinary medicine, Suez Canal University, Egypt.

Keywords: Disease resistance; Growth curve; Lethal Dose 50; lipopolysaccharides; host bactericidal activity; complement activity.

Tenacibaculosis induction in the Senegalese sole (*Solea senegalensis*) and studies of *Tenacibaculum maritimum* survival against host mucus and plasma

Abstract

Tenacibaculum maritimum, the aetiological agent for marine tenacibaculosis, is one of the most significant pathogens that threaten Senegalese sole, *Solea senegalensis* (Kaup), aquaculture. Since no immersion challenge with *T. maritimum* has been reported previously for this flatfish species, the present study aimed to optimise bacterial yields as well as to establish a challenge model for tenacibaculosis induction. Several approaches were performed to optimise bacterial culture conditions, including treatment with non-ionic surfactants, detergents, celullase hydrolysis and strong shacking. A prolonged bath challenge was performed for 24 h under two different temperatures, 16 and 23 ºC. Moreover, mucus and plasma bactericidal activities against *T. maritimum* were also assessed. Culturing bacteria with strong and continuous shaking provided suitable culture conditions to obtain higher bacterial yields without aggregation or fluctuation, contrarily to most other treatments that showed a huge amount of bacterial aggregates. A prolonged bath method for 24 h, without skin or gill scarification, was considered suitable for disease induction with high mortality rates. Moreover, data regarding mucus and plasma bactericidal activities suggested that there is a lack of host innate immune response against *T. maritimum* or that this particular pathogen presents evading strategies against Senegalese sole.

1. Introduction

Global aquaculture is a major responsible for fish supply as food and its importance has been increasing during recent years. Southern Europe in particular offers high potential for this activity and Senegalese sole, *Solea senegalensis* (Kaup), presents itself as an opportunity for the Mediterranean aquaculture diversification. However, disease outbreaks appear to be the most significant limitation to the development of the industry (Morais *et al.* 2014). One of the most significant pathogens that threaten Senegalese sole aquaculture is the Gram-negative bacterium *Tenacibaculum maritimum*, the aetiological agent for marine tenacibaculosis (Toranzo *et al*. 2005). Likewise other filamentous bacteria, *T. maritimum* presents many obstacles to laboratory work, resulting in a tendency for aggregation, fluctuation and adhesion to all kind of substrates, thus revealing difficulties in detection and recognition of its colonies (Yamamoto *et al*. 2010). The colonies usually exhibit active spreading (swarming) and polymorphism, making the enumeration of bacteria by classical methods very difficult. Therefore, many attempts for isolation and cultivation of filamentous bacteria in pure culture have been done. Ramothokang *et al*. (2003) assessed different methods for effective isolation and cultivation of filamentous bacteria which have been associated with activated sludge bulking. Their study revealed that cellulase hydrolysis appears to be an unsuccessful isolation method, while using a 100% concentrated non-ionic surfactant alleviated cell clumping without any damage to the bacterial cells. Caldara *et al*. (2012) observed that mucin biopolymers have the ability to limit bacterial surface attachment and biofilm formation without killing or trapping bacteria. Moreover, Simões (2011) suggested that adding Tween 80 to the culture medium may prevent *T. maritimum* aggregation.

The establishment of an effective experimental design for exposure of healthy animals to infection is a basic research practice in animal health research. It is of major importance to identify responsible putative microorganisms in disease occurrence. Many studies have already explored different approaches as challenge models with *T. maritimum* in several fish species including red seabream, *Pagrus major* (Temminck & Schlegel), blackhead seabream, *Acanthopagrus schlegeli* (Bleeker), European seabass, *Dicentrarchus labrax* (L.), rainbow trout, *Oncorhynchus mykiss* (Walbaum), greenback flounder, *Rhombosolea tapirina* (Günther), and striped trumpeter, *Latris lineata* (Forster), revealing that mortalities widely differ depending on the method applied (Wakabayashi *et al*. 1984; Baxa, *et al*. 1987; Bernardet *et al*. 1994; Soltani *et al*. 1996). For instance, Atlantic salmon, *Salmo salar* (L.) exposed to gill abrasion and infected with a high concentration of *T. maritimum* (4×10^{11} cells per fish) showed increased mortalities, suggesting the abrasion as main promoting factor for disease induction (Powell *et al*. 2004; Powell *et al*. 2005). Moreover, other authors suggested that infection may preferably occur through the mouth and tail

and that challenge by bathing methods is not effective unless the skin was scarified or abraded (Wakabayashi *et al*. 1984; Baxa *et al*. 1987). In contrast, Avendaño-Herrera *et al*. (2006) proposed a prolonged bath challenge with *T. maritimum* as an effective infection model for turbot but not for Senegalese sole. It has been recently suggested that a subcutaneous route may reproduce the disease in a faster and more reliable way than the intraperitoneal one (Faílde *et al*. 2013), although most previous studies showed that both intramuscular and intraperitoneal routes were not effective in disease induction in several fish species (Wakabayashi *et al*. 1984; Alsina & Blanch 1993; Avendaño-Herrera *et al*. 2006). To the best of our knowledge, no immersion challenge with *T. maritimum* has been successfully performed in Senegalese sole. Therefore, the present work aims to optimize bacterial culture conditions to obtain maximum growth without aggregation, as well as to establish an optimal and reproducible challenge model for Senegalese sole using different *T. maritimum* virulent isolates.

2. Material and Methods

2.1. Experimental fish

Experiments were performed by trained scientists and following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes.Healthy Senegalese sole were obtained from a local fish farm, located in North-West Portugal, with no history of tenacibaculosis. Large-sized fish (176.5 ± 14.3 g) were used for mucus and blood sampling to assure the required sample volume, while fish weighing 25.5 ± 5.9 g were used for infection trials. Prior to the trials, fish were maintained in a recirculating aerated seawater (salinity 33 ± 1) system with mechanical and biological filtration where dissolved oxygen was maintained around 90%, water temperature at 21 ± 1 ºC, and a 12 h light/12 h dark photoperiod was adopted. Fish were fed to apparent satiety with commercial pellets (Skretting LE-2 ELITE, Burgos, Spain).

2.2. Bacterial strains

Three virulent *T. maritimum* strains (ACC20.1, ACC13.1 and ACC6.1) isolated from Senegalese sole in a local fish farm (Portugal) were used during experimental trials. These isolates belong to the serotype O3 described for *T. maritimum* (Avendaño-Herrera *et al*. 2005) and were 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 used. The recovery of all bacterial strains was achieved using marine agar (Laboratorios CONDA, Spain) and *Flexibacter maritimus* medium (FMM; Pazos *et al*. 1996) at 25 °C for 48-72 h. Molecular

diagnosis of all bacterial strains was performed by polymerase chain reaction (PCR) as described by Avendaño-Herrera *et al*. (2004).

2.3. Bacterial culture optimization

Trials were carried out under different conditions as follows:

2.3.1. Non-ionic surfactants treatment. Igepal CA-630 (Sigma, Germany) is a non-ionic surfactant that alleviates cell clumping without any obvious damage to the cells. Different concentrations (0.1%, 1%, 10% and 100%) were mixed to marine broth (Laboratorios CONDA, Spain) according to Ramothokang *et al*. (2003). Briefly, marine broth was pre-treated with a ten-fold dilution of Igepal CA-630 after adjusting the proper concentrations, followed by vigorous shaking for 2 min using a vortex. The mixture was autoclaved at 121 ºC for 15 min. Additionally, 5 mL of Tween 80 (10%; Sigma) were added to 45 mL of marine broth, autoclaved and stored at room temperature until used, following the procedure of Simões (2011).

2.3.2. Cellulase hydrolysis treatment. Cellulase is an enzyme complex capable of decomposing cellulosic polysaccharides into smaller fragments. Briefly, the enzyme was dissolved in distilled water at a concentration of 1 mg L^{-1} according to Ramothokang *et al*. (2003). Then, 1 mL of the enzyme suspension and 4 mL of a 0.05M acetic acid (Sigma) were added to 45 mL of marine broth, subsequently sterilized by filtration.

2.3.3. Detergent treatment. A stock solution of sodium dodecyl sulfate (SDS 1%; Sigma) was prepared and stored at room temperature. Then, the SDS stock solution was added to marine broth at 1%, 5% and 10% to achieve graded concentrations in a final volume of 50 mL, followed by sterilization by filtration.

2.3.4. Another bacterial culture approach was performed by inoculating the bacterial strains directly into sterile marine broth or *Flexibacter maritimus* broth (FMB) without supplementation. *T. maritimum* was inoculated into 50 mL marine broth kept in 500 mL flask followed by incubation with continuous strong shaking.

For all above culture conditions, bacteria were pre-cultured in 150 mL sterile marine broth at 25 °C for 48 h and 60 rpm. Since many flocculent aggregates were observed under this particular culture condition, and preparation of a homogeneous bacterial suspension was not possible, a recommendation suggested by Yamamoto *et al*. (2010) was followed. Therefore, pre-cultured bacteria for 48 h were submitted to a sedimentation process for 5 min to let the aggregates settle and only the upper part of the broth culture was used. Thereafter, all culture conditions described above were inoculated with 200 µL of this pre-

cultured bacterial suspension previously adjusted to an absorbance of 0.8 at 600 nm. All culture conditions started with an initial volume of 50 mL and were incubated at 25 ºC for 72 h with continuous shaking at 60 rpm, except for the approach specified in section 2.3.4 which was 140 rpm.

Optical density readings were taken every two hours of incubation and performed in duplicates for each strain at 600 nm. Moreover, bacterial counts were manually performed by means of a Helber count chamber for each absorbance reading.

2.4. Lipopolysaccharides extraction and purification from *T. maritimum* strains

Lipopolysaccharides (LPS) were extracted by hot phenol-water according to the method described by Rezania *et al*. (2011) with slight modifications. 100 mL bacterial suspensions were centrifuged at 10,000 x *g* for 5 min and washed twice with 0.15 M PBS (pH=7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Pellets were then re-suspended in 10 mL PBS and sonicated on ice for 10 min. To eliminate protein and nucleic acids contaminants, the samples were treated with proteinase K, DNase and RNase prior to the extraction step. Proteinase K (100 µg mL^{-1}; Roche, Mannheim, Germany) was added to the mixture and the tubes were left for one additional hour at 65°C. Subsequently, the mixture was treated with RNase (40 µg mL^{-1}; Roche) and DNase (20 µg mL^{-1}; Roche) in the presence of 1 µL mL^{-1} 20% $MgSO_4$ and 4 µL mL^{-1} chloroform and incubated overnight at 37 °C. For the next step an equal volume of hot (65-70 °C) 90% phenol was added to the mixtures followed by vigorous shaking at 65-70 °C for 30 min, cooled on ice and then transferred to 50 mL polypropylene tubes and finally centrifuged at 3500 × *g* for 30 min. The supernatants were then transferred to 50 mL conical centrifuge tubes and extra phenol phases were removed using 20 mL hot (65-70°C) distilled water. Sodium acetate at 0.5 M final concentration and 10 volumes of 95 % ethanol were added to the extracts and samples were stored at -20 °C overnight to precipitate LPS. Tubes were then centrifuged at 3500 x *g* 4 °C for 30 min and pellets were re-suspended in 1 mL distilled water, followed by extensive dialysis (Snake Skin dialysis tubing of 10 K MWCO, Thermo Fischer Scientific) against distilled water at 4 ºC. Purified LPS, without any residual phenol, was lyophilized, its dry weight determined and re-suspended in PBS to a final concentration of 2 mg mL^{-1}, being kept at -20 ºC until used. Visualization was achieved by SDS-PAGE (12%) electrophoretic resolution of 20 µg purified LPS and consequent staining following the improved silver stain protocol described by Zhu *et al*. (2012).

2.5. Pathogenicity assays

Acclimated Senegalese sole weighing 25.5 ± 5.9 g were randomly distributed by four closed recirculated seawater systems comprised by six flat-bottomed aquaria (0.05 m^2; 6.5 L). Each aquarium contained groups of 6 fish with aerated sea water and were left to acclimate for one week prior to bacterial challenge. Fish from three of those systems were inoculated with each *T. maritimum* strain at three final concentrations in duplicates: $2.6\times10^{5-7}$, $9.6\times10^{5-7}$, and $4.8\times10^{5-7}$ for ACC6.1, ACC13.1 and ACC20.1, respectively. Bacterial inocula were prepared with sterile marine broth according the method described in section 2.3.4. A prolonged bath method was applied as follows. The recirculation system was stopped and the water volume in each aquarium was lowered. Then, in two different experiments, fish were inoculated with the bacteria in 1 L of seawater (salinity 33 ± 1) at 16 ± 1 ºC or 23 ± 1 ºC with strong aeration for 24 h. Afterwards, the rearing water in each aquarium was changed three times and the recirculation system was re-established. Another group of 36 fish was maintained in the fourth recirculated seawater system under the same conditions. Those fish were inoculated with a sterile saline solution instead of bacteria and served as control. Dead and moribund fish were removed from each aquarium daily and analysed to re-isolate the pathogen. Humane endpoints were stablished for moribund fish and for those showing ulcerative lesions or necrotized skin, since preliminary trials showed that those fish were not able to recover from the disease. The amount of each bacterial strain able to kill 50% of the challenged fish (LD50) was calculated according to the method of Reed & Müench (1938). Any survivors at the end of the experiments were sacrificed by an anaesthetic overdose (1 mL L^{-1}; 2-phenoxiethanol, Sigma) and examined for detection of *T. maritimum*.

2.6. Bacteriological assays

External lesions from dead and moribund fish were examined with wet preparations under a light microscopy for detection of bacterial motility. Samples from external lesions and kidneys were also seeded on marine agar and FMM plates and incubated at 25 ºC for 24-72 h for bacteriological analysis (Pazos *et al.* 1996). Pure colonies were identified using morphological, physiological and biochemical tests according to Santos *et al*. (1999), Gram staining, and results were finally confirmed by nested PCR as previously described elsewhere (Avendaño-Herrera *et al*. 2004). Mucus and tissue samples (mainly head-kidney) from moribund fish were also collected for pathogen identification by nested PCR as described above.

2.7. Mucus and plasma collection

Acclimated Senegalese sole weighing 176.5 ± 14.3 g were anesthetized with 2-phenoxyethanol (1 mL L^{-1}; Sigma) and used for mucus and blood sampling. Mucus samples were carefully scraped from the anterior to the posterior ocular and blind sides using a sterile spatula then diluted 1:6 times with sterile TBS solution (50mM TRIS HCL, 150 mM NaCl adjusted at pH 8). Afterwards, the mixture was thoroughly mixed and centrifuged at 1500 rpm for 10 min and 4 ºC. The supernatant was then collected and sterilised using syringe filters (pore size 0.2 µm; Sarstedt, Germany). For plasma collection, blood withdrawal was performed from the caudal vessel of individual fish using heparinised syringes and thereafter centrifuged at 10,000 x *g* for 10 min and 4 ºC. An aliquot of plasma was also heat-inactivated at 47 °C for 20 min according to Sakai (1981). This procedure was followed to determine the possible involvement of complement components in the plasma bactericidal activity. Mucus and plasma samples were stored at -80 °C until used.

2.8. Assessment of mucus and plasma bactericidal activities against *T. maritimum*

The bactericidal assay was carried out using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described elsewhere (Machado *et al*. 2015). Briefly, 20 µL of mucus or plasma samples were added to duplicate wells of a U-shaped 96-well plate. Marine broth was added to some wells instead of samples and served as positive control. To each well, 20 µL of the three *T. maritimum* strains (ACC6.1, ACC13.1, and ACC20.1; 1×10^8 bacteria mL^{-1}) in marine broth were added and the plate was incubated for 0, 1, 3, 6 and 18 h at 25 ºC. To each well, 25 µL of MTT (1 mg mL^{-1}; Sigma-Aldrich) were added and incubated for 10 min at 25 ºC 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 (Sigma-Aldrich). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was calculated from analytical triplicates and expressed as percentage of bacteria surviving in relation to the number of bacteria from positive controls (100%).

2.9. Data analysis

All results are expressed as means ± standard deviation (SD). Data were analysed for normality and homogeneity of variance and transformed when necessary. All the results expressed as percentage were previously arcsine transformed (Zar 1999). Data were analysed by two-way ANOVA followed by Tukey's post hoc test to identify differences among experimental treatments. All statistical analyses were performed using the computer package Statistica 12 for windows. The level of significance used was $P \leq 0.05$ for all statistical tests.

3. Results

3.1. Bacterial growth

T. maritimum cultured in marine broth supplemented with Igepal CA-630, cellulase and SDS revealed bacterial aggregation and fluctuation under particularly all conditions tested, while marine broth supplemented with Tween 80 suppressed bacterial growth. *T. maritimum* supplemented with 100% Igepal CA-630 did not reveal any bacterial fluctuation, but a decrease in growth was observed when compared to bacteria cultured with no supplementation and strong shacking. Although all bacterial strains cultured with marine broth and FMB with strong shacking showed no fluctuation or aggregation and higher yields compared to the other culture conditions assessed, a decrease in growth was observed in bacteria cultured in FMB compared to those grown in marine broth. *T. maritimum* cultured in marine broth with strong shaking showed a similar exponential growth for all bacterial strains, between time points 6 and 48 h, with maximum growth yields observed after 48 h of incubation, which translated into 4.9×10^8, 4.8×10^8 and 4.85×10^8 cells for ACC6.1, ACC13.1 and ACC 20.1, respectively, and was considered the log phase. The initial slow growth observed between 0 and 6 h was regarded as the lag phase, whereas the stationary phase was observed between 48 and 50 h. After 50 h of incubation the optical density of all bacterial strains began to decrease indicating the beginning of the decline phase.

3.2. Pathogenicity assays

The mortality of the experimentally infected Senegalese sole was assessed for 2 and 4 weeks post inoculation in experiments at high and low temperature, respectively. Fish exposed to all *T. maritimum* strains at 16 ºC did not show any mortality or pathological symptom (results not shown), whereas Senegalese sole exposed at 23 ºC presented different degree of mortalities, depending on bacterial concentration. The strain ACC6.1 produced higher mortalities in a shorter time than ACC13.1 or ACC20.1. In fact, all fish infected with the higher ACC6.1 dose died within 4 days following inoculation. Most fish died during the first week in the case of those exposed to ACC6.1 (LD_{50} of 2.6×10^5; Fig. 1A), while mortalities in those exposed to strains ACC13.1 (LD_{50} of 9.6×10^5; Fig. 1B) and ACC20.1 (LD_{50} of 4.8×10^5; Fig. 1C) extended for up to 12-15 days.

To elucidate possible differences at the level of LPS composition that could explain the observed differences in fish mortality, the LPS of each strain was extracted, purified and visualized by direct staining following separation by SDS-PAGE. Samples from all *T. maritimum* strains revealed a characteristic dark staircase (ladder-like) pattern of bands with a similar profile (data not shown).

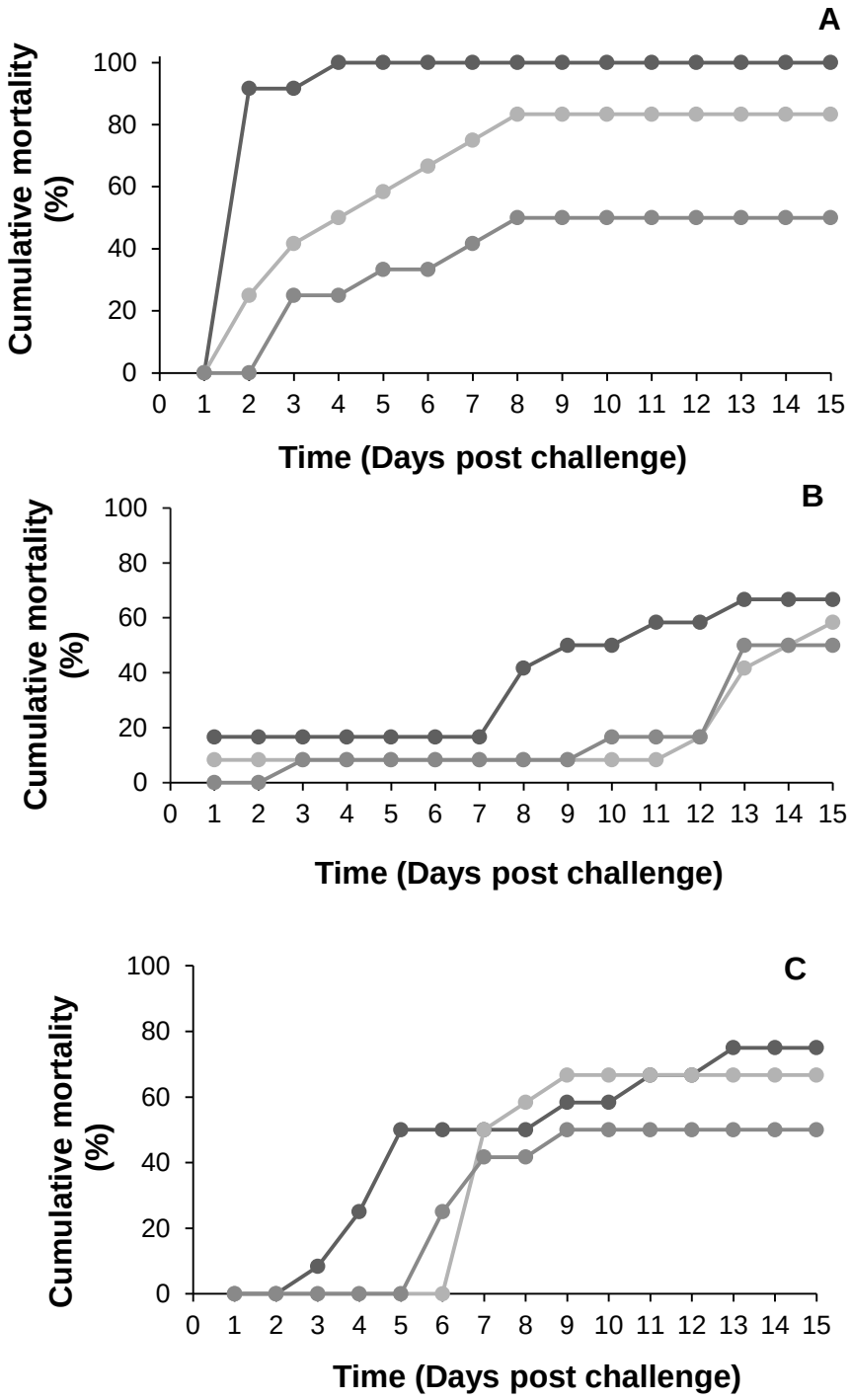

Fig.1. Cumulative mortality of Senegalese sole submitted to a prolonged bath method with different doses of *T. maritimum* strains: (A) ACC6.1 (2.6×10^7 cells mL^{-1}(●), 2.6×10^6 cells mL^{-1} (●) and 2.6×10^5 cells mL^{-1} (●); (B) ACC13.1 (9.6×10^7 cells mL^{-1} (●), 9.6×10^6 cells mL^{-1} (●) and 9.6×10^5 cells mL^{-1} (●); (C) ACC20.1 (4.8×10^7 cells mL^{-1} (●), 4.8×10^6 cells mL^{-1} (●) and 4.8×10^5 cells mL^{-1} (●).

All infected fish showed skin ulcerations mainly in the ocular side, tail rots, red mouth, corneal opacity and fin erosions (Fig. 2A and 2B). Internally, all fish exhibited pale gills, friable livers and severely congested kidneys (Fig. 2C).

All bacterial strains were successfully isolated from external and internal lesions on marine agar and FMM media after 48 h incubation period. On FMM plates, all colonies from each bacterial strain appeared pale yellow with rhizoid edges and exhibited swarming, while on marine agar plates colonies were flattened and strongly adherent. All samples from external and internal tissues revealed positive results for conventional and nested PCR with amplification bands of 1088 bp (data not shown).

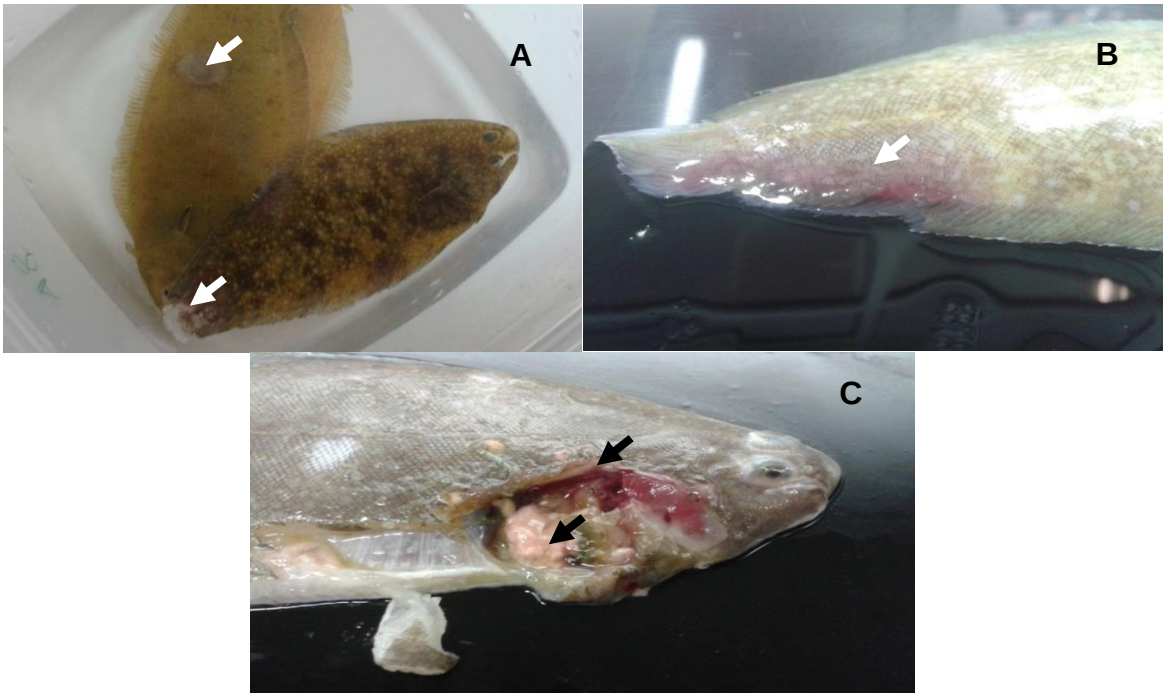

Fig.2. Macroscopic lesions from experimentally infected Senegalese sole with *T. maritimum*. Arrows show (A) severe tail rot and ulcerative lesion on the ocular side; (B) redness, necrotized skin and fin erosion; and (C) friable liver and congested kidney.

3.3. Mucus and plasma bactericidal activities

The mucus bactericidal activity showed significant differences for all *T. maritimum* strains over time, with the highest activity being observed at 6 h following incubation (Fig. 3). After 18 h, mucus samples exhausted their bactericidal activity since values started dropping to levels similar to those observed at 3 h. The 2-way ANOVA also indicated a significant difference among strains with higher values in strain ACC13.1 compared to strain ACC20.1 (results not shown). No interactions were observed among treatments.

Regarding plasma bactericidal activity, untreated samples showed a similar pattern to those observed from mucus with higher levels at 6 h following incubation compared to

time 0 and 1 h. Still, that increase was only significant against strain ACC6.1 (Fig. 4A) compared to strains ACC13.1 and ACC20.1 (Figs. 4B and 4C, respectively). Moreover, bactericidal activity from heat-inactivated plasma decreased significantly at 6h following incubation with strain ACC6.1 (Fig. 4A) compared to untreated plasma, whereas samples incubated with strains ACC13.1 and ACC20.1 only showed a slight and no significant decrease (Figs. 4B and 4C). No interactions were observed among treatments.

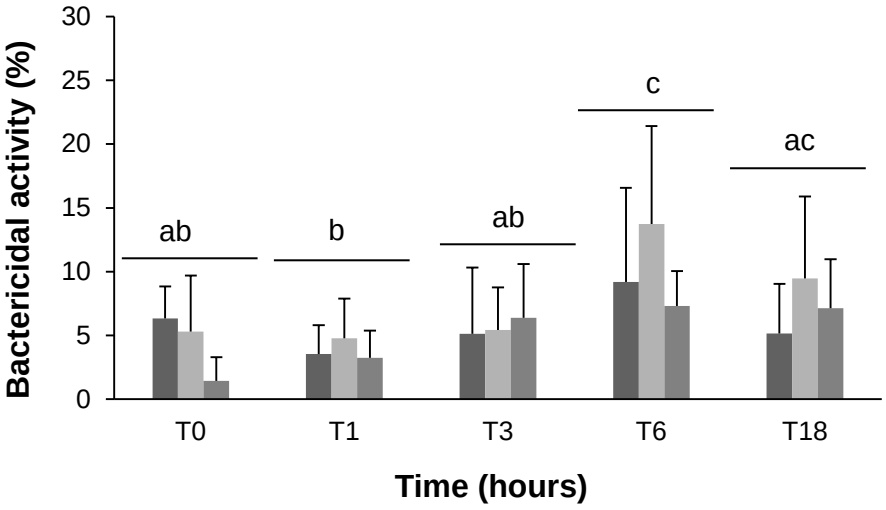

Fig.3. Bactericidal activity of Senegalese sole mucus against three *T. maritimum* strains: ACC 6.1(■), ACC 13.1(□) and ACC 20.1(■). Data are presented as means ± SD (n = 10). Different letters mean significant differences between strains for the same concentration (two-way ANOVA; $P \leq 0.05$).

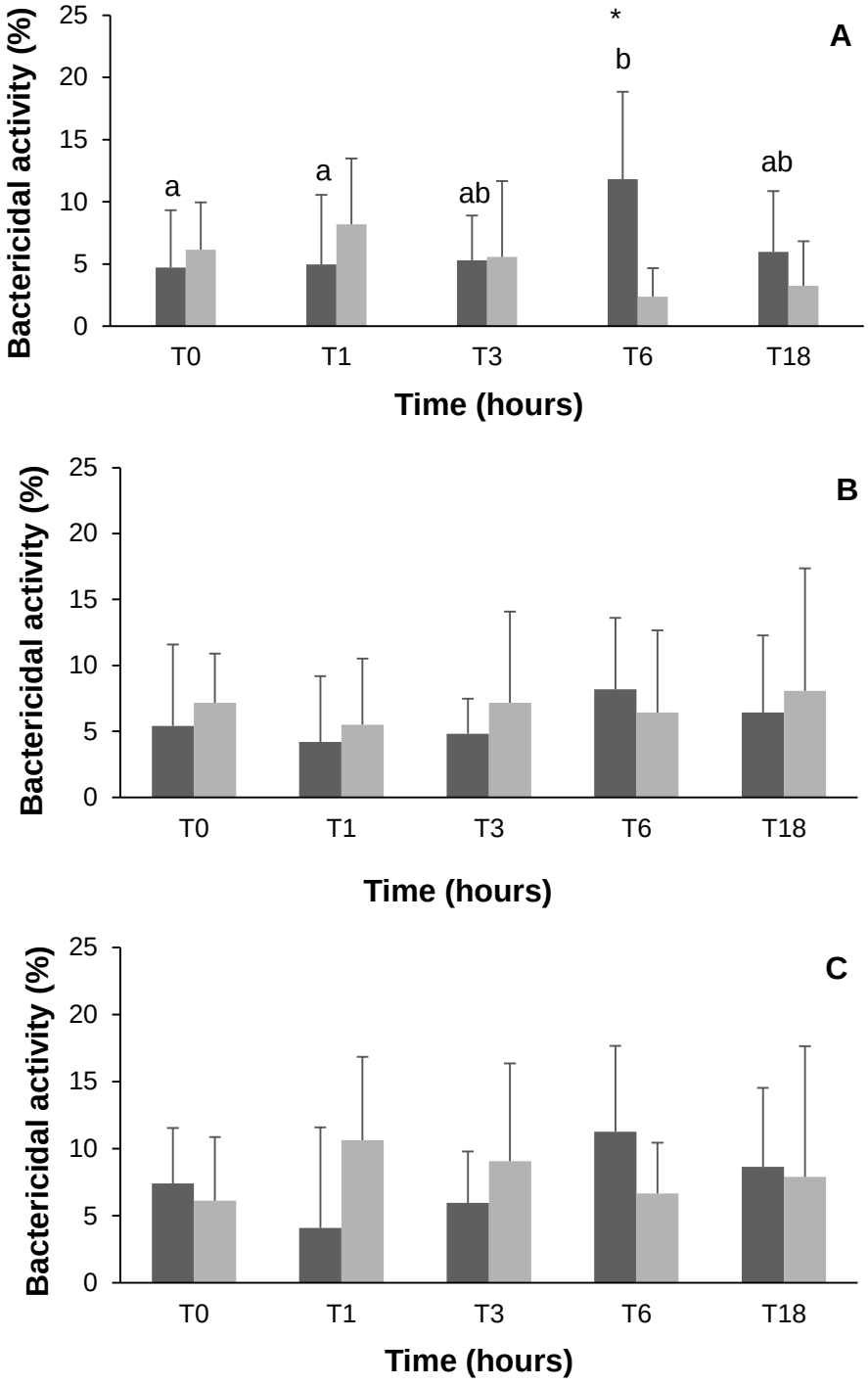

Fig.4. Bactericidal activity of Senegalese sole plasma (■) or heat-inactivated plasma (■) against three *T. maritimum* strains: (A) ACC6.1; (B) ACC13.1; (C) ACC20.1. Data are presented as means ± SD (n = 10). Different letters mean significant differences regarding incubation time for each treatment, while asterisk denotes significant differences between plasma samples for a given time (two-way ANOVA; $P \leq 0.05$).

4. Discussion

To the best of our knowledge, this is the first study presenting data regarding bacterial culture optimization and challenge procedures for *T. maritimum* against Senegalese sole. The current study revealed that using treatments such as cellulase hydrolysis, detergents and non-ionic surfactants translated in bacterial aggregation at almost any concentration. Although Igepal CA-630 prevented bacterial fluctuation, it negatively affected bacterial growth and cell wall integrity reflecting a non-representative bacterial inoculum for an experimental infection. The best method to avoid bacterial aggregation with a significant amount of bacterial yields was to inoculate bacteria directly into marine broth at 25 °C for 48 h with vigorous shaking. The role of strong agitation in preventing bacterial slime formation and aggregation was already reported by Burchard *&* Schwarz (1989) and Sorongon *et al*. (1991). These authors observed that static liquid cultures revealed detectable slimy manifested by increasing culture viscosity, while during shaking conditions, some of the extracellular polymers constituting the slime were sloughed off and, therefore, increased bacterial hydrophobicity which in turn demotivates surface bacterial adhesion and biofilm formation. Similarly, van-Gelderen *et al*. (2010) suggested that aeration in broth culture produced an effective inoculum in the challenge as it prevents bacterial fluctuation. The present work reports a standard growth curve for the three *T. maritimum* strains under study. Similar results were observed by Simões (2011), although with relatively lower bacterial yields compared to those presently obtained. Those differences are probably related to distinct culture conditions.

In the current study, several attempts and preliminary trials were performed regarding *T. maritimum* infectivity and pathogenicity against Senegalese sole, but further information is still required. For instance, Senegalese sole exposed to the pathogen at 16 ºC were not infected, whereas incubation periods of 18 h or with normal aeration also ended in failure (results not shown). Temperature and strong aeration appear to act as key physical players and those parameters are thus suggested as critical factors that should be taken into consideration during experimental infection of Senegalese sole with *T. maritimum*. Therefore, results from the present study suggest that a prolonged bath for 24 h together with high water temperature (23 ± 1 ºC) and strong aeration appear to be an effective challenge model with no need for skin or gill scarification. The role of temperature as a promoting factor in disease induction is not novel and was already reported in many other studies. For instance, Holt *et al*. (1975) stated that experimental infection of steelhead trout, *Salmo gairdneri* (Richardson), or coho salmon, *Oncorhynchus kisutch* (Walbaum), with *Flavobacterium columnare* from 12 to 20 °C resulted in increased mortalities. Moreover, the adhesive capacity of the highly virulent *F. columnare* to gill tissue was also enhanced at higher temperatures (Decostere *et al*. 1999). According to Yamamoto *et al*.

(2010), relatively stable mortalities were observed in Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), exposed to *T. maritimum* at a temperature range between 17 and 26 ºC, but variable and lower mortalities were observed below 17 ºC and above 26 ºC.

In the present study, the detection of *T. maritimum* in different host tissues suggests that the body surface can be considered the primary site of *T. maritimum* infection, which gives rise to ulcerative skin as previously reported by other authors (Toranzo *et al*. 1993; Magariños *et al*. 1995). Results from this study are also congruent with those obtained by Vilar *et al*. (2012) and Faílde *et al*. (2014) who stated that *T. maritimum* easily spreads to the internal organs due to its ability to proliferate and produce enzymes once it reaches the dermis, probably through eroded skin. All bacterial strains used in this study presented a similar LPS profile and belong to the serotype O3 previously described for this species (Avendaño-Herrera *et al.* 2005, 2006), but a different degree of virulence was observed among the various strains. However, whether LPS plays or not a pivotal role on *T. maritimum* pathogenicity still needs to be unravelled.

It is well known that the primary infection site of *T. maritimum* is the body surface of several fish species (Magariños *et al*. 1995; Avendaño-Herrera *et al*. 2006). To our knowledge, antimicrobial activities of Senegalese sole mucus and plasma against *T. maritimum* have not been reported before. In the present study, both mucus and plasma samples presented a relatively low bactericidal capacity, suggesting that Senegalese sole does not contain adequate compounds, either local (i.e. mucus) or systemically (i.e. plasma), with potent bactericidal activity to kill *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, *Scophthalmus maximus* (L.), seabream, *Sparus aurata* (L.), and seabass, thus overcoming the mucus antimicrobial activities. In the current study, results also suggest that mucus and plasma bactericidal activities appear to be time-dependent increasing 6 h following infection. These results suggest a need for a prolonged bath method to induce the disease. Interestingly, bactericidal activity increased systemically at 6 h only against strain ACC6.1, which were more virulent. In fact, this particular *T. maritimum* strain also showed to be highly virulent against turbot (Avendaño-Herrera *et al*. 2006).

5. Conclusion

The present study showed that heat-inactivated plasma samples presented similar bactericidal activities to untreated plasma, suggesting that the complement system has no major role against *T. maritimum*, at least through the alternative pathway. This resistance to the alternative complement system may be attributed to the presence of a LPS O-chain

compound that contain an unusual linkage ([R]-2-hydroxyglutaric acid residue), which enhances biofilm formation and seems to be unique for *T. maritimum* (Vinogradov *et al*. 2003). Similarly, Wiklund & Dalsgaard (2002) reported that both virulent and avirulent strains of *Flabobacterium psychrophilum* (one member of the same *T. maritimum* family), previously isolated from rainbow trout, have the ability to resist rainbow trout serum alternative complement activity. Although *T. maritimum* ability to resist both local and systemic bactericidal host responses could also be related to certain evolving strategies to withstand fish innate immunity as recorded in many other Gram negative pathogens (Rooijakkers & Strijp 2007), the mechanisms of *T. maritimum* evasion still remain to be elucidated.

In summary, the present study provides detailed information about bacterial culture optimization and challenge procedures for tenacibaculosis induction in Senegalese sole. Culturing bacteria with strong and continuous shaking provide suitable culture conditions to obtain higher bacterial yields without aggregation and fluctuation. A prolonged bath method for 24 h without skin or gill scarification was considered suitable for disease induction resulting in high mortality rates at high temperatures. Finally, data regarding mucus and plasma bactericidal activities against *T. maritimum* suggest both a lack of host innate immune responses against this particular pathogen or evading strategies of *T. maritimum* against Senegalese sole, which require further investigations. These results can help understanding the mechanism of *T. maritimum* infection and assist future studies to increase vaccine efficiency.

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Chapter 3

In vitro assessment of Senegalese sole (*Solea senegalensis*) immune responses against different *Tenacibaculum maritimum* strains

Mahmoud Mabrok[1,2,3], Rita Azeredo[1,4], Luísa Valente[1,2], António Afonso[1,2], Benjamin Costas[1,2]

[1]CIIMAR/CIMAR, Centro Interdisciplinar de Investigacão Marinha e Ambiental, Universidade do Porto, Porto,Portugal.

[2]ICBAS–UP, Instituto de Cîencias Biomedicas Abel Salazar, Universidade do Porto, Porto, Portugal.

[3]Department of Fish Diseases and Management, Faculty of Veterinary medicine, Suez Canal University, Egypt.

[4]Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal.

Keywords: *T. maritimum*, innate immune parameters, nitric oxide, reactive oxygen species, killing capacity, gene expression.

M. Mabrok

In vitro assessment of Senegalese sole (*Solea senegalensis*) immune responses against different *Tenacibaculum maritimum* strains

Abstract

Currently, little is known about *T. maritimum* evading strategies and many aspects regarding the host-pathogen interaction are still not fully elucidated. Hence, the present study aims to assess Senegalese sole cellular immune responses following stimulation with either live or UV killed *T. maritimum* through both functional (e.g. superoxide anion and nitric oxide production, leucocytes killing capacity) and gene expression approaches. Senegalese sole head-kidney leucocytes were isolated and exposed to several live or inactivated *T. maritimum* strains during 4 h, 12 h, 24 h and 48 h. Results from the present study did not reveal significant changes in superoxide anion and nitric oxide production in leucocytes exposed to different bacterial strains. UV killed *T. maritimum* strains induced higher nitric oxide production by leucocytes in contrast to the lower superoxide anion release induced by live strains. Moreover, lactate dehydrogenase activity was assessed and results suggested some evidence for necrotic cell death induction mainly during the first 4 h following bacterial inoculation. Regarding gene expression, stimulation with live strains induced an increase in interleukin 1β (IL1β), hepcidin antimicrobial peptide (HAMP1), cyclooxygenase 2 (COX2) and g-type lysozyme (gLYS) transcripts at 4 h, which decreased similarly until 48h. Although interleukin 10 (IL10) expression levels presented a similar pattern, an upregulation was observed at 48 h post stimulation. Moreover, the expression levels of IL1β, COX2, HAMP1 and IL10 from host cells stimulated with inactivated bacterial strains increased more than those from leucocytes exposed to live bacteria. Finally, the downregulation of inflammatory and iron regulating genes as well as the extensive destruction of phagocytes were considered important tools in bacterial pathogenesis.

1. Introduction

Senegalese sole (*Solea senegalensis*) is considered one of the most important cultured marine species in Portugal. However, its cultivation has been hampered by infectious diseases responsible for high mortalities in its farming production. *Tenacibaculum maritimum* in particular is the Gram-negative bacteria responsible for marine tenacibaculosis (formerly flexibacteriosis) and presents itself as one of the most serious pathogens that affect sole farming resulting in severe economic losses. Fish possess several innate, acquired humoral and cell-mediated immune responses to resist bacterial diseases (Ellis 1999). In particular, the host immune system can recognize certain pathogen molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and peptidoglycan via a limited number of germ line-encoded pattern recognition receptors (PRRs), including the family of Toll-like receptors (TLRs) as the most extensively studied (Akira *et al*. 2006). Most Gram-negative bacteria are simultaneously recognized by several PRRs. For instance, LPS is the main component of the outer cell membrane of the Gram-negative bacteria which binds to the co-receptor CD14 expressed at the surface of the host cell. The binding complex enhances transferring of LPS to the accessory molecule MD2, which is considered the extracellular domain of TLR4 (Akira *et al*. 2006). Peptidoglycan and bacterial membrane proteins are recognized by TLR2 (Fisette *et al*. 2003, Massari *et al*. 2002), whereas the genomic unmethylated CpG DNA which plays an important role during Gram-negative microbial infection can be recognized by TLR9 with the assistance of other PRRs (Bafica *et al*. 2005). Following recognition, a multitude of intracellular signaling pathways are activated, including adaptor molecules, kinases, transcription factors and a wide range of pro-inflammatory responses (Akira & Takeda 2004, Akira *et al*. 2006).

The professional phagocytes, neutrophils and macrophages, form a major defense against infection. Fish macrophages and neutrophils both produce reactive oxygen species (ROS) during the respiratory burst on contact with bacteria or during phagocytosis, as well as nitric oxide, as potent bactericidal agents (Secombes 1996). Following generation of the superoxide anion (O_2^-), it is further converted into an array of ROS including hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), hypochlorous acid (OCl^-) and peroxynitrite ($ONOO^-$) by spontaneous catalytic enzymes (Borregaard & Cowland 1997, Ischiropoulos *et al*. 1992, Sheppard *et al*. 2005). Neutrophils are considered tissue-destructive cells, responsible for inflammatory tissue damage during acute infections. They also have ability to release various antimicrobial molecules and extracellular traps (NETs) with important roles on infection and inflammation (Kumar & Sharma 2010). Neutrophils also contain large amounts of

myeloperoxidase (MPO) which is present in their cytoplasmic granules (Afonso *et al*. 1997). The MPO has ability to generate bactericidal ions that can destruct bacterial cells by halogenation (Klebanoff & Clark 1978).

Several assays have been conducted so far to study the macrophages phagocytic activity against invading pathogens. For instance, the capacity of *Pasteurella piscicida* strains to survive contact with macrophages obtained from rainbow trout (*Oncorhynchus mykiss*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) was evaluated by Skarmeta *et al*. (1995). Those authors stated that the production of O_2^- increased within few hours of macrophage incubation with the same bacteria and exhibited a potent bactericidal activity. Other authors already reported the response of Senegalese sole primary head-kidney leucocytes following stimulation with two different *T. maritimum* strains (ACC6.1 and ACC13.1) and revealed an increased ROS and NO production in cells exposed to inactivated bacterial strains (Costas *et al.* 2014). However, that study did not involve cell host interactions with live bacteria. Therefore, the aim of the current study is to gain deeper insights on the cellular innate immune responses of Senegalese sole leucocytes following inoculation with either live or UV killed *T. maritimum*.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

The *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) used in the present study were isolated from Senegalese sole and belong to the serotype O3 described for *T. maritimum* (Avendaño-Herrera *et al*. 2005). Bacteria were kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and kept frozen at - 80 °C until being used. The recovery of all bacterial strains was achieved using marine agar (MA; Laboratorios CONDA, Spain) and *Flexibacter maritimus* medium (FMM) at 25 °C for 48-72 h. Bacteria were inoculated in marine broth according to procedures described in **Chapter 2**, harvested in the late logarithmic phase and adjusted to 10^8 CFU mL^{-1}. When required, bacteria were killed by ultraviolet (UV) exposure for 4 h. Loss of bacterial viability following treatment was confirmed by plating on MA plates and resulting cultures revealed no bacterial growth.

2.2. Experimental fish

Healthy Senegalese sole weighing around 200 g were obtained from a commercial fish farm located in north-west Portugal with no history of recent tenacibaculosis. Fish were maintained

in recirculating aerated sea water, at 18–20 °C and natural photoperiod (12 h light/12 h dark). Fish were euthanized with 2-phenoxyethanol (1.5 mL L^{-1} sea water; Sigma, Germany) before isolation of cells. Water quality was maintained with mechanical and biological filtration, and fish were fed to apparent satiety with commercial pellets. Only healthy fish, as indicated by their activity and exterior appearance, were used for leucocyte collection.

2.3. Isolation of head-kidney leucocytes

Senegalese sole head-kidney leucocytes (HKL) were isolated according to Secombes (1990) and Costas *et al*. (2011). Briefly, the head-kidney was removed under aseptic conditions, pushed through a 100-µm nylon mesh and suspended in Leibovitz L-15 medium (L15; Gibco, United Kingdom) supplemented with 2% fetal bovine serum (FBS; Gibco), penicillin (P, 100 IU mL^{-1}; Gibco), streptomycin (S, 100 µg mL^{-1} , Gibco) and heparin (20 units mL^{-1}; Sigma). The cell suspensions were then loaded onto a 34:51% Percoll (Sigma) density gradient and centrifuged at 400 × *g* at 4 °C for 40 min. The band of cells lying at the interface of the Percoll gradient was collected and washed three times at 400 × *g* and 4 °C for 5 min in L15, 0.1% FBS and P/S. Cell viability was determined by the trypan blue exclusion test. Cells were counted in a hemocytometer and adjusted to 1.5 x 10^7 cells mL^{-1} in L15, 0.1% FBS and P/S. Afterwards, cells were plated in 24 or 96 well plates at 500 or 100 µl per well, respectively. After overnight incubation at 18 ºC, the non-adherent cells were washed off and the monolayers were treated as described below.

2.4. Nitric oxide assay

The nitric oxide (NO) production of HKL was conducted according Neumann *et al.* (1995). This method is based on the Griess reaction that quantifies the nitrite content of the leucocytes supernatants, because NO is an unstable molecule and degrades to nitrite and nitrate. To stimulate HKL for NO production, the leucocyte monolayer was incubated with UV killed or live *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) at 10^8 CFU mL^{-1}. HKL incubated with medium alone were regarded as controls. After 72 h of incubation at 18 °C, 50 µL of the supernatants were removed from individual wells and placed in a separate 96-well plate. Afterwards, 100 µL of 1% sulphanilamide (Sigma) in 2.5% phosphoric acid was added to each well, followed by 100 µL of 0.1% N-naphthyl-ethylene-diamine (Sigma) in 2.5% phosphoric acid. Optic density was determined at 540 nm. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

2.5. ROS assay

ROS from stimulated HKL were measured according to Secombes (1990). The principle of the current assay is based on the reduction in ferricytochrome C method for the detection of O_2^-, and processed after the cells were incubated with L15 5% FBS at 18 °C for 24 h. Afterwards, the leucocyte monolayers were washed twice with phenol red-free Hank's balanced salt solution (HBSS, Gibco). Then, 100 µL suspensions of ferricytochrome C solution (2 mg ferricytochrome C mL^{-1} diluted in phenol red-free HBSS) containing 10^8 CFU mL^{-1} of UV killed or live *T. maritimum* strains were added. Ferricytochrome C solution containing 10 µg mL^{-1} of phorbol myristate acetate (PMA, Sigma) was used as a stimulus for the respiratory burst and served as a positive control. Ferricytochrome C with PMA or *T. maritimum* and 0.725 mg mL^{-1} of superoxide dismutase (SOD; Sigma) was used to confirm the specificity of the reaction and served as negative controls. Triplicates for each treatment were displayed. Plates were read 30 min post stimulation, and were then taken on a Synergy HT (BioTek) microplate spectrophotometer at 550 nm. Optical densities were converted to nmol of O_2^- values obtained by multiplying 15.87 as described by Pick (1986).

2.6. Killing assay

The HKL bactericidal capacity was measured by a colorimetric method based on the reduction of MTT [3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide; Sigma] (Graham *et al*. 1988). Briefly, leucocyte monolayers were incubated with L15 5% FBS at 18 °C for 24 h. Subsequently, adherent cells were washed twice with phenol red-free HBSS and 100 µL of live *T. maritimum* strains (10^8 CFU mL^{-1}) were added. The plates were then centrifuged at 150 × *g* for 5 min at 18 °C to bring the bacteria into contact with leucocytes. After that, the plates were incubated at 18 °C for 0 h (T_0), 3 h (T_3) and 5 h (T_5). At the end of each incubation period, the supernatants were removed and the killing stopped by lysing the HKL with 50 µL of cold sterile distilled water. Then, 100 µL of marine broth (Laboratorios CONDA) were added to promote the growth of surviving bacteria and incubated at 25 °C during 48 h. Afterwards, 10 µL of MTT (5 mg mL^{-1}) were added to each well and the plates read after 15 min on a Synergy HT microplate spectrophotometer at 600 nm. The average values of triplicates were then estimated. The differences between the optical density reading at T_0, T_3 and T_5 represent the degree of bactericidal activity:

$$\frac{T_0 \text{ MTT reduction} - T_3 \text{ or } T_5 \text{ MTT reduction}}{T_0 \text{ MTT reduction}} \text{ X } 100$$

2.7. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is an enzyme with wide tissue distribution in the body and its high concentration is recognized as an indicator of cell destruction and damage. The main principle is that lactate dehydrogenase (LDH) catalyses the reduction of pyruvate by NADH, according the following reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

The LDH activity was determined by a commercial kit (SPINREACT, Spain) and conducted according the manufacture's specifications with slight modifications. Briefly, leucocyte monolayers were stimulated with UV killed or live *T. maritimum* as described above. After bacterial inoculation, 10 µL of the supernatant were collected at 4 h, 12 h, 24 h and 48 h and incubated with 90 µL of the kit's working reagent in 96-well plates for 1 min at 25 °C. After that, the absorbance was measured at 340 nm wave length for 3 min with 1 min interval. The difference between absorbances and the average absorbance difference per minute was calculated: delta A min^{-1}. LDH concentration is expressed in units per litre of sample, where one international unit (IU) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions.

2.8. Gene expression analysis

Leucocyte monolayers in 24-well plates were washed twice with phenol-red free HBSS and incubated with L15 5% FBS at 18 °C for 24 h. Afterwards, UV killed and live *T. maritimum* strains were added as described above and incubated 4 h, 12 h, 24 h and 48 h at 18 °C for the assessment of gene expression. Extraction of total RNA was conducted using TRIzol Reagent (Sigma) following manufacturer's specifications. Subsequently, 8 µg of total RNA per biological sample were treated with DNase I (Promega, Madison, USA) to remove contaminating DNA, and first-strand cDNA was synthesized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) following manufacturer's instructions.. Quantitative PCR assays were performed with the Mastercycler RealPlex4 PCR System (Eppendorf) using 1 µL cDNA (1:5 dilution) seeded in 10 µL of iQ SYBR green Supermix (BioRad) and 0.4 µL (10 µM) of each specific primer in a final volume of 20 µL. The specific primers (Table 1) used for amplification were designed using Primer 3 software according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). The efficiency of the primer pairs was analyzed in serial five-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA (Livak & Schmittgen 2001). Efficiency values for

interleukin 10 (IL10), hepcidin antimicrobial peptide (HAMP1), interleukin 1β (IL1β), lysozyme (gLYS), and cyclooxygenase 2 (COX2) were -3.19, -2.7, -3.19, -3.2 and -3.17, respectively. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. All reactions were carried put as technical duplicates. The Ct values were compared through the comparative Ct method to the geometric mean derived from the expression of Senegalese sole 40S ribosomal protein S4 (RPS4) gene (Livak & Schmittgen 2001). The expression of the candidate genes was normalized using the RPS4 of Senegalese sole as a housekeeping gene, as it showed a constant expression level and was constitutively expressed independently of treatments. Fold change units were calculated by dividing the normalized expression values of cells from different treatments by the normalized expression values of the control (unstimulated cells).

Table1. Primers sequences of the genes analyzed by real time PCR.

Gene name	Symbol	Accession number	Efficiency	MT	Product Length	Primers sequence
Riposomal protein S4	RPS4	AB291557.1	97.2	67	83	R: GTGAAGAAGCTCCTTGTCGGCACCA F: AGGGGGTCGGGGTAGCGGATG
G-type lysozyme	gLYS	FR872377.1	105.4	57.7	89	R: CTCATTGCTGGCATCATCTC F: TACGCTCCTCTGCTTGGATT
Interleukin 1β	IL1β	KU695545	105.8	60.4	127	R: ACTGAGGCCAGACCTGTAGC F: TGACGGTTGACAGACAGCTT
Interleukin 10	IL10	AIBJXQL *	105.8	60.6	78	R: CCGTCTTTGTGTTATTTCTCCAACAG F: TGGAGTTCAGCTTTGTGATGTCA
Hepcidin antimicrobial peptide 1	HAMP1	AB455099.1	105.8	58	109	F: AAACTGAGCAGCGTCTGACA R: TGAATGCCTTCATCTTCACG
Cyclo-oxygenase-2	COX-2	AJ630649.1	105.8	61	160	F: CATTCTTTGCCCAGCACTTCACC R: AGCTTGCCATCCTTGAAGAGTC

* Transcript sequences retrieved from *Solea* DB database (http://

www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadp_ifapa/home_page)

2.9. Statistical analysis

All results are expressed as means ± standard deviation (SD). Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene's test and, when necessary, they were transformed before being treated statistically. A non-parametric Kruskal-Wallis test, followed by multiple comparison tests, was used when data did not meet parametric assumptions. Data were analyzed by two-way ANOVA. All procedures were followed by Tukey post hoc through paired-comparisons to identify differences among experimental treatments. All statistical analyses were performed using the computer package statistical 12 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests. All results expressed as a percentage were previously arcsine transformed (Zar 1999). Data from NO and LDH assays are presented as fold change levels, calculated by dividing each treatment value by the mean value from controls (unstimulated cells). Fold values higher than (0) were considered an increase.

3. Results

3.1. NO assay

Although NO production in Senegalese sole HKL increased following exposure to both live and inactivated bacteria, UV killed bacterial strains augmented NO levels from host cells in a higher degree than those exposed to live bacteria and regardless bacterial strain (Fig. 1). NO production by HKL exposed to UV killed ACC6.1 strain increased 2.4 fold than that induced by live one, while in the case of ACC13.1 and ACC20.1 killed strains were recorded as 1.7, 1.5 fold increases, respectively.

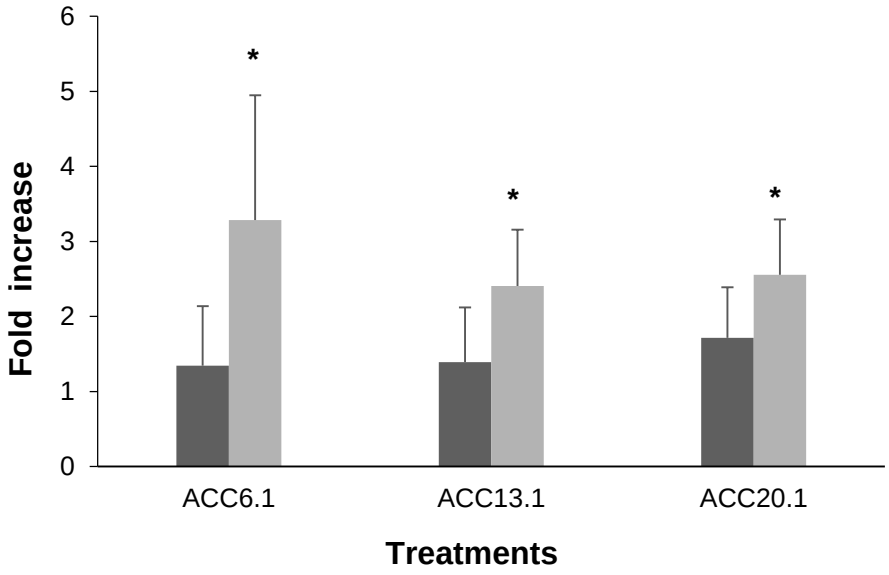


Fig.1. Nitric oxide production (µM) of Senegalese sole, *Solea senegalensis*, head-kidney leucocytes expressed as fold increase following exposure to 10^8 CFU mL $^{-1}$ of UV killed (▒) and live (■) *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1). Data are presented as means ± SD (n = 8). Asterisk means significant differences between killed and live bacterial strains (two-way ANOVA; $P \leq 0.05$).

3.2. ROS assay

Senegalese sole HKL increased ROS production following exposure to both UV killed and live bacterial strains in a different manner, with slightly higher values in host cells stimulated with live bacteria. HKL exposed to ACC6.1, ACC13.1 and ACC20.1 live strains presented 1.47, 1.9 and 2.02 fold increases, respectively. Moreover, ROS produced due to bacterial stimuli was lower than that produced by PMA which served as positive control. Differences among bacterial strains did not reveal significant changes (Fig. 2).

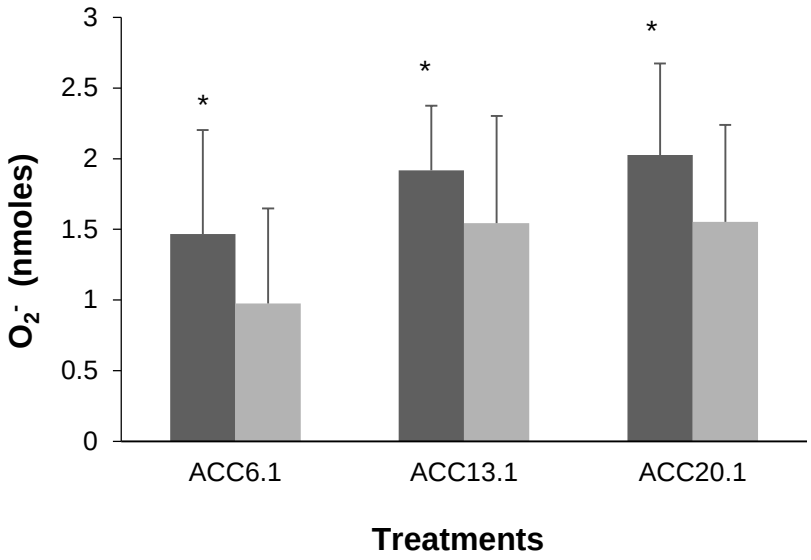


Fig.2. ROS production (nmoles) of Senegalese sole, *Solea senegalensis*, head-kidney leucocytes following exposure to 10^8 CFU mL^{-1} UV killed ( ) and live ( ) *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1). Data are presented as means ± SD (n = 8). Asterisk means significant differences between UV killed and live bacterial strains (two-way ANOVA; $P \leq 0.05$).

3.3. Killing assay

Bactericidal activity was observed in HKL exposed against strain ACC13.1, with higher values compared to HKL inoculated with strain ACC6.1 (Fig. 3). Moreover, HKL incubated with strain ACC20.1 did not show significant variation compared to those exposed to strains ACC6.1 and ACC13.1. There were no significant variations regarding incubation time for each strain.

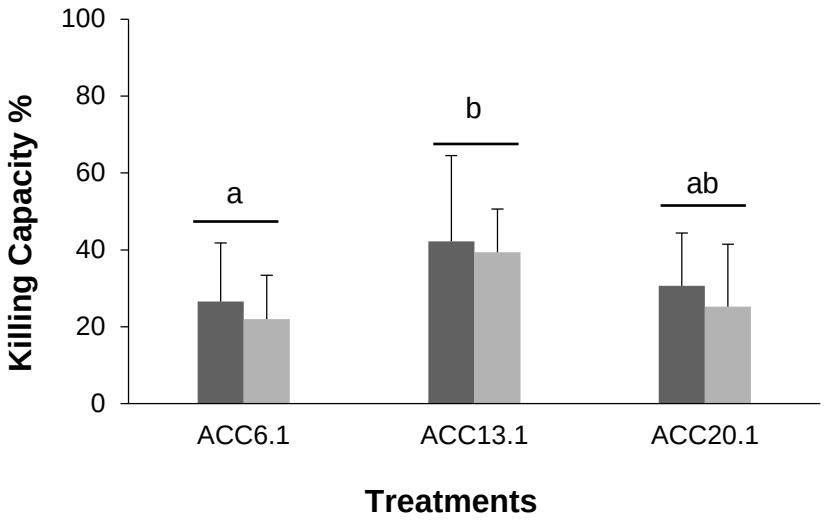


Fig.3. Senegalese sole, *Solea senegalensis*, head-kidney leucocytes killing capacity (%) against 10^8 CFU mL^{-1} of three *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) after 3 h (■) and 5 h (□) incubation period. Data are presented as means ± SD (n = 8). Different letters indicate significant differences between bacterial strains (two-way ANOVA; $P \leq 0.05$).

3.4. Lactate dehydrogenase (LDH) assay

HKL revealed variations in LDH activity following stimulation with both live and UV killed strains at 4 h following inoculation (Fig. 4). HKL inoculated with live strain ACC6.1 showed the highest LDH values with significant differences compared to HKL exposed to inactivated cells (Fig. 4A). Whereas a similar pattern of increase in LDH activity can be observed in time, HKL exposed to live strain ACC20.1 increased LDH levels at 48 h compared to those cells exposed during 12 and 24 h, and also compared to HKL inoculated with the UV killed strain at the same time (Fig. 4C). Comparing to unstimulated cells (control), HKL showed significant increases in LDH activity after 12 h and 24 h of exposure to either live or UV killed strains, however, at 4h and 48 h significant increases in LDH were only recorded in those exposed to live ACC6.1 and ACC20.1 strains, respectively.

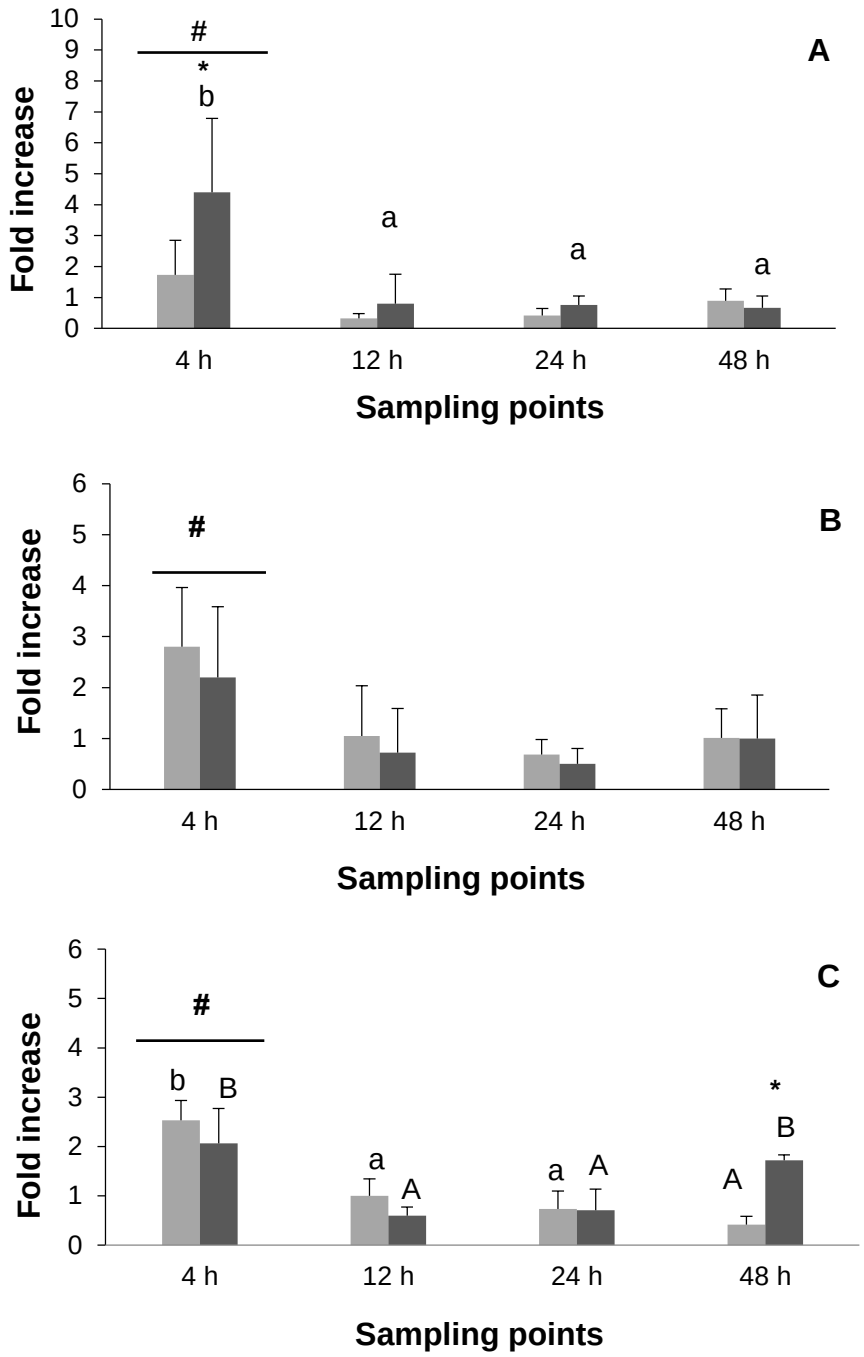


Fig.4. LDH activity (presented as fold increase) from Senegalese sole head-kidney leucocytes following stimulation of with 10^8 CFU mL^{-1} of UV killed (□) or live (■) *T. maritimum* ACC6.1 (A), ACC13.1 (B) and ACC20.1 (C) strains. Data are presented as means ± SD (n = 4). Different letters indicate significant changes due to live (capital) or UV killed (small) strains, while # stands for differences regarding incubation time, and asterisk means changes between UV killed and live bacterial strains for each particular sampling point (two-way ANOVA; $P \leq 0.05$).

3.5. Gene expression

A similar pattern of gLYS expression was observed in HKL exposed to live or UV killed *T. maritimum* strains. Host cells stimulated with live strains showed an increase in gLYS transcripts already at 4 h following inoculation, with the highest levels observed in HKL exposed to strain ACC6.1 (Fig. 5A). Moreover, gLYS mRNA expression decreased in time until values close to zero at 48 h. HKL exposed to UV killed *T. maritimum* strains presented a relatively constant gLYS mRNA expression level, with the exception of HKL exposed to strain ACC13.1, which presented an increase in gLYS transcripts at 24 h compared to cells exposed during 4 or 48 h (Fig. 6A). While gLYS mRNA expression was higher in HKL inoculated with live bacteria at 4 h, the opposite was observed after 12, 24 and 48 h.

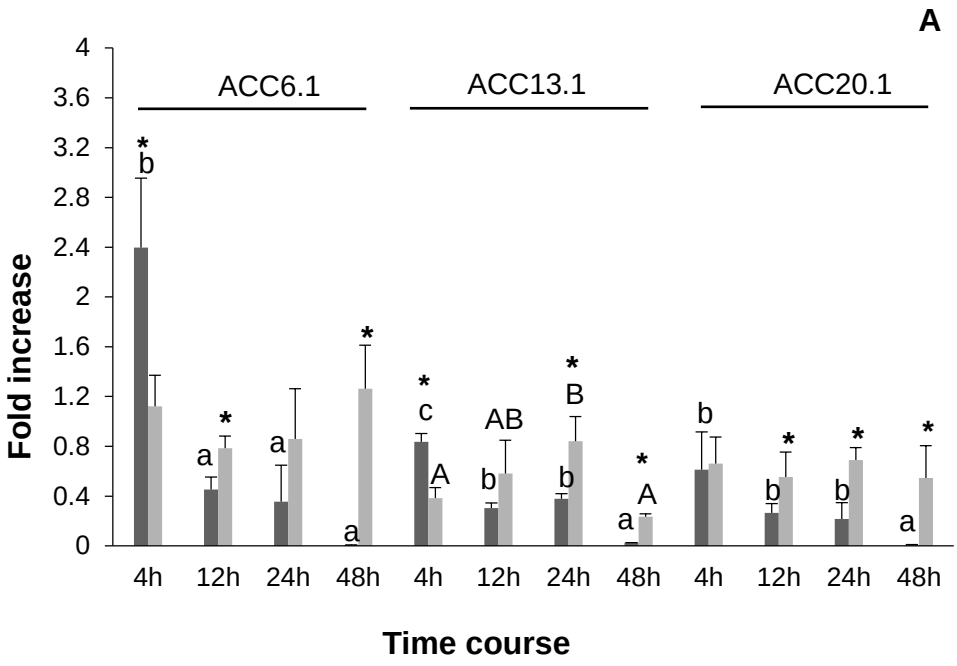
COX2 expression levels did not change in HKL exposed to live bacteria whereas a marked increase was observed at 12 and 24 h in host cells inoculated with the inactivated strains ACC6.1 and ACC13.1 and at 12, 24 and 48 h in HKL inoculated with the inactivated strain ACC20.1 (Fig. 5B). Moreover, host cells exposed to the UV killed pathogen presented higher COX2 mRNA levels compared to those inoculated with live bacteria at most incubation times.

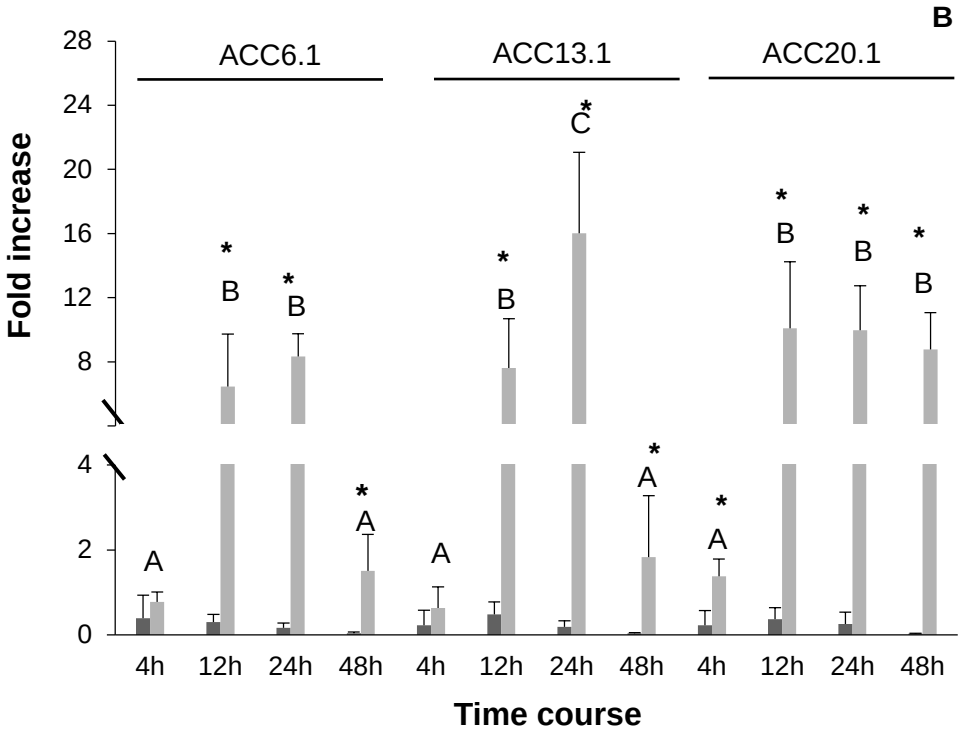
HKL exposed to live bacteria showed a similar inverted U-shaped pattern of expression levels for HAMP1 mRNA with peak values at 12 h (Fig. 5B). Moreover, host cells exposed to UV killed bacteria increased HAMP1 transcripts already at 4 h after inoculation peaking at 12 or 14 h depending on the bacterial strain (Fig. 5B). Similarly to that observed for COX2 mRNA levels, HKL exposed to the inactivated pathogen presented higher HAMP1 transcripts compared to those inoculated with live bacteria at most incubation times.

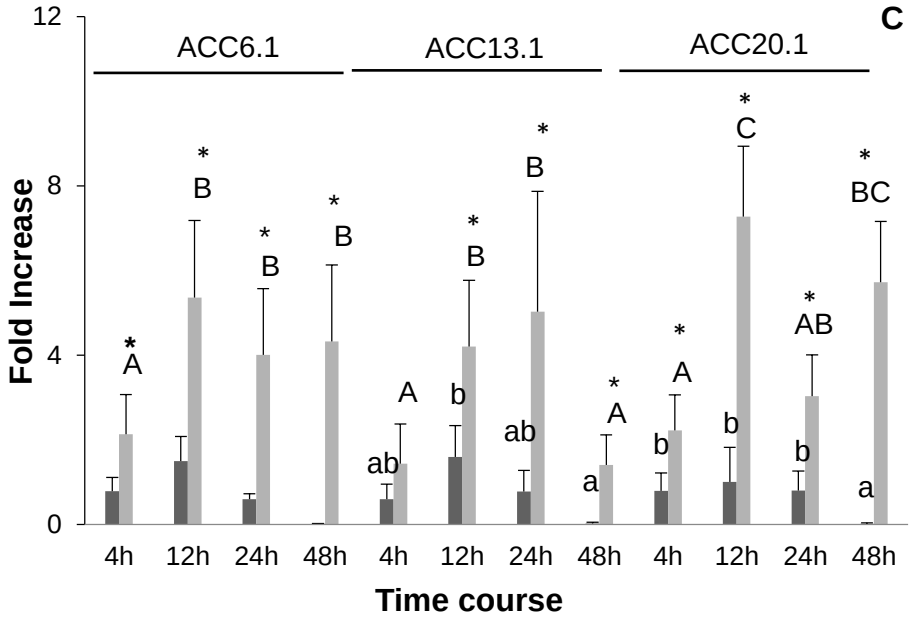
The pro-inflammatory cytokine IL1β presented a similar expression pattern to that observed for the COX2 gene in Senegalese sole HKL exposed to both live and UV killed bacteria. For instance, in case of host cells exposed to live ACC6.1 strain, a slight increase around 0.5-fold was observed after 4 h, followed by a 5-fold increase at 12 h and a sharp decrease in expression levels at 24 h and 48 h following bacterial inoculation (Fig. 5D). Concerning host cell responses to the inactivated pathogen, an exponential increase was observed over time with peak values at 24 h after bacterial inoculation followed by a dramatic decrease after 48 h (Fig. 5D). HKL exposed to the inactivated pathogen presented higher IL1β transcripts compared to those inoculated with live bacteria for all incubation times.

IL10 increased around 10-fold in HKL inoculated with UV killed bacterial strains already at 4 h followed by a decrease until 3-4 fold levels at 12 h compared to unstimulated cells. While those levels remained constant in HKL exposed to the inactivated strain ACC20.1, another increase in IL10 transcripts was observed at 48 h and at 24 and 48 h in host cells exposed to

UV killed strains ACC6.1 and ACC13.1 (Fig. 5E). Similarly to that observed for COX2 and HAMP1 mRNA levels, HKL exposed to the inactivated pathogen presented higher expression values compared to those inoculated with live bacteria at most incubation times. Although a slight increase in IL10 mRNA expression levels was observed HKL exposed to live strains ACC6.1 and ACC20.1 at 4 h, those levels only increased significantly at 24 h in host cells inoculated with strain ACC20.1 and at 48 h for those exposed to strains ACC6.1 and ACC13.1. Moreover, HKL incubated with live strain ACC20.1 showed similar IL10 expression levels compared to host cells exposed to live strains ACC6.1 and ACC13.1 at 48 h (Fig. 5E).




B
ACC6.1
ACC13.1
ACC20.1
Fold increase
28
24
20
16
12
8
4
2
0
4h
12h
24h
48h
Time course


C
ACC6.1
ACC13.1
ACC20.1
Fold Increase
12
8
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4h
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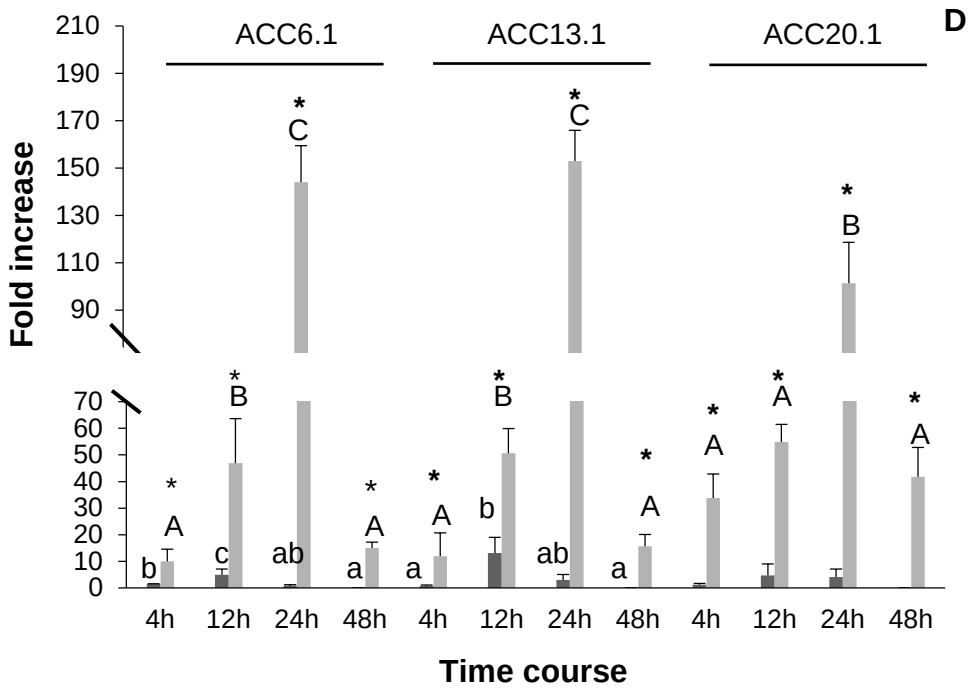
24h
48h
Time course


D
ACC6.1
ACC13.1
ACC20.1
Fold increase
210
190
170
150
130
110
90
70
60
50
40
30
20
10
0
4h
12h
24h
48h
Time course

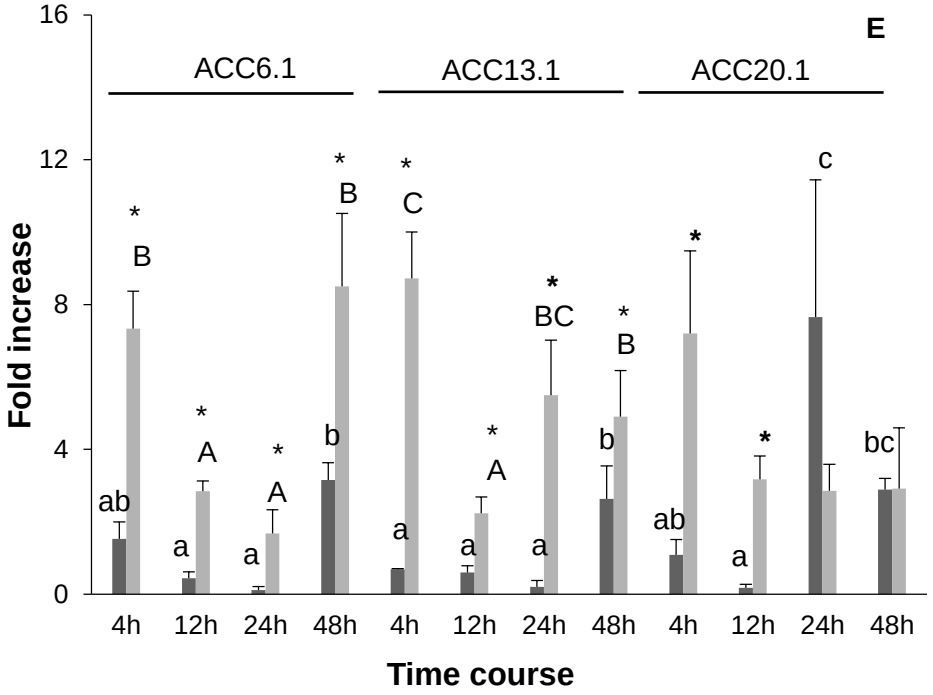

E
ACC6.1
ACC13.1
ACC20.1
Fold increase
16
12
8
4
0
4h
12h
24h
48h
Time course

Fig.5. Quantitative expression of g-type lysozyme (gLys; A), cyclooxygenase 2 (COX2; B), hepcidin antimicrobial peptide 1 (HAMP1; C), interleukin 1β (IL1β; D) and interleukin 10 (IL10; E) in Senegalese sole head-kidney leucocytes following stimulation with 10^8 CFU mL^{-1} of live (■) and UV killed (□) *T. maritimum* strains during 4 h, 12 h, 24 h and 48 h. Data are presented as means ± SD (n = 4). Bars represent the fold increase in expression of each gene as compared to the control group (unstimulated cells), previously normalized to endogenous 40S ribosomal protein S4 expression levels. Different letters indicate significant changes due to live (capital) or UV killed (small) strains, while asterisk means changes between UV killed and live bacterial strains for each particular sampling point (two-way ANOVA; $P \leq 0.05$).

4. Discussion

During microbial invasion, macrophage lineage cells eliminate invading pathogens through a battery of antimicrobial responses. Therefore, it is not surprising that many pathogens, especially highly virulent ones, can evade host immune responses. For successful invasion and colonization, certain pathogens display several evading mechanisms including the ability to suppress phagocytes oxidative burst by producing detoxifying enzymes or depressing the activity of some enzymatic components (John *et al.* 2001, Ng *et al.* 2004, Davis *et al*. 2007). Gram-negative bacteria possess cell wall proteins, LPS and polysaccharide capsules which may have remarkable and functional roles in their virulence mechanisms (Evelyn 1996, Croxatto *et al*. 2007). Moreover, sialic acid on the external surface of some bacterial pathogens such as *Flavobacterium columnare* suppresses the alternative complement pathway activation and therefore attenuated the host immune response (Ourth & Bachinski 1987). According to Bader & Shotts (1998), *Flavobacterium psychrophilum, F. columnare* and *Flexibacter maritimus* are closely related and it was supposed that *T. maritimum* could share the same characteristic cell wall components and virulence mechanisms of *F. columnare*.

To our knowledge, there are few available data regarding the interactions between *T. maritimum* and host cells. Results from the present study showed similar host cell responses against *T. maritimum* strains belonging to the same serotype. Although these results appear to be not in agreement to that observed by Costas *et al*. (2014), who reported an increased ROS and NO production in Senegalese sole HKL inoculated (10^6 CFU mL^{-1}) with strain ACC6.1 compared those exposed to strain ACC13.1, those differences between studies could be related to a different amount of bacteria in each inoculum. A similar outcome was also observed in rainbow trout (*Oncorhynchus mikiss*) and goldfish (*Carassius auratus*)

macrophages exposed to *Pasteurella piscicida* and *Mycobacterium marinum*, respectively (Skarmeta *et al*. 1995, Grayfer *et al*. 2011). Results from the present study also reported different ROS and NO production by HKL when inoculated with live or inactivated bacteria. While Senegalese sole HKL appear to slightly increase ROS against live *T. maritimum* strains compared inactivated ones, NO production showed the opposite pattern. A similar outcome was observed by Grayfer *et al.* (2011) when goldfish macrophages were infected with live or inactivated *M. marinum*, whereas Billings (2006) reported that NO production was not affected following treatment of channel catfish (*Ictalurus punctatus*) kidney cells with live *F. columnare*. Moreover, a similar result was reported by Chen *et al*. (2015) who stated that injection of heat-killed *Vibrio harveyi* increased the concentration of NO in the hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) in a higher significant manner when compared to that of the live one. In the present study, it is tempting to speculate that *T. maritimum* appears to present the ability to evade host phagocyte responses. ROS production against live bacteria was relatively low and close to that produced by HKL exposed to the inactivated pathogen. Although one evading strategy could be linked to the elimination of reactive radicals through antioxidant enzymes such as catalase. The present study also suggests that this evasion strategy may not require viable bacteria. Therefore, it is hypothesized that *T. maritimum* cell wall components may redirect the host immune response through distinct TLR signalling pathways from those desired for proper pathogen elimination. This hypothesis would be further supported by the low IL1β and COX2 mRNA expression levels observed in Senegalese sole HKL exposed to live *T. maritimum* strains. Additionally, the expression level of gLys gene in the current study was downregulated in Senegalese sole HKL following stimulation with either UV killed or live strains. Similarly, Ren *et al*. (2015) stated that challenge with *F. columnare* decreased the expression level of three lysozyme genes (Lyg1, Lyc and Lyg) in the mucus of catfish *(Ictalurus punctatus*) shortly after 2 h of infection. Therefore, the downregulation of that gene may be the results of the virulent factors of *T. maritimum*, to suppress the host immune response.

Indeed, *T. maritimum* strains used in the present study appear to present similar evading strategies against Senegalese sole phagocytes. However, results from the present study showed an increased bactericidal activity of Senegalese sole HKL against strain ACC13.1 compared to strain ACC6.1. This increased survival from strain ACC6.1 could be related to its higher virulence (according to Chapter 2), and seems to be related to the increased LDH activity observed in cells inoculated with live ACC6.1 after 4 h. It seemed that the killing

mechanism(s) of ACC6.1 live strain required active protein synthesis, since no significant variation in enzyme production was recorded following stimulation with UV killed strain when compared to unstimulated cells at the same particular time.

An augmentation in LDH activity suggests pore formation in the host cell membrane (Pei & Ficht 2004), and thus emphasizes the hypothesis of higher bacterial virulence. Similarly, Peselis (2011) recorded cell necrosis and apoptosis in Atlantic salmon kidney cells following incubation with either *F. columnarae* bacterial cells, or bacterial culture supernatants. Moreover, a similar increase in cell death was observed in HKL inoculated with the other strains (either live or UV killed) after 12 h and 24 h which could be due to an increased amount of free radicals following HKL activation. In fact, a dangerous outcome from the combination of increased NO formation and augmented generation of superoxide anion is the formation of peroxinitrite, which has a high cytotoxic potency (Roth 2007). In addition, the observed cytotoxicity post stimulation with UV killed *T. maritimum* strains could also be considered as consequence of host-pathogen interaction. During bacterial infection apoptosis and necrosis of host cells function as a defense mechanism by destroying the site of pathogen replication (Skovgaard 2012).

Bacteria are known to stimulate macrophages to induce IL1β expression (Ingerslev *et al*. 2006, Panigrahi *et al*. 2007). In the present study, a marked increase in the expression of IL1β up to 150-fold was observed following stimulation with UV killed strains after 24 h whereas live strains only induced a slight increase at 4 h and 12/24 h. Therefore, live *T. maritimum* seems to possess an evading strategy which could be related to the activation of different host PRR to avoid the development of inflammation. In fact, COX2 was irresponsive or even downregulated. Downregulation of channel catfish skin mucus immune related genes following challenge with *F. columnare* could bring some insights regarding the evading strategy of flavobactereacae members against fish immune responses (Ren *et al*. 2015). Although, the expression profile of IL1β was extremely high in Senegalese sole cells after stimulation with UV killed strains, the later failed to enhance ROS production. These finding revealed that the IL1β–Myd88 axis and NADPH oxidase–mediated ROS signalling are two independent pathways that differentially regulate neutrophil migration during inflammation. A similar result was obtained in a Zebrafish after induction of inflammation (Yan *et al*. 2014).

Among the examined gene, IL10 showed a higher expression level at 48 h, 12 h and 48 h following stimulation with ACC6.1, ACC13.1 and ACC20.1 live strains, respectively. During the rest of the sampling points, it was downregulated. In contrast, the expression level post stimulation with UV killed was significantly higher at all sampling points; with a most

prominent increase at 4 h. IL10 is a multifunctional cytokine which plays an important role in disease control. It can be generated by several immune cells, including T cells, B cells, macrophages or monocytes, NK cells, keratinocytes, eosinophils, mesangial cells, epithelial cells and tumour cells (Zdanov 2004). In fish, it has been reported that IL10 can be expressed following bacterial infection or LPS treatment (Savan *et al*. 2003, Pinto *et al*. 2007, Tanekhy *et al*. 2009). In the current study, an inverse linear relationship between IL10 and IL1β and COX2 was observed, suggesting IL10 role as anti-inflammatory cytokine. Similar expression patterns of those genes were observed in several tissues of Indian major carp (*Catla catla*), following bacterial inoculation and LPS treatment (Swain *et al*. 2012). Furthermore, the role of IL10 in the suppression of inflammatory responses that was elicited by activated macrophages, and prevention of tissue damage following infection was briefly elucidated (Moore *et al*. 2001).

Hepcidin presents a dual role as an antimicrobial peptide that acts against Gram-negative and Gram-positive bacteria (Shike *et al*. 2002), or can be involved in the central regulation and haemostasis of iron absorption and iron recycling by macrophages (Nemeth *et al*. 2004, Rodrigues *et al.* 2006, Shi *et al*. 2006). Its upregulation in mammals has been found to be associated with IL1β, IL6, LPS and iron overloading (Lee *et al*. 2004). In the present study, the increased expression of HAMP1 following stimulation with UV killed strains could be considered a predictable action to maximize the local iron storage, deny bacteria access to the metal and thereby limiting the spread of infection. The dual role of hepcidin as antimicrobial peptide and iron metabolism regulator during microbial infection has been fully investigated in mammals (Krause *et al*. 2000) and fish (Rodrigues *et al*. 2006, Douglas *et al*. 2003, Hirono *et al*. 2005). On the other hand, the lower expression of hepcidin following stimulation with live strains could be either attributed to the capacity of the pathogen to express high-affinity iron uptake mechanisms (Avendaño-Herrera *et al*. 2006), or to their capability to overcome the antimicrobial nature of that molecule. Furthermore, it reflectes the role of iron in bacterial virulence, as these bacteria displays, at least, two different systems of iron acquisition including production of an unidentified siderophore and utilization of heme groups as an iron source by a direct binding (Avendaño-Herrera *et al*. 2005).

5. Conclusions

Senegalese sole head kidney leucocytes stimulate ROS and NO production following exposure to live and UV killed strains in a different manner. Pathogen exposed to ultraviolet radiation modulates the fish cellular immune responses. Our results elucidate specific

interaction diversity between evaluated bacterial strains and sole immune parameters. The diversity was based on the ability of such strain to withstand host cellular responses. Evidence for the induction of necrotic cell death following stimulation with different treatments was conducted and revealed that the necrotic factor is actively produced by that viable bacteria and the killing mechanism(s) requires active bacterial protein synthesis.

T. maritimum appears to present the ability to evade host phagocyte responses, being the elimination of reactive radicals through antioxidant enzymes such as catalase the most implemented tool. However, further investigation is still required. Although most studies were focused on protein characterization and enzyme activities, no studies have examined the gene expression profile in Senegalese sole HKL following stimulation with *T. maritimum*. In this regard, our study sets for the first time an *in vitro* model to investigate the expression patterns of some immune-related genes post stimulation. Notably, most of the selected genes were rapidly responded to the stimulus, and showed totally different expression patterns. The observed downregulation of the most of evaluated genes after stimulation with live bacterial strains can provide early insight for the pathogen evading strategy. In addition the antagonistic action between IL10 and IL1β gave further indication about the anti-inflammatory property of IL10 and its role in disease control.

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Chapter 4

In vitro assessment of Senegalese sole (*Solea senegalensis*) immune responses following stimulation with extracellular products and lipopolysaccharides from *Tenacibaculum maritimum*

M. Mabrok

In vitro assessment of Senegalese sole (*Solea senegalensis*) immune responses following stimulation with extracellular products and lipopolysaccharides from *Tenacibaculum maritimum*

Abstract

The present study aimed to study the effects of *T. maritimum* extracellular products (ECPs) and lipopolysaccharides (LPS) against Senegalese sole head-kidney leukocytes (HKL) through functional approaches (i.e. superoxide anion and nitric oxide production). Furthermore, the cytotoxic activity of such stimuli against HKL was evaluated. Senegalese sole HKL were isolated and exposed to several concentrations of ECPs and LPS from 3 *T. maritimum* strains belonging to the same serotype during 24 h. The current study revealed that *T. maritimum* strains belonging to the same serotype produced a similar ECPs profiles at either 24 or 48 h of culture. Senegalese sole HKL produced reactive oxygen species (ROS), while failed to produce nitric oxide (NO) following stimulation with ECPs. The ROS production was found to be concentration dependent, with the highest production observed following stimulation with 10 μg mL^{-1}, whereas the lowest one was recorded following stimulation with 100 μg mL^{-1}. Moreover, HKL did not increase ROS nor NO production following exposure to LPS from *T. maritimum* and *E. coli*, which appear to be related to the low LPS concentration employed. The cytotoxic activity of both ECPs and LPS from *T. maritimum* against HKL was assessed and results showed an increase in host cell death. Moreover, the potent cytotoxicity displayed by *T. maritimum* ECPs against Senegalese sole HKL may give some insights regarding the mechanism by which this bacterium induces cell death.

Keywords: antigens, *Tenacibaculum maritimum,* ECPs, LPS, reactive oxygen species, nitric oxide.

1. Introduction

Knowledge on the pathogenicity, the pattern of lesions and the portal of entry of an organism are essential for understanding disease kinetics. Although several studies have examined the different physical characteristics and molecular heterogeneity of various *Tenacibaculum maritimum* isolates (Avendaño-Herrera *et al*. 2004), it is still necessary to determine their effect on the host. The interaction between microorganisms and phagocytic cells is a crucial determinant of the outcome of the disease process. Despite of macrophages create several antimicrobial compounds to fight most of the infiltrating pathogens, many pathogens have evolved not only to overcome these multifaceted microbicide responses but also to survive within macrophages. *Vibrio anguillarum* has ability to survive inside seabass (*Dicentrarchus labrax* L.) phagocytes, interfering with respiratory burst, and downregulating the expression of the apoptotic genes such as caspase-3 and caspase-9 (Sepulcre *et al*. 2007). Furthermore, *Vibrio tapetis* and its extracellular products induced great changes in *Ruditapes philippinarum* haemocytes including minimization of cell viability and phagocytic activity, changing the morphological size and the internal composition (Allam & Ford 2006). The ability of the pathogen to induce a disease is presumably not confined to host immune status, but rather additionally related to the survival and evading strategies implemented by this pathogen. Most pathogenic bacteria produce a variety of extracellular toxins or enzymes that are associated with their virulence including adhesins, hemolysins, cytotoxins, antiphagocytic factors, proteases, and complement resistant factors (Allan & Stevenson 1981). Additionally, other bacteria have the ability to sequester iron using fur regulating genes, penetrate the epithelial cells, and survive or multiply inside phagocytic cells (Ljungh & Wadstrom 1981).

The proteomic analysis of *Flavobacterium columnare* outer membrane defined several proteins including sarcosine-insoluble lipoproteins (Liu *et al*. 2008), metalloprotease (Bertolini & Rohovec 1992, Dumpala *et al*. 2010), and chondroitin AC lyase (Griffin 1991, Teska 1993), suggesting to be virulence factors contributing to host cell necrosis. Moreover, the crude extracellular preparation (CEP) of *Flavobacterium psychrophilum* recovered from rainbow trout (*Oncorhynchus mykiss*) necrotic myositis has the ability to degrade gelatin and type II collagen, secrete a protein complex with zinc metalloprotease-like activity which induces severe muscular necrosis following intramuscular injection (Ostland *et al*. 2000). It has been suggested that the virulence of bacteria belonging to the Flavobacteriaceae group, including *T. maritimum*, is associated with the extracellular proteins (ECPs), subcellular components, hemolysin, and lipopolysaccharides (Austin & Austin 1999). All *T. maritimum* described isolates contain a variety of analogous proteins with molecular masses between 14.4 and 97.4 kDa of potent proteolytic activities (Pazos

1997). *T. maritimum* strains, including the non-motile avirulent one (NUF1129), have shown the ability to produce chondrotinase and gelatinase. Rahman *et al*. (2014) suggested that the expression of some proteolytic enzymes during the microbial invasion is fundamentally required for successful colonization and bacterial virulence. Therefore, the current study aimed to analyze extracellular proteins profile of different *T. maritimum* strains of the same serotype at different phases of growth.

Gram-negative bacteria possess complex outer membrane molecules known as lipopolysaccharides (LPS), which consist of three categories: lipid A, the core polysaccharide, and an O-antigen side chain (Al-Hendy *et al*. 1992). Lipid A was considered endotoxin in some bacterial species while O-antigen side chain has been mentioned as a virulence factor in other pathogens (Al-Hendy *et al*. 1992, Amaro *et al*. 1997). The profiling analysis of *T. maritimum* LPS revealed that the O-chain compound contained an unusual linkage ([R]-2-hydroxyglutaric acid residue), which seems to be unique for this bacterium (Vinagradov *et al*. 2003). The role of that compound in biofilm formation, colonization, and cell death induction was briefly evaluated (Baxa *et al.* 1988, Vinagradov *et al*. 2003). To the best of our knowledge, little is known about *T. maritimum* pathogenicity, and there were no reports about *in vitro* activity of *T. maritimum* ECPs and LPS. Therefore, the aim of the current study is to gain deeper insights on the cellular innate immune responses of Senegalese sole head-kidney leukocytes following inoculation with those antigens. Furthermore, the cytotoxic activity of such stimulus against HKL was also evaluated.

2. Material & Methods

2.1. Bacterial strains

The *T. maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) used in the present study were isolated from Senegalese sole and belong to the serotype O3 described for *T. maritimum* (Avendaño-Herrera *et al*. 2005). Bacteria were kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and kept frozen at - 80 °C until being used. The recovery of all bacterial strains was achieved using marine agar (MA; Laboratorios CONDA, Spain) and *Flexibacter maritimus* medium (FMM) at 25 °C for 48-72 h.

2.2. Extracellular products (ECPs) extraction

The ECPs from *T. maritimum* strains were prepared following inoculation of each bacterial strain into sterile marine broth (Laboratorios CONDA, Spain) for 24 or 48 h at 25 ºC according to procedures described in **Chapter *2***. The harvested bacterial suspensions were then centrifuged at 4000 × *g* for 30 min at 4 ºC and the culture supernatants were

subsequently filtered using 0.2 µm pore size filters (Sarstedt). Cell-free supernatants were concentrated 75 fold using Amicon ultra-15 centrifugal filter units (Merck Millipore, Germany) according to the manufacturer´s protocol and stored at -80 °C.

Direct SDS-PAGE analysis of filtered culture supernatants from *T. maritimum*, collected following 24 and 48 h of culture, was performed after trichloroacetic acid (TCA) precipitation. Briefly, proteins from 1.5 ml aliquots of cell-free culture supernatants were precipitated with 10% (w/v) TCA for 30 min on ice and recovered by centrifugation. Protein pellets were washed in 10% (w/v) TCA, recovered by centrifugation, washed with acetone, allowed to dry, solubilized in SDS-sample buffer and subjected to SDS-PAGE. The latter was performed in a 10% polyacrylamide gel using the Laemmli discontinuous buffer system (Laemmli 1970).

To determine total proteins from the bacterial culture supernatants, a bicinchoninic acid Pierce assay kit (BCA; Thermo Fischer Scientific, USA) was employed using bovine serum albumin as a standard. Bacterial culture supernatants or ECPs were adjusted at 1 mg mL^{-1} and stored at - 80 ºC until being used.

2.3. Lipopolysaccharides extraction and purification

Lipopolysaccharides (LPS) were extracted by hot phenol-water according to the method described by Rezania *et al*. (2011) with slight modifications. 100 mL bacterial suspensions were centrifuged at 10,000 × *g* for 5 min and washed twice with 0.15 M PBS (pH=7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Pellets were then re-suspended in 10 mL PBS and sonicated on ice for 10 min. To eliminate protein and nucleic acids contaminants, the samples were treated with proteinase K, DNase and RNase prior to the extraction step. Proteinase K (100 µg mL^{-1}; Roche, Mannheim, Germany) was added to the mixture and the tubes were left for one additional hour at 65°C. Subsequently, the mixture was treated with RNase (40 µg mL^{-1}; Roche) and DNase (20 µg mL^{-1}; Roche) in the presence of 1 µL mL^{-1} 20% $MgSO_4$ and 4 µL mL^{-1} chloroform and incubated overnight at 37 °C. For the next step an equal volume of hot (65-70 °C) 90% phenol was added to the mixtures followed by vigorous shaking at 65-70 °C for 30 min, cooled on ice and then transferred to 50 mL polypropylene tubes and finally centrifuged at 3500 × *g* for 30 min. The supernatants were then transferred to 50 mL conical centrifuge tubes and extra phenol phases were removed using 20 mL hot (65-70°C) distilled water. Sodium acetate at 0.5 M final concentration and 10 volumes of 95 % ethanol were added to the extracts and samples were stored at -20 °C overnight to precipitate LPS. Tubes were then centrifuged at 3500 × *g* and 4 °C for 30 min and pellets were re-suspended in 1 mL distilled water, followed by extensive dialysis (Snake Skin dialysis tubing of 10 K MWCO, Thermo Fischer Scientific) against distilled water at 4 ºC. Purified LPS, without any

residual phenol, was lyophilized, its dry weight determined and re-suspended in PBS to a final concentration of 2 mg mL^{-1}, being kept at -20 ºC until used. Visualization was achieved by SDS-PAGE (12%) electrophoretic resolution of 20 µg purified LPS and consequent staining following the improved silver stain protocol described by Zhu *et al*. (2012).

2.4. Isolation of head-kidney leukocytes

Head kidney leukocytes (HKL) of sex healthy Senegalese sole were collected and isolated according to Secombes (1990) and Costas *et al*. (2011). Briefly, the head kidney was removed under aseptic conditions, pushed through a 100-µm nylon mesh and suspended in Leibovitz L-15 medium (L15; Gibco, United Kingdom) supplemented with 2% fetal bovine serum (FBS; Gibco), penicillin (P, 100 IU mL^{-1}; Gibco), streptomycin (S, 100 µg mL^{-1}; Gibco) and heparin (20 units mL^{-1}; Sigma). The suspensions were then loaded onto a 34:51% percoll (Sigma) density gradient and centrifuged at 400 × *g* at 4 °C for 40 min. The band of cells lying at the interface of the percoll gradient was collected and washed three times at 400 × *g* and 4°C for 5 min in L15, 0.1% FBS and P/S. The viable cell concentration was determined by the trypan blue exclusion test. Cells were counted in a hemocytometer and adjusted to 1.5 x 10^7 cells mL^{-1} in L15, 0.1% FBS and P/S. After overnight incubation at 18 ºC, the non-adherent cells were washed off and the monolayers were treated as described below.

2.5. Stimulation of Senegalese sole leukocytes

ECPs and LPS from *T. maritimum* strains (ACC6.1, ACC13.1, and ACC20.1) as well as LPS from *E. coli* were used to determine Senegalese sole cellular immune responses including ROS and NO. Furthermore, their cytotoxicity against HKL was conducted using MTT assay. Therefore, 100 µL of L-15 5 % FBS containing ECPs (10, 50 or 100 µg mL^{-1}) or LPS (10 µg mL^{-1}) were added to the cell monolayers. HKL in L-15 5 % FBS alone served as controls.

2.5.1. Nitric oxide (NO) production

NO production of HKL was conducted according Neumann *et al.* (1995). This method is based on the Griess reaction that quantifies the nitrite content of the leukocytes supernatants, because NO is an unstable molecule and degrades to nitrite and nitrate. Following stimulation with different antigens for 72 h at 18 °C, 50 µL of the supernatants were removed from individual wells and placed in a separate 96-well plate. Afterward, 100 µL of 1% sulphanilamide (Sigma) in 2.5% phosphoric acid was added to each well, followed by 100 µL of 0.1% N-naphthyl-ethylene-diamine (Sigma) in 2.5% phosphoric

acid. Optic density was measured at 540 nm. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

2.5.2. Reactive oxygen species (ROS) production

ROS from stimulated HKL were measured according to Secombes (1990). The principle of the current assay is based on the reduction in ferricytochrome C method for the detection of O_2^- and performed after the cells were incubated with L15 5% FBS at 18 °C for 24 h. Afterwards, the leukocyte monolayers were washed twice with phenol red-free Hank's balanced salt solution (HBSS, Gibco). Then, 100 µL suspensions of ferricytochrome C solution (2 mg ferricytochrome C mL^{-1} diluted in phenol red-free HBSS) containing different antigens as indicated above were added. Ferricytochrome C solution containing 10 µg mL^{-1} of phorbol myristate acetate (PMA, Sigma) was used as a stimulus for the respiratory burst and served as a positive control. Ferricytochrome C with PMA or different *T. maritimum* antigens and 0.725 mg mL^{-1} of superoxide dismutase (SOD; Sigma) was used to confirm the specificity of the reaction and served as negative controls. Triplicates for each treatment were displayed. Plates were read 30 min post stimulation, and were then taken on a Synergy HT (BioTek) microplate spectrophotometer at 550 nm. Optical densities were converted to nmol of O_2^- values obtained by multiplying 15.87 as described by Pick (1986).

2.5.3. Cytotoxic assay

Cytotoxic effects of *T. maritimum* antigens on Senegalese sole HKL were measured by a colorimetric method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) (Graham *et al*. 1988). Briefly, HKL monolayers were incubated with L15 5% FBS at 18 °C for 24 h. Subsequently, adherent cells were washed twice with phenol red-free HBSS and 100 µL of L15 5% containing different antigens or medium alone were added as indicated above. Then, 96-well plates were incubated at 18 °C for 24 h, and 10 µL of MTT (5 mg mL^{-1}) were added to each individual well. The plates were slightly agitated and the plates read after 15 min on a Synergy HT microplate spectrophotometer at 600 nm. The average values of triplicates were then estimated. The differences between the optical density reading for each treatment and control represented the degree of cytotoxic activity.

2.6. Statistical analysis

All results are expressed as means ± standard deviation (SD). Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also

verified using the Levene's test and, when necessary, they were transformed before being treated statistically. A non-parametric Kruskal-Wallis test, followed by multiple comparison tests, was used when data did not meet parametric assumptions. Data obtained following stimulation with ECPs were analyzed by two-way ANOVA, whereas that obtained following stimulation with LPS were analyzed by one-way ANOVA. All procedures were followed by Tukey post hoc through paired-comparisons to identify differences among experimental treatments. All statistical analyses were performed using the computer package statistical 12 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests. All results expressed as percentage were previously arcsine transformed (Zar 1999). Data from NO assay are presented as fold change levels (means ± SD), calculated by dividing each treatment value by the mean value from controls (unstimulated cells). Fold values higher than (0) were considered an increase.

3. Results

3.1. ECPs and LPS profiles

Samples from all *T. maritimum* strains displayed the same extracellular protein profiles with more characteristic clear bands after 48 h of incubation (Fig. 1). The samples showed banding patterns from 97.2 kDa down to14.3 kDa. Major single bands are located around 21, 30, 45, 67, and 75 kDa.

LPS profiles indicated that all samples from all *T. maritimum* strains revealed a characteristic dark staircase (ladder-like) pattern of bands with a similar profile (data not shown).

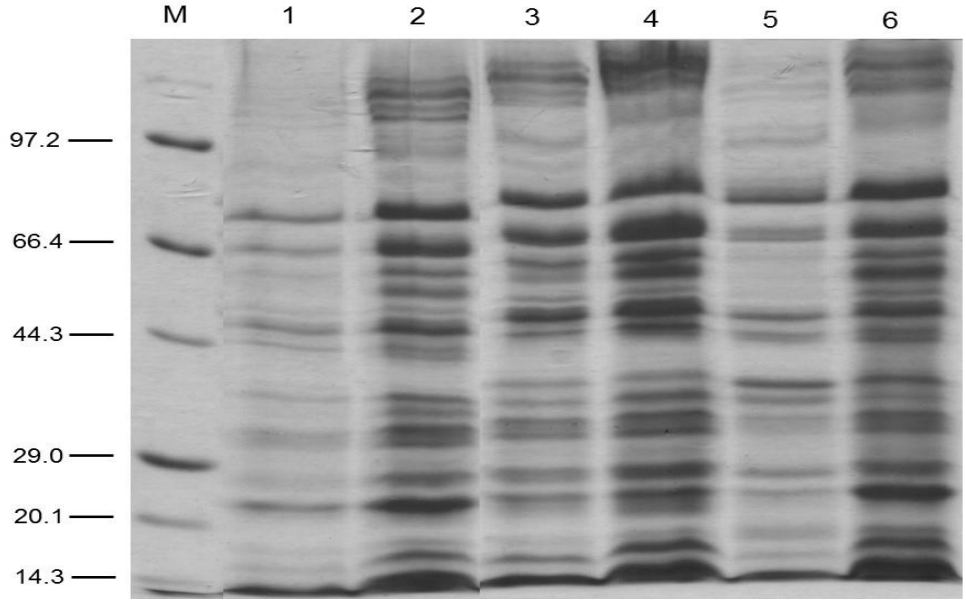

Fig.1. SDS-PAGE analysis of concentrated culture supernatants from *Tenacibaculum maritimum* after 24 or 48 h of culture in marine broth. Lane 1: strain ACC6.1 at 24 h; lane 2: strain ACC6.1 at 48 h; lane 3: strain ACC13.1 at 24 h; lane 4: strain ACC13.1 at 48 h; lane 5: strain ACC20.1 at 24 h; lane 6: strain ACC20.1 at 48 h. The numbers on the left indicate the position of the molecular weight standards (in kilodaltons).

3.2. NO assay

Senegalese sole HKL presented a slight NO production following exposure to ECPs even at the highest concentration. Moreover, ECPs from different bacterial strains induced similar NO responses within each particular concentrations (Fig. 2). Likewise, there was no significant variation in NO production in Senegalese sole HKL exposed to LPS from different *T. maritimum* strains (Fig. 3).

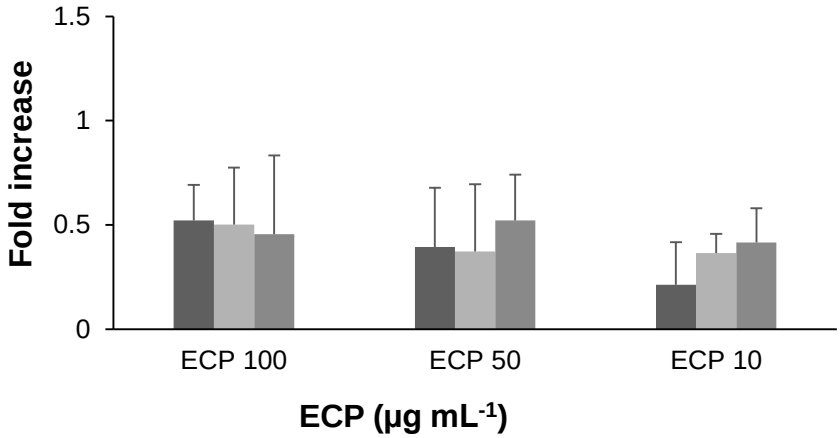

Fig.2. Nitric oxide production (µM) of Senegalese sole head-kidney leukocytes following exposure to ECPs of ACC6.1 (■), ACC13.1 (■), and ACC20.1 (■) *T. maritimum* strains at different concentrations (100, 50, and 10 µg mL^{-1}). Data are presented as fold increase values compared to unstimulated cells and presented as means ± SD (n = 6).

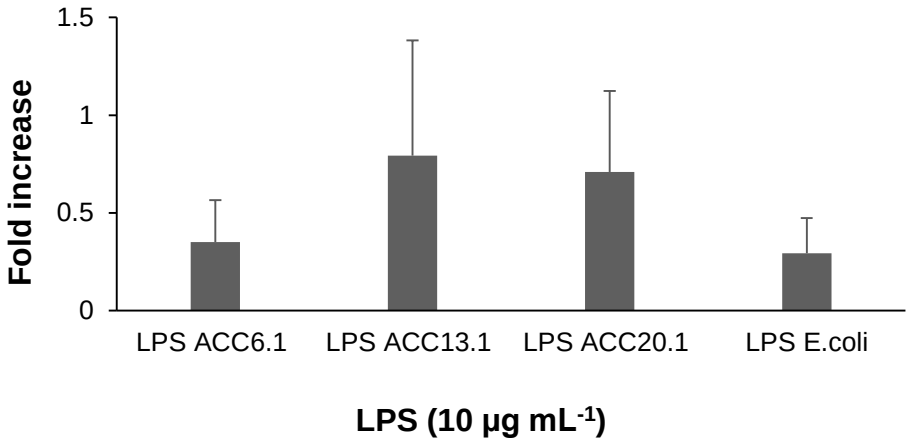

Fig.3. Nitric oxide production (µM) of Senegalese sole head-kidney leukocytes following exposure to LPS of three different strains of *T. maritimum* (10 µg mL^{-1}; ACC6.1, ACC13.1, and ACC20.1) and *E. coli* (10 µg mL^{-1}). Data are presented as fold increase values compared to unstimulated cells and presented as means ± SD (n = 6).

3.3. ROS assay

ROS produced by Senegalese sole HKL following stimulation with PMA, which served as a positive control, was extremely higher than that triggered following exposure to both ECPs and LPS (results not shown). Senegalese sole HKL increased ROS levels in a different fashion depending on ECPs concentration, with lower values produced by cells exposed to the highest ECPs (100 µg mL^{-1}) in contrast to higher levels in HKL exposed to the lowest (10 µg mL^{-1}) concentration (Fig. 4). On the other hand, HKL exposed to LPS from both *T. maritimum* and *E. coli* showed a similar pattern of ROS production with no significant changes (Fig. 5).

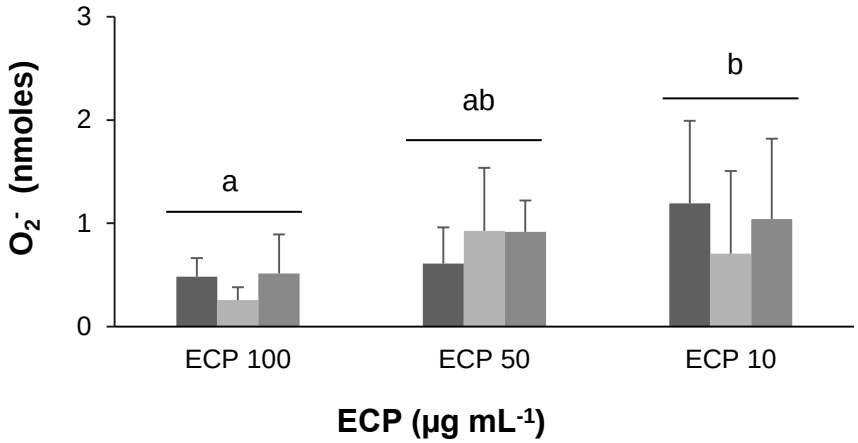

Fig.4. ROS production (nmoles) of Senegalese sole head-kidney leukocytes following exposure to ECPs from ACC6.1 (■), ACC13.1 (■), and ACC20.1 (■) *T. maritimum* strains at different concentrations (100, 50, and 10 µg mL^{-1}). Data are presented as means ± SD (n = 6). Different letters indicate significant differences among ECPs concentrations (two-way ANOVA; $P \leq 0.05$).

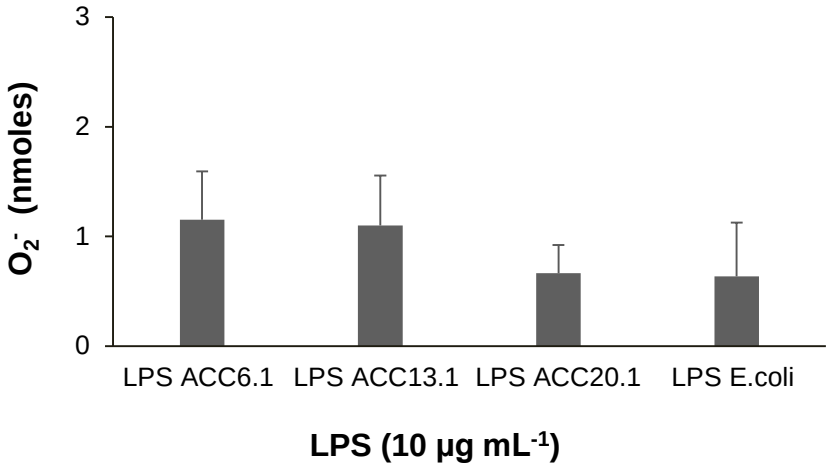

Fig.5. ROS production (nmoles) of Senegalese sole head-kidney leukocytes following exposure to LPS from three different strains of *T. maritimum* (ACC6.1, ACC13.1, and ACC20.1) and *E. coli* at a concentration of 10 µg mL^{-1}. Data are presented as means ± SD (n = 6).

3.4. Cytotoxicity assay

All ECPs and LPS treatments induced cytotoxicity in Senegalese sole HKL. Regardless bacterial strains, ECPs reduced HKL viability in a dose dependent manner (Fig. 6). A potent cytotoxicity was observed in HKL exposed to the highest ECPs concentration (100 µg mL^{-1}), in contrast to lower levels observed in host cells exposed to the lowest one (10 µg mL^{-1}). Concerning LPS, a slight cytotoxicity was observed with no significant variations in HKL exposed to different *T. maritimum* strains and *E. coli* (Fig. 7).

Through light microscopy, it was observed that HKL incubated with L15 medium alone retained a typical morphology with round and intact with smooth edges (Fig. 8A). Following incubation with ECPs at the highest concentration, there was evidence of internal vacuolization, cellular detachment and elongation, and cell wall deterioration (Fig. 8B), and the most prominent feature was cell clustering and aggregation (Fig. 8C).

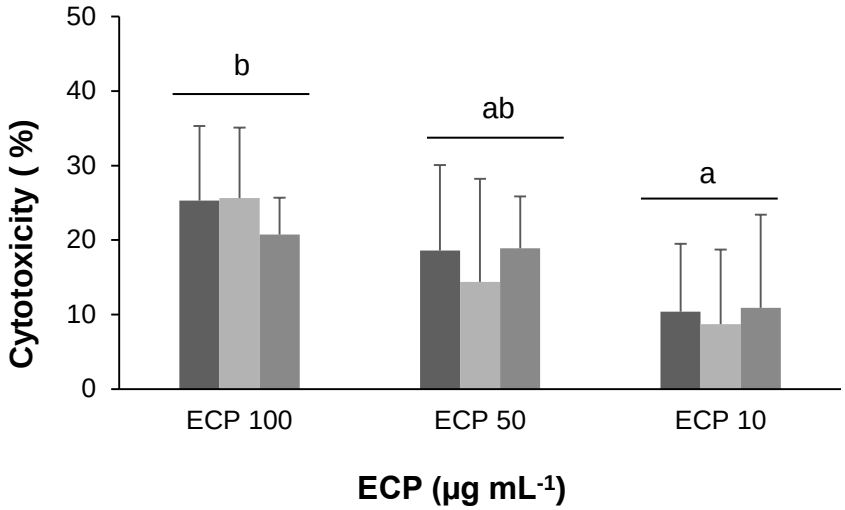

Fig.6. Cytotoxic activity of ACC6.1 ( ), ACC13.1 ( ), and ACC20.1 ( ) *T. maritimum* ECPs against Senegalese sole head-kidney leukocytes at different concentrations (100, 50, and 10 µg mL^{-1}). Data are presented as means ± SD (n = 6). Different letters indicate significant differences between ECPs concentrations (two-way ANOVA; $P \leq 0.05$).

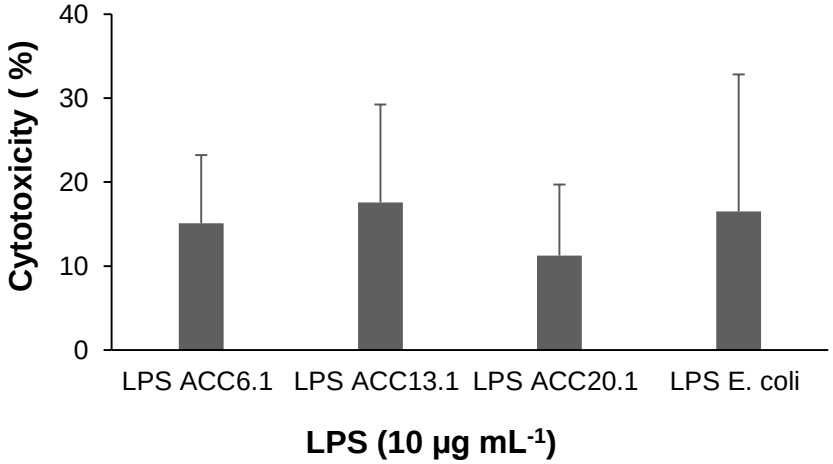

Fig.7. Cytotoxic activity of LPS of different *T. maritimum* strains (ACC6.1, ACC13.1, and ACC20.1) and *E. coli* against Senegalese sole head-kidney leukocytes at a concentration of 10 µg mL^{-1}. Data are presented as means ± SD (n = 6).

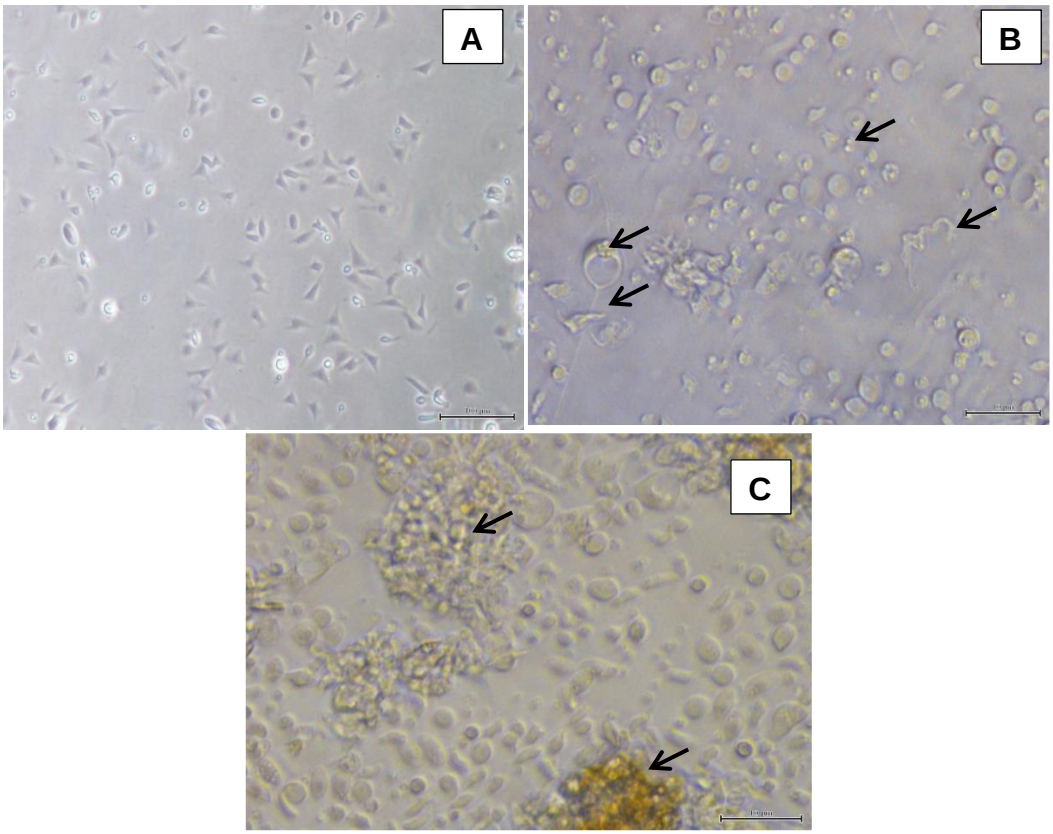

Fig.8. Light microscopy view of Senegalese sole head-kidney leukocytes following incubation with L15 medium (A) or *T. maritimum* ECPs at a concentration of 100 µg mL^{-1} for 24 h. Different arrows referred to increased vacuolization, cellular elongation and cell wall deterioration (B), as well as cell clustering and aggregation (C).

4. Discussion

ECPs have been implicated as important virulence factors for many invading pathogens. A first prerequisite for the successful invasion of the host tissue is the ability of pathogens to produce extracellular proteolytic enzymes, advancing them to withstand host defense, utilizing host tissue as a source of nutrition which in role enabling them to survive and replicate within the host (Ellis 1991, Falkow 1991, Stanley *et al*. 1994). Based on cell wall analysis, previous studies on *T. maritimum* isolates using whole cell protein profiling revealed a relative homogeneity among strains (Bernardet *et al*. 1994). The proteomic and immunoblotting analysis of *T. maritimum* outer membrane was studied by Avendaño-Herrera *et al*. (2004), who reported that all strains contributed a plentiful number of the same bands between 14.2 and 66 kDa. Similarly, Pazos (1997) observed similar patterns of protein profiles among *T. maritimum* isolates with a molecular mass in the range of 14.4 -97.4 kDa. Results from the current study indicated that *T. maritimum* strains belonging to the same serotype produced similar ECPs profiles, in agreement with that previously

reported by other authors. Contrary to the current study, van-Gelderen *et al*. (2010) observed heterogeneity in ECPs from 18 *T. maritimum* isolates. Heterogeneity of ECPs was also observed in other marine pathogenic bacterial species including *Aeromonas salmonicida* (Gudmundsdóttir 1996). In the present study, no variations in protein profiling regarding the time of incubation (either 24 h or 48 h) were observed. Similar results were obtained by Newton *et al*. (1997) who stated that the expression of the two proteases of *F. columnare* LA 88-173 did not change regarding the culture condition and the time factor, as the two proteases with apparent molecular masses of 53.5 and 58 kDa were seen earlier after one day of inoculation, and remain constant all over the experiment. In contrast, Bertolini & Rohovec (1992) stated that the extracellular proteases of *Flabobacterium columnare* changed slightly regarding the time factor. Similarly, Secades *et al*. (2001) recorded variations in the production of the *Flabobacterium psychrophilum* Fpp1 enzyme which was classified as one member of metalloproteases. The authors attributed these variations to many factors such as calcium concentration, culture growth phase, and culture temperature. Nevertheless, *T. maritimum* ECPs profiles from the above mentioned previous works and the present study appear to be too complex and remind whole bacterial cell proteins profiles. This scenario could be attributed to a spontaneous process of bacterial cell death and proliferation which still requires further investigation.

ECPs contain several enzymes and toxins and their roles not only confined to disease induction but also, extended to be involved in pro-inflammatory responses. It has been suggested that bacterial ECPs are more important than the bacterial cell to the inflammatory reaction (Mutoloki *et al*. 2006). In the current study, Senegalese sole HKL presented the same pattern of NO production following exposure to both ECPs and medium alone (control), with no significant variation among strains. These results are in agreement to that obtained by Billings (2006) who stated that *F. columnare* ECPs failed to increase NO production after incubation with channel catfish (*Ictalurus punctatus*) phagocytes. Similarly, Villamil *et al*. (2003) observed a reduction in NO production following stimulation of turbot head-kidney macrophages with higher doses of *Vibrio pelagius* ECPs. In contrast, Yano (1996) stated that catfish head-kidney cells produce NO following exposure to *Edwardsiella ictaluri* and attributed that effect to the bacterial ECPs. Contrary to mammals, extremely high concentrations of LPS are needed to induce leukocytes immune responses in different fish species (Hirono *et al.* 2004). It is known that lower vertebrates, mostly fish and amphibians, are resistant to the toxic effects of LPS (Berczi *et al*. 1966), suggesting that the TLR4 associated molecules (CD14, LY96 (MD-2) and TICAM2 (TRAM)) that are required for the TLR4-mediated response to the endotoxic moiety of LPS in mammals, may be absent or may perform different functions in fish.

Based on that argument, it is hypothesized that the low sensitivity of Senegalese sole HKL to LPS exposure was probably due to the low concentration used. Similar results were obtained by Iliev (2005) who observed great resistance and lowered sensitivity in leukocytes of several fish species to LPS activation.

Senegalese sole HKL increased ROS production following exposure to the lowest ECPs concentrations with no changes when stimulated with LPS. In contrast, Billings (2006) reported that ECPs from *F. columnarae* have no effect on channel catfish PMA-stimulated cells for hydrogen peroxide production. It was observed that the higher concentration of ECPs suppressed ROS production while the lower one triggered that response. In the present study, it is tempting to speculate that *T. maritimum* appears to present the ability to evade host phagocyte responses, being the production of immunosuppressive extracellular toxins or enzymes most prominent tools. A similar evading strategy was observed in other pathogens including *Renibacterium salmoninarum* (Bandin *et al*. 1993). On the other hand, the increased ROS production in HKL from the present study could be also related the lower cytotoxicity observed in host cells exposed with the lowest ECPs concentration.

To the best of our knowledge, this is the first study showing the cytotoxic activity of ECPs and LPS from *T. maritimum* as antigens against Senegalese sole HKL. The current study revealed that both ECPs and LPS induce cytotoxicity after 24 h of incubation in a different manner. Light microscopic observation showed that over a period of 24 h, HKL cells incubated with bacterial ECP at higher concentration (100 µg mL^{-1}) exhibited morphological changes such as detachment, enlargement, cell wall deterioration and increased vacuolization while those incubated with medium alone remained small, intact, with smooth edges, typical normal cell morphology. Similarly, Xiang *et al*. (2008) observed a condensation of nuclear chromatin, fragmentation of genomic DNA and formation of apoptotic bodies following activation of goldfish (*Carassius auratus*) lymphocytes with various doses of LPS. Moreover, Peselis (2011) observed increased vacuolization and cell wall deterioration in Atlantic salmon kidney (ASK) cells following stimulation with higher appropriate multiplicity of infection MOI dose of *F. columnarae*. The author also added that the lethality of *F. columnare* culture supernatant suggested that the pathogen secretes one or more toxins into its environment that cause rapid death to ASK. The role of ECPs from *T. maritimum* in host cell death and bacterial pathogenicity was previously mentioned by Baxa *et al*. (1988) and Rahman *et al.* (2014). Furthermore, van-Geldern *et al*. (2009) illustrated that *T. maritimum* ECPs have a direct effect *in vivo* rather than *in vitro* in Atlantic salmon, and their virulence appears to be not only confined to toxins production but also included extracellular phosphatase, phosphoamidas and proteases such as hemolysin, chondrotinase and gelatinase.

5. Conclusions

T. maritimum strains belonging to the same serotype produced a variety of ECPs profiles. Senegalese sole HKL increased ROS while failed to induce NO production following exposure to *T. maritimum* ECPs. Moreover, HKL appear to be irresponsive following exposure to LPS. Moreover, *T. maritimum* ECPs displayed a potent cytotoxicity against Senegalese sole HKL giving thus some insights regarding the mechanism by which this bacterium induces cell death.

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Chapter 5

Cellular, humoral and mucosal innate immune responses in Senegalese sole, *Solea Senegalensis* (Kaup), challenged with *Tenacibaculum maritimum*

M. Mabrok

Cellular, humoral and mucosal innate immune responses in Senegalese sole, *Solea Senegalensis* (Kaup), challenged with *Tenacibaculum maritimum*.

Abstract

Tenacibaculosis, caused by *Tenacubaculum maritimum*, continues to inflict substantial losses among marine water cultured species including Senegalese sole. However, the immune mechanisms involved in this disease in fish are still poorly understood. The present study aimed to investigate the skin mucus terminal carbohydrate composition, several immune-related enzymes (i.e. lysozyme, peroxidase, proteases and antiproteases), haemolytic activity of complement and the bactericidal activity in skin mucus and plasma of Senegalese sole in a time-course basis following bath challenge with *T. maritimum*. The haematological profile and the kinetics of cell migration post infection were also considered. Lectin binding results suggest that skin mucus contains in order of abundance, α-D-mannose, α-D-glucose, N-acetylneuraminic acid and N-acetyl-D-galactosamine. Passive changes in the glycosylation pattern following bacterial challenge were observed and may consider an influential process to overcome host mucosal immunity. In general, our results demonstrated a delay in the skin mucus immune response compared to that found in plasma. A significant increase in skin mucus lysozyme, complement, protease and antiprotease activities were observed at the end of the experiment (14 days post challenge). Interestingly, the higher activity of these enzymes was positively related with the skin mucus bactericidal capacity, suggesting that these enzymes play an important role in the defence against Gram-negative bacteria. The haematological profile revealed a significant increase in peripheral lymphocytes in challenged fish after 14 days following infection. Although the present study showed inflammatory processes and immune parameters alteration following challenge with *T. maritimum*, the route of entry and the survival strategy of this particular pathogen still not fully elucidated and required further investigations.

Keywords: Time-course, tenacibaculosis, glycosylation pattern, innate immune system, skin mucus, teleosts.

1. Introduction

Disease outbreak has all the earmarks of being the most critical impediment on the advancement of the Senegalese sole (*Solea senegalensis*) commercial production, with the bacterial disease most often diagnosed (Morais *et al*. 2015). The immune system is an exceptionally advanced framework that capacities to outfit living beings with the capacity to oppose pathogenic microorganisms and described by two pathways, innate and acquired immunity. The innate immunity contributes to a large number of humoral and cellular factors which assume an imperative part as the first line of defense against invading pathogens (Ellis 1999). Bacterial adhesion to tissues of the fish is an important issue during the initial stage of infection (Thune *et al*. 1993, Toranzo & Barja 1993). Therefore, physical barriers are considered the first line of defense against microbial infections (Shephard 1994, Ellis 2001, Olivares-Fuster *et al*. 2011). Skin of teleost fish is unique and diverse in terms to the mucus secretion which involves immune defenses (Fast *et al.* 2002, Salinas *et al*. 2011). Mucus forms a biofilm insulating layer that protects the underlying epithelium from damage. Interactions between this mucus layer and bacteria are important in disease progression. It consists mainly of water and glycoproteins of oligosaccharides nature responsible for gel formation (Perez-Vilar & Hill 1999). Oligosaccharides in particular are thought to interfere with microbial adhesion and therefore, prevent glycoprotein degradation by the act of microbial proteases (Abraham *et al*. 1999, Ascencio *et al*. 1998, Mantle & Husar 1993). Furthermore, mucin carbohydrates act as receptors that play a fundamental role in either pathogens elimination or settlement and colonization (Cone 2009, Estensoro *et al*. 2013). Subsequently, all suspended and trapped particles, bacteria or viruses are removed from the mucus layer by the act of water current (Mayer 2003). The protective role of the mucus has been elucidated in several fish species including rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*) and Atlantic salmon (*Salmo salar*) (Fast *et al.* 2002), turbot (*Scophthalmus maximus*) and aye (*Plecoglossus altivelis*) after being challenged with *Vibrio anguillarum* (Fouz *et al*. 1990, Kanno *et al.* 1989), gilthead seabream (*Sparus aurata*) and European seabass *(Dicentrarchus labrax)* after being challenged with *Pasteurella piscicida* and *Flexibacter maritimum* (Magariños *et al.* 1995).

Once the superficial barriers are breached, an inflammatory reaction is prompted with a definitive inundation of phagocytes that have powerful bactericidal properties (Ellis 2001). The role of neutrophils and macrophages in phagocytosis, debasement of attacking microorganisms as well as different patterns of localization and mobilization into the infected areas is all around reported in several fish species (Steinhagen & Jendrysek 1994, Secombes 1996, Matsuyama *et al*. 1999, do Vale *et al*. 2002). Both macrophages and neutrophils can produce reactive oxygen species (ROS) during the respiratory burst

on contact with organisms (Campos-Perez *et al*. 1997, Costas *et al*. 2014). Furthermore, there are various soluble compounds including (complement, transferrins, antiproteases, lysozyme, lectins, C-reactive protein) that likewise increment in reaction to attacking microorganisms, making up the complement system considered the most vital guard element (Ellis 2001). Most of these parameters are present in fish mucus and plasma and have been used as indicators for disease induction (Magnadóttir 2006). The fish complement cascades are usually synthesized as inactive precursors which serve either as enzymes or as binding proteins upon activation by certain foreign substances. Activation of complement is initiated by different stimulus, including LPS which is the main constituent of Gram-negative bacterial cell wall. The activation is substantially initiated via three pathways: the classical pathway (CCP) (Holland & Lambris 2002), alternative pathway (ACP) and lectin pathway (LCP) (Sakai 1992). The functions of complement are numerous, but it is most well-known for its bactericidal activities, bacterin neutralization, and detoxifications, cytotoxic activities against heterologous cells, opsonization and recruitment of phagocytic cells to the site of inflammation (Ellis *et al*. 1981, Sakai 1992, Griffin 1983). Regardless of endeavors made in the last decades, there are impressive gaps concerning the route of entry and survival strategies of *Tenacibaculum maritimum* and little is known about host immune responses against this particular pathogen. Therefore, the current study aims to investigate both systemic and mucosal immune responses of Senegalese sole challenged with *T. maritimum*. Moreover, the modulation in the skin mucus glycosylation patterns in Senegalese sole exposed to *T. maritimum* will also be assessed using a lectin-binding assay.

2. Material and methods

2.1. Bacterial culture condition and inoculum preparation

T. maritimum strain ACC6.1 isolated from the Senegalese sole in a local fish farm (Póvoa de Varzim, Portugal) 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 (Laboratorios CONDA, Spain) at 25 °C for 48 h. For inoculum preparation, the bacteria were harvested and inoculated into 50 mL of marine broth (MB) for additional 48 h under the same temperature with continuous shaking (140 rpm). Exponentially growing bacteria were harvested by centrifugation at 4000 × *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}) as described in **Chapter 2**.

2.2. Experimental fish

Healthy Senegalese sole were obtained from a commercial fish farm (Torreira, Portugal) with no history of tenacibaculosis. Fish weighing 25.5 ± 5.9 g were maintained in a recirculated aerated sea water (salinity 33 ± 1) system, where dissolved oxygen was maintained around 90%, water temperature at 21 ± 1 °C, and a 12 h light/12 h dark photoperiod was adopted. Water quality was maintained with mechanical and biological filtration, and the fish were fed daily 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. Only healthy fish, as indicated by their activity and external appearance, were used in the experiments.

2.3. Experimental design

Experiments were performed by trained scientists and following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes. Twelve groups of seven fish each were randomly distributed in 2 identical recirculated water systems composed of 6 tanks filled with 8 L of aerated sea water. Thereafter, fish were left to acclimate for five days prior to bacterial challenge. Subsequently, fish in one of those systems (6 tanks, n = 42) were bath challenged according to **Chapter 2** with a final bacterial concentration of 2.7 x 10^5 CFU mL^{-1} in the rearing water. Fish in the other system (6 tanks, n = 42) were challenged with sterile marine broth (MB) and served as controls. One fish was then removed from each tank at the following times after bacterial challenge: 0 h, 4 h, 24 h, 48 h, 3 days, 7 days and 14 days, and skin mucus, blood and plasma were collected as described below. All groups were maintained in both systems for up to 14 days at 23 ± 1 °C. 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.

2.4. Sampling procedures

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. Briefly, the ocular and blind sides of both control and challenged fish were gently scraped by using a cell scraper with enough care to avoid contamination with urogenital and/or intestinal excretions. Collected mucus samples were then centrifuged at 1500 × *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 withdrawal was performed from the caudal

vein using heparinized syringes. An aliquot of homogenized blood was used for total white blood cells counting and blood smears, whereas the remaining blood was centrifuged at 10,000 × *g* during 10 min at 4 °C and the resulting plasma stored at -80 °C until assayed. Immediately after blood collection, blood smears were performed from homogenized blood, air dried, and stained with Wright's stain (Haemacolor; Merck) after fixation with formol-ethanol (10% of 37% formaldehyde in absolute ethanol). Detection of peroxidase activity was conducted as described by Afonso *et al*. (1998) in order to facilitate detection of neutrophils. The slides were examined (1000 x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. The absolute value (x 10^4 mL^{-1}) of each cell type was then calculated.

2.5. Innate immune parameters

2.5.1. Lysozyme activity

Lysozyme activity was measured based on the turbidimetric method described by Swain *et al*. (2007) with some modifications. Briefly, 20 µL of skin mucus or 15 µL of plasma samples were placed in flat-bottomed 96-well plates in duplicate. To each well, 180 µL of freeze-dried *Micrococcus lysodeikticus* (0.5 mg mL^{-1}) previously dissolved in 0.05M sodium phosphate buffer with pH 6.2 was added as lysozyme substrate. 180 µL of 0.04 M sodium phosphate buffer free *Micrococcus lysodeikticus* was added and served as sample blank. Two wells with 200 µL of 0.04M sodium phosphate buffer were used as a standard blank. The reduction in absorbance at 450 nm was measured after 20 min at 35 ºC in a Synergy HT microplate reader. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.04 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve, and expressed as µg mL^{-1}.

2.5.2. Alternative complement pathway (ACP) activity

The ACP was measured only in mucus samples due to technical constrains and according to Sunyer & 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. Rabbit red blood cells (RaRBCs; Probiologica Lda, Portugal) were used for ACP determination. RaRBCs were washed three to four times in GVB and then re-suspended in GVB to a final concentration 2.5 x 10^8 cells mL^{-1}. Twenty µL of RaRBCs suspension were mixed with 40 µl of serially diluted skin mucus in Mg-EGTA-GVB buffer. The same volume of RaRBCs was mixed with cold sterile distilled water which induced 100% hemolysis and served as a positive control, whereas RaRBCs mixed

with Mg-EGTA-GVB buffer served as spontaneous control. 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 hemolysis was estimated by measuring the optical density (OD) of the supernatant at 414 nm in a Synergy HT microplate reader (Biotek). The ACH_{50} units were defined as the concentration of skin mucus giving 50% hemolysis of RaRBCs.

2.5.3. Anti-protease activity

Total anti-protease activity was determined by the ability of skin mucus or plasma to inhibit trypsin activity according to Machado *et al.* (2015) with some modifications for mucus samples. Briefly, 50 µL of skin mucus or 10 µL of plasma samples were incubated 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 °C in polystyrene microtubes. To the incubation mixture, 60 or 100 mL of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) and 125 mL of azocasein (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3; Sigma) were added to skin mucus and plasma, respectively, and incubated for 1 h at 22 °C. Finally, 250 mL of trichloroacetic acid were added to each microtube and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 x *g* for 5 min at room temperature. Afterwards, 100 mL of the supernatant was transferred to a 96 well-plate that previously contained 100 mL of NaOH (40 mg mL^{-1}) per well. The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer in place of skin mucus or plasma and trypsin served as blank whereas the reference sample was phosphate buffer in place of skin mucus or plasma. The percentage inhibition of trypsin activity compared to the reference sample was calculated. All analyses were conducted in duplicates.

2.5.4. Protease activity

The protease activity was measured only in mucus samples due to technical constrains and using the azocasein hydrolysis assay according to Ross *et al.* (2000). Briefly, 100 µL of skin mucus were incubated with an equal volume of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0) containing azocasein (20 mg mL^{-1}, Sigma) for 24 h at 30 ºC. Thereafter, the reaction was stopped by adding 10% trichloroacetic acid and the mixture was vigorously shacked and centrifuged at 10,000 × *g* for 10 min. The supernatants were then transferred to a 96-well plate in triplicates containing 100 µL per well of NaOH (40 mg mL^{-1}), and the OD was read at 450 nm using a Synergy HT microplate reader. Skin mucus was either replaced by trypsin (5 mg mL^{-1}, Sigma) which served as positive control (100% of protease activity), or by phosphate buffered, which served as negative control (0% activity). The protease activity was calculated using the following formula:

Protease activity (%) = ((Reading Abs- Negative control Abs) / Positive control Abs) x100.

2.5.5. Peroxidase activity

The peroxidase activity in skin mucus or plasma was estimated according to Quade & Roth (1997). Briefly, 30 µL of skin mucus or 15 µL of plasma samples were diluted in 120 µL or 135 µL of Hank's buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat bottomed 96-well plates, respectively. Afterwards, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (Sigma) and 50 µL of 5 mM H_2O_2 were added to each well. The color-change reaction was stopped after 2 min by adding 50 µL of 2 M sulphuric acid, and the OD was measured at 450 nm in a Synergy HT microplate reader. Samples without skin mucus or plasma were used as a blank. The peroxidase activity (units mL^{-1} plasma) was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

2.5.6. Bactericidal activity

The bactericidal assay was carried out using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay as described elsewhere (Machado *et al*. 2015). Briefly, 20 µL of skin mucus or plasma samples were added to duplicate wells of a U-shaped 96-well plate. HBSS was added to some wells instead of skin mucus or plasma, and served as positive control. To each well, 20 µL of the *T. maritimum* strain (ACC6.1; 1×10^8 bacteria mL^{-1}) in marine broth (Laboratorios CONDA, Spain), or *Photobacterium damselae* subsp. *piscicida* (PP3; 1×10^6 bacteria mL^{-1}) in tryptic soya broth (BD, France) were added and the plates were incubated for 2.5 h at 25 ºC. To each well, 25 µL of MTT (1 mg mL^{-1}; Sigma-Aldrich) were added and incubated for 10 min at 25 ºC to allow the formation of formazan. Plates were then centrifuged at 2000 × *g* for 10 min and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma-Aldrich). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was calculated from analytical triplicates and expressed as percentage of bacteria surviving in relation to the number of bacteria from positive controls (100%).

2.6. Determination of the terminal glycosylation pattern

Glycosylation pattern in the skin mucus was determined by lectin ELISA as described previously (Neuhaus *et al*. 2007). Briefly, 100 µL of skin mucus (diluted 1:5 in 50 mM carbonate- bicarbonate buffer, pH 9.6) were placed in flat- bottom 96-well in duplicates and incubated overnight at 4 °C. Samples were then rinsed three times with PBS-T (20 mM phosphate saline buffer and 0.05% Tween 20, pH 7.3), blocked for 2 h at room temperature with blocking buffer (3% BSA in PBS-T) and rinsed again with PBS-T. Subsequently, the samples were incubated for 1h with 20 mg per well of biotinylated lectins (Table 1; Sigma), washed and incubated streptavidin conjugated to horseradish-

peroxidase (HRP, 1:1000 Life Technologies). After exhaustive rinsing with PBS-T the samples were developed using 100 mL of a 0.42 mM solution of 3, 30, 5, 50-tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 mL of 2 M H_2SO_4. The plates were read at 450 nm in a plate reader (FLUO star Omega, BMG Labtech). Negative controls consisted of samples without mucus or without lectins, whose OD values were subtracted from each sample value. Data are presented as the OD at 450 nm.

Table 1: Lectins used in this study, their acronym and sugar-binding specificities.

Acronym	Lectin source	Sugar binding specificity
UEA I	*Ulex europeaus*	α-L-Fucose
Con A	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
WFA	*Wisteria floribunda*	N-acetyl-D-galactosamine
SNA	*Sambucus nigra*	N-acetylneuraminic acid
LEA	*Lycopersicon esculentum*	N-acetyl-β-D-glucosamine

2.7. Statistical analyses

All results are presented as means ± standard deviation (SD). Data were analyzed for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test) and, when necessary, they were log-transformed before being treated statistically. Data were analyzed by two-way ANOVA followed by Tukey tests to identify significantly different between groups. A non-parametric Kruskal-Wallis test, followed by multiple comparison tests, was used when data did not meet parametric assumptions. All statistical analyses were conducted using the computer package STATISTICA 12 software for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

3. Results

3.1. Pathogenicity assay

No mortalities were recorded in Senegalese sole bath challenged with a LD_{50} dose of *T. maritimum*. However, some specimens showed caudal fin erosions, red mouth, detached skin and faint gills (results not shown).

3.2. Host cell responses against *T. maritimum*

Peripheral lymphocyte numbers in challenged fish remained similar to control individuals throughout the trial until 14 days of challenge, where a significant lymphocytosis was observed in bath challenged specimens (Fig. 1A). Those levels were also higher compared to infected fish at time 0 h, while no changes were observed in control individuals during the whole trial. Similarly, circulating thrombocytes remained similar between control and challenged fish (Fig. 1B). Moreover, a significant increase in peripheral neutrophil numbers was observed in the challenged fish at times 0 h, 48h and 3 days post challenge (Fig. 1C). Similarly, an increase in circulating monocytes was also observed at times 0 h and 24 h post challenge (Fig. 1D). Regardless time factor, challenged fish showed a marked neutrophilia (P = 0.001) and monocytosis (P = 0.004) compared to control groups, with no changes throughout the trial.

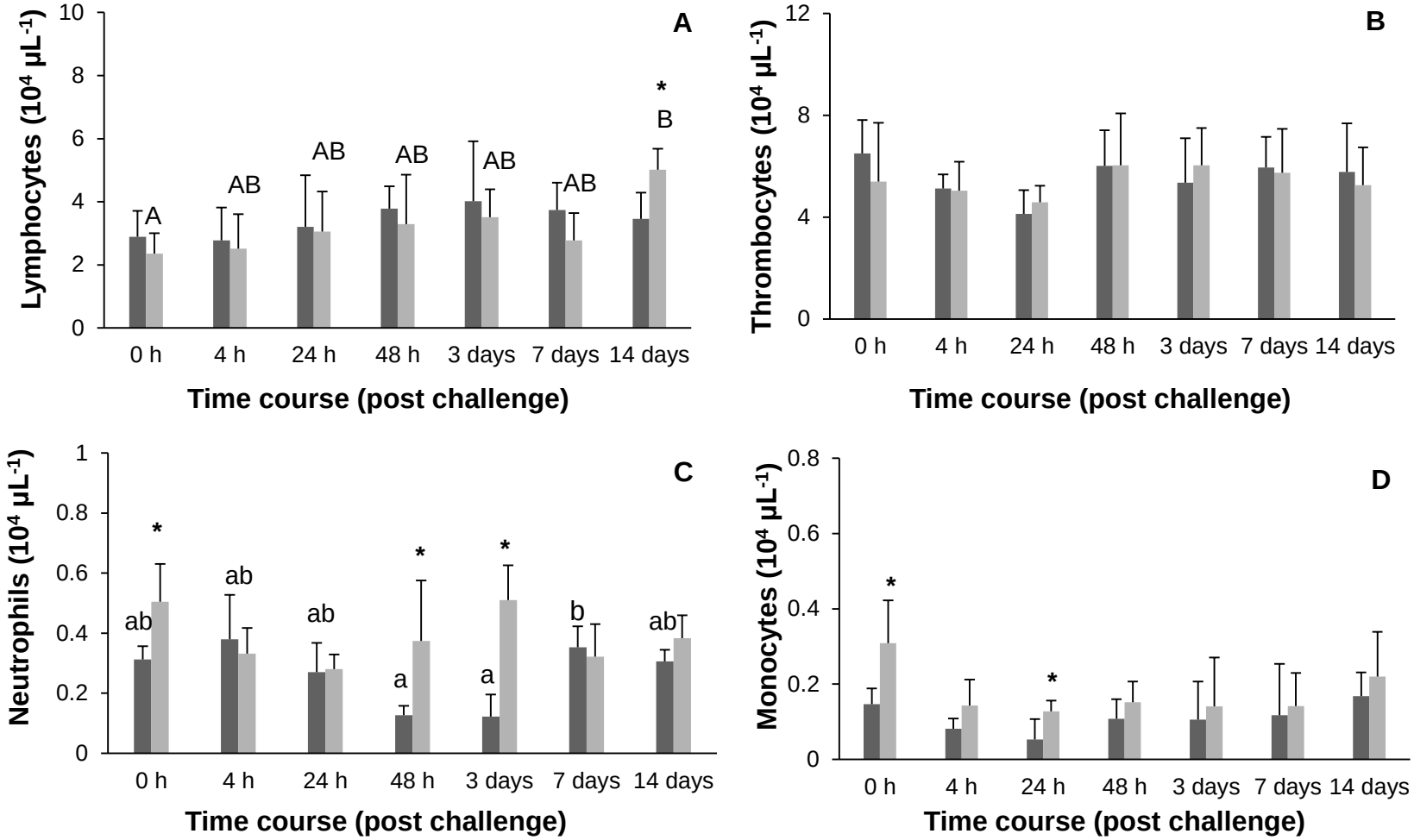


Fig.1. Proportion of peripheral lymphocytes (A), thrombocytes (B), neutrophils (C) and monocytes (D) of Senegalese sole following bath challenge with marine broth (MB) as a control (■) and *T. maritimum* ACC6.1 with concentration 2.7 x 10^5 CFU mL^{-1}(■) during 14 days. Data (10^4 μL^{-1}) are presented as means ± SD (n = 6). Different small letters mean significant differences among control groups, while capital letters mean significant differences among challenged groups regarding the time-course post challenge. Single asterisk denotes significant differences between control and challenged group for a given time (two-way ANOVA; $P \leq 0.05$).

3.3. Innate immune responses post challenge

3.3.1. Lysozyme activity

The kinetics of skin mucus lysozyme activity of both challenged and control groups are presented in Fig. 2A. While no changes were observed in control fish, bath challenged individuals increased mucosal lysozyme activity compared to controls at 14 days following infection and compared to infected fish at earlier times. A similar outcome was also observed for plasma lysozyme levels, which increased already at day 3 in infected individuals following bath challenge (Fig. 2B).

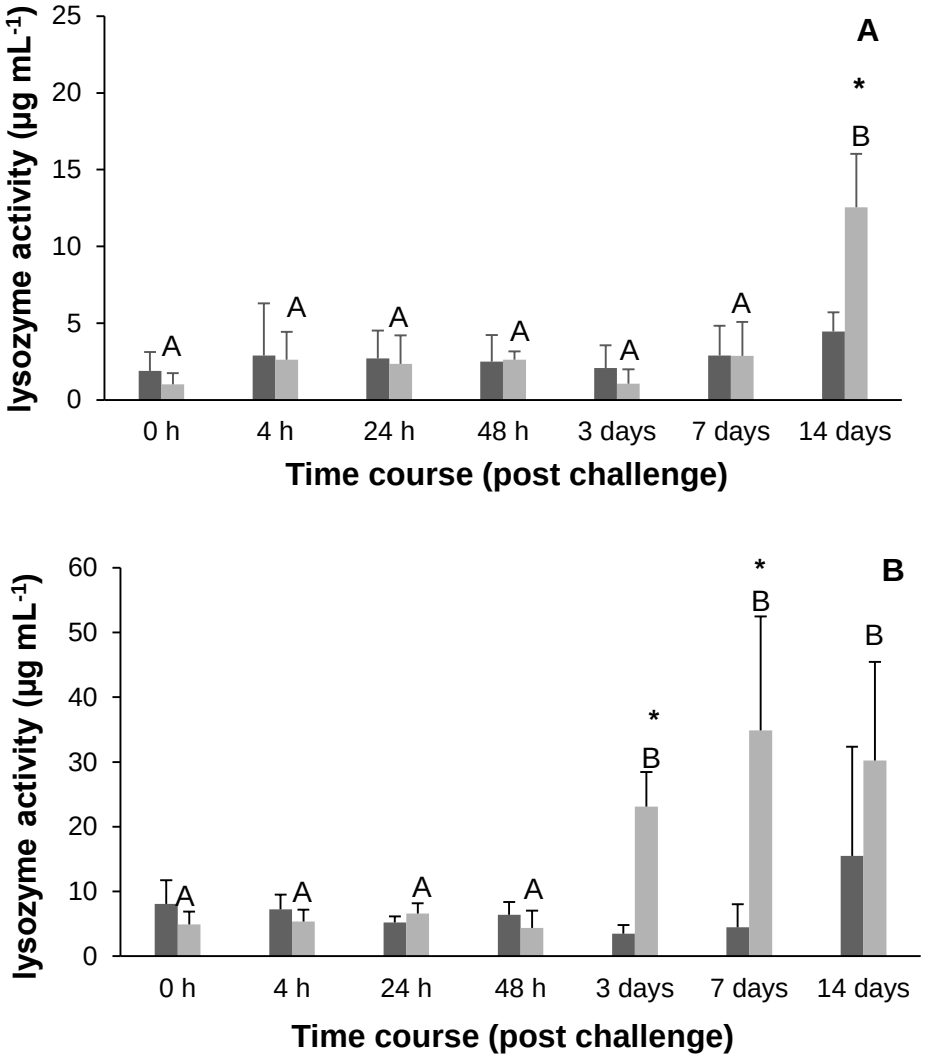


Fig.2. Lysozyme activity (µg mL^{-1}) in Senegalese sole skin mucus (A) and plasma samples (B) collected from control (■) and infected (□) groups challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.2. ACP activity

The kinetics of skin mucus ACP activities are presented in Fig. 3. While no changes were observed within control and infected throughout the experimental trial, bath challenged individuals presented a decrease in ACP activity at times 4 and 24 h in contrast to the increase observed at time 14 days compared to control individuals.

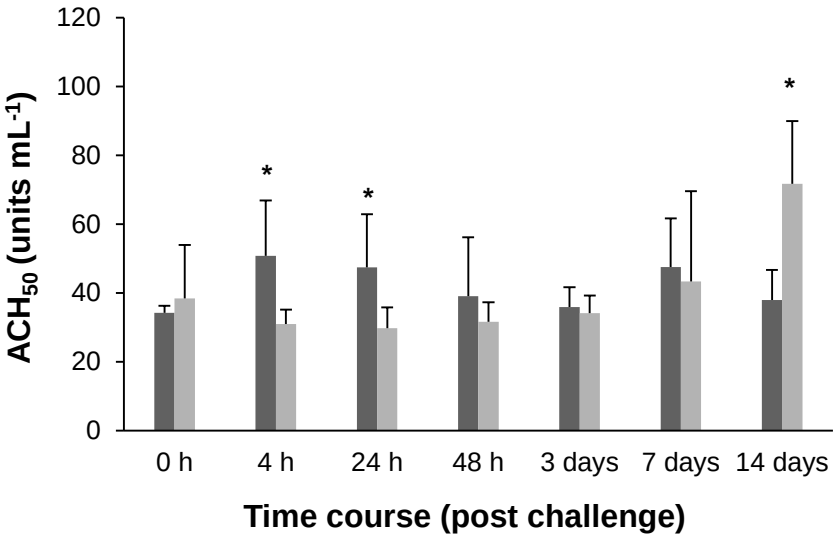


Fig.3. Alternative complement pathway activity (units mL^{-1}) in Senegalese sole skin mucus samples from control (■) and infected groups (□) challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Single asterisk denotes significant differences between control and challenged group for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.3 Anti-protease activities

The kinetics of skin mucus anti-protease activity are presented in Fig. 4A. Skin mucus from infected groups increased anti-proteases activity at 4 h and 14 days post challenge compared to control individuals. Moreover, a decreased anti-protease activity was observed in control fish at 4 h compared to those sampled at times 0 h, 24 h, and 3, 7, and 14 days. Regarding infected fish, anti-protease activity presented a similar pattern to that observed for control fish mucosal lysozyme with increased levels at 14 days following infection compared to infected fish at earlier times.

Regarding plasma samples, a decrease in anti-protease activity was observed at 14 fays post challenge in control groups, while a slight increase was observed in infected individuals at times 0 h and 14 days compared to control groups (Fig. 4B).

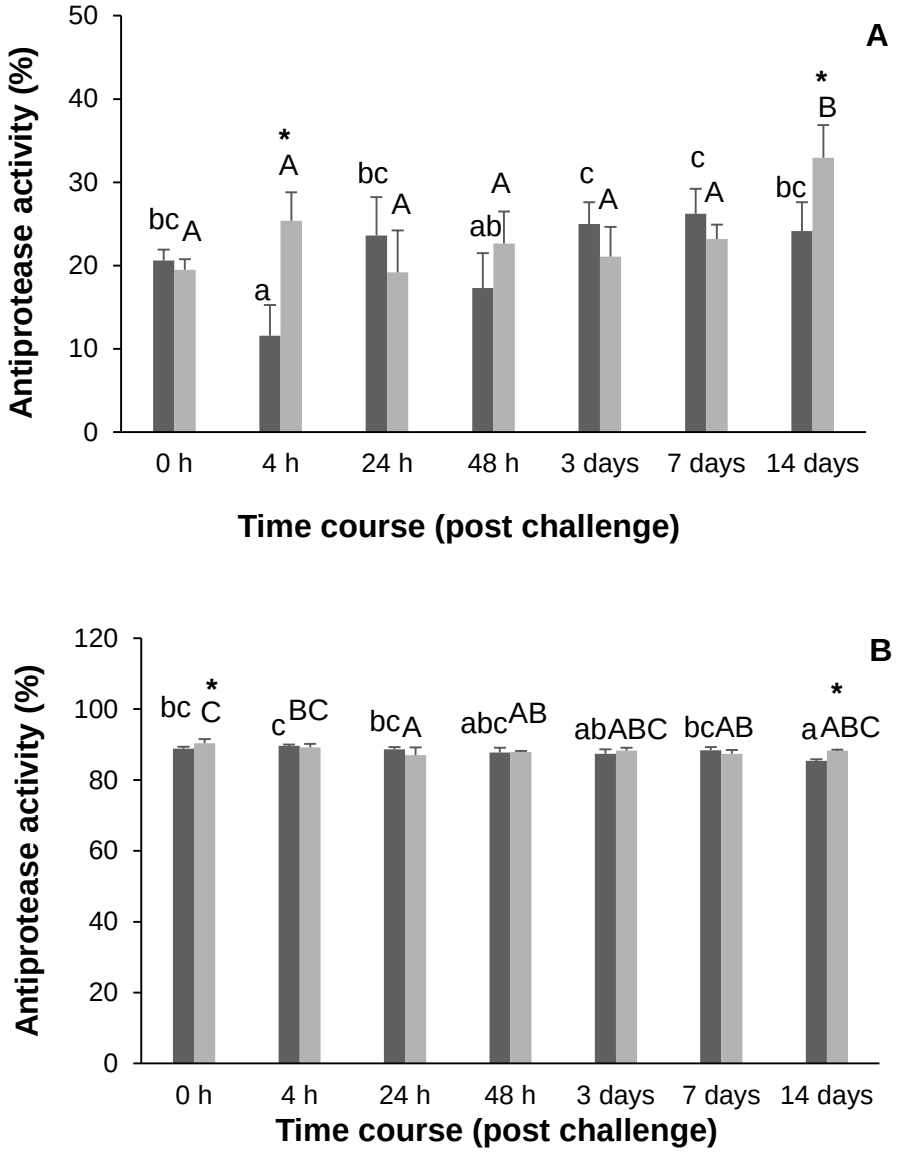


Fig.4. Anti-protease activity (%) in Senegalese sole skin mucus (A) and plasma samples (B) collected from control (■) and infected (□) groups challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.4. Protease activity

The kinetics of skin mucus protease activity are presented in Fig. 5. The skin mucus of both control and infected groups displayed a similar pattern with higher protease activities observed during the early sampling points. Moreover, the protease activity in fish

challenged with *T. maritimum* increased at 4 h and 14 days post infection compared to controls.

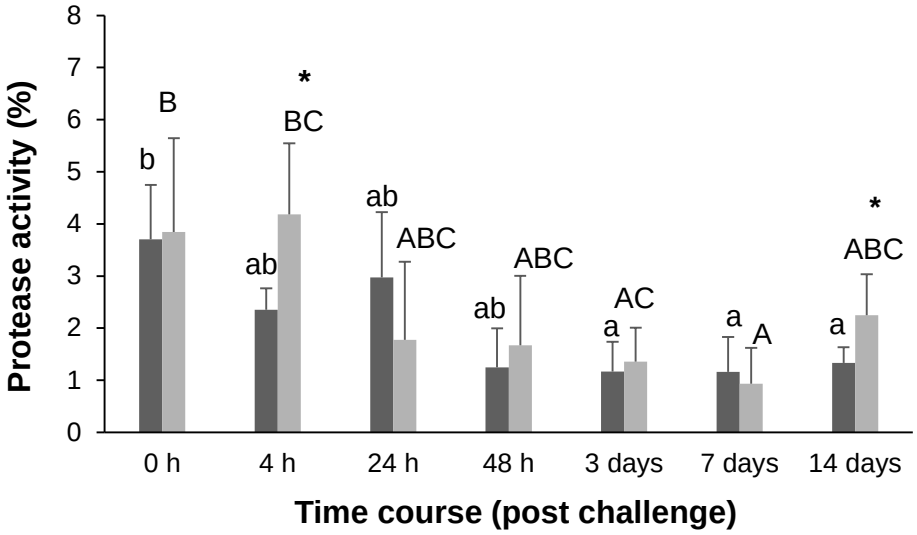


Fig.5. Protease activity (%) in Senegalese sole skin mucus collected from control (■) and infected (□) groups challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.5. Peroxidase activity

The kinetics of skin mucus peroxidase activity are presented in Fig. 6A. The skin mucus of both control and infected groups displayed a similar pattern with higher peroxidase activities observed at the end of the trial. Moreover, the peroxidase activity in control fish increased at 24 h compared to infected individuals at the same time and to their counterparts at times 4 h, 48 h, and 3 and 7 days.

Regarding plasma peroxidase activity, a similar increase was observed in both control and infected groups at the end of the trial (Fig. 6B). However, infected fish already increased those levels at 48 h following bath challenge being significantly different from control groups at 48 h and 7 days.

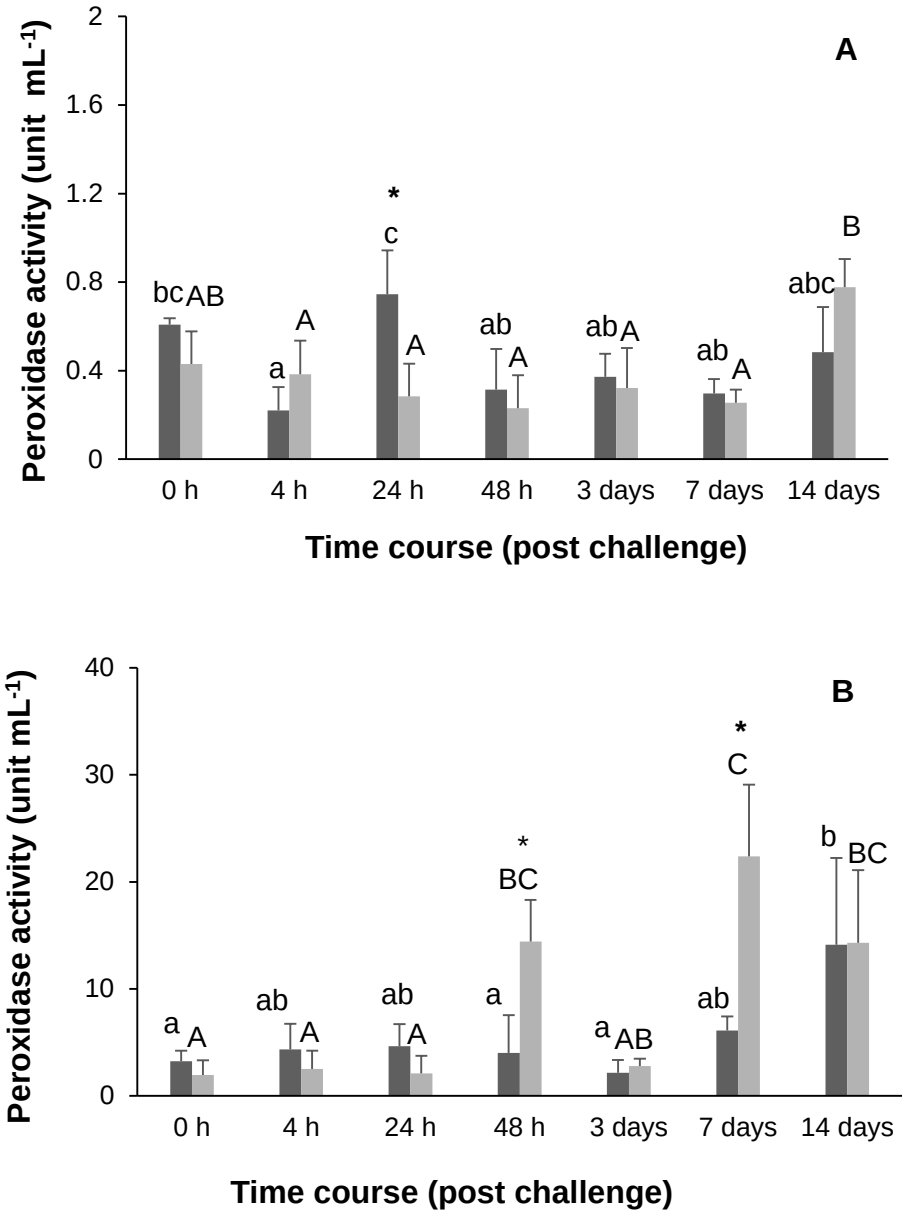


Fig.6. Peroxidase activity (units mL^{-1}) in Senegalese sole skin mucus (A) and plasma samples (B) collected from control (■) and infected group (□) challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.6. Bactericidal activity

The kinetics of the skin mucus bactericidal activities against *T. maritimum* and *P. damselae* subsp. *piscicida* are presented in Figs. 7A and 7B, respectively. In both cases, the skin mucus of the control group displayed higher bactericidal activities at earlier times.

For instance, skin mucus bactericidal activity from control fish against *T. maritimum* increased than at 0 h and 48 h compared to infected fish, whereas against *P. damselae* subsp. *piscicida* augmented at 4 h and 48 h. In contrast, the skin mucus from challenged groups increased the bactericidal activity against both bacteria after 14 days.

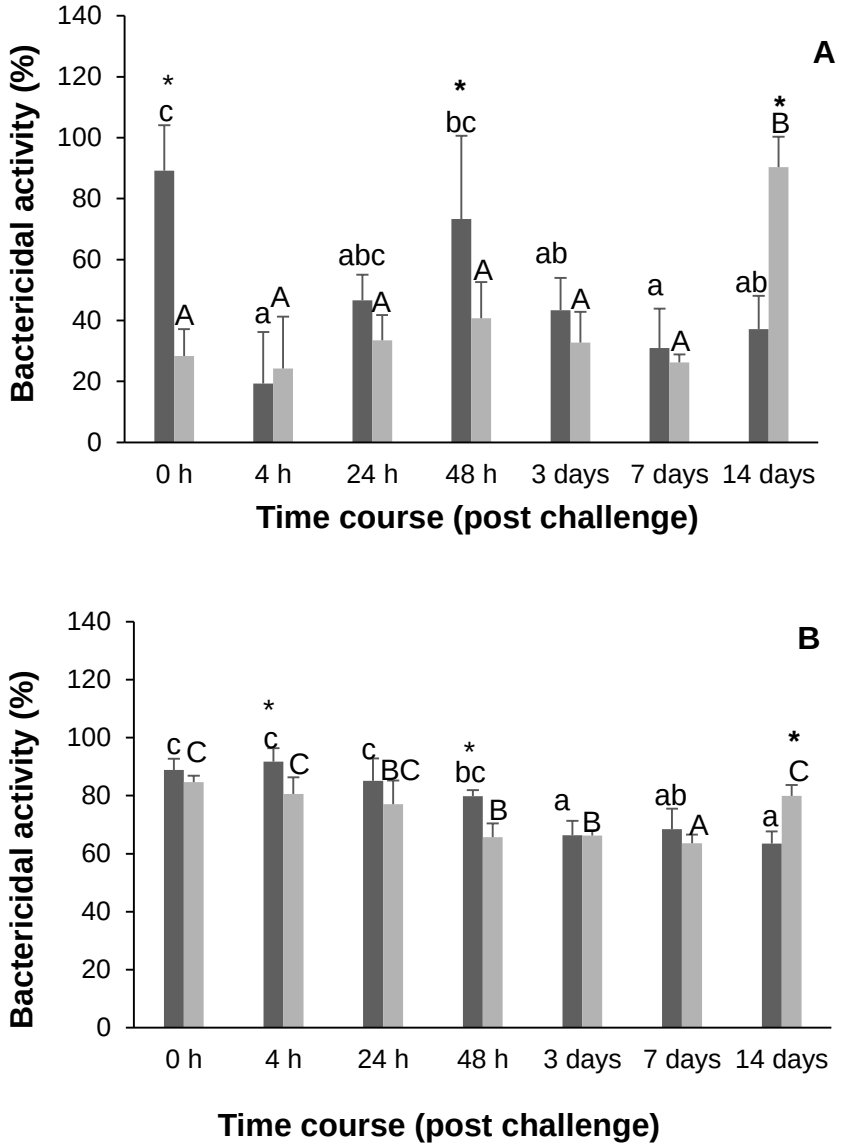


Fig.7. Bactericidal activity (%) against *T. maritimum* (A) and *Photobacterium damselae* subsp. *piscicida* (B) in Senegalese sole skin mucus samples collected from control (■) and infected group (□) challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

The kinetics of the plasma bactericidal activities against *T. maritimum* and *P. damselae* subsp. *piscicida* are presented in Figs. 8A and 8B, respectively. In general, plasma bactericidal activities from both control and infected groups were lower compared to skin mucus bactericidal activity. Moreover, plasma from challenged groups increased bactericidal activity against both bacteria at 24 h and 14 days in the case of *T. maritimum*, and at 0 h and 3 and 14 days against *P. damselae* subsp. *piscicida*.

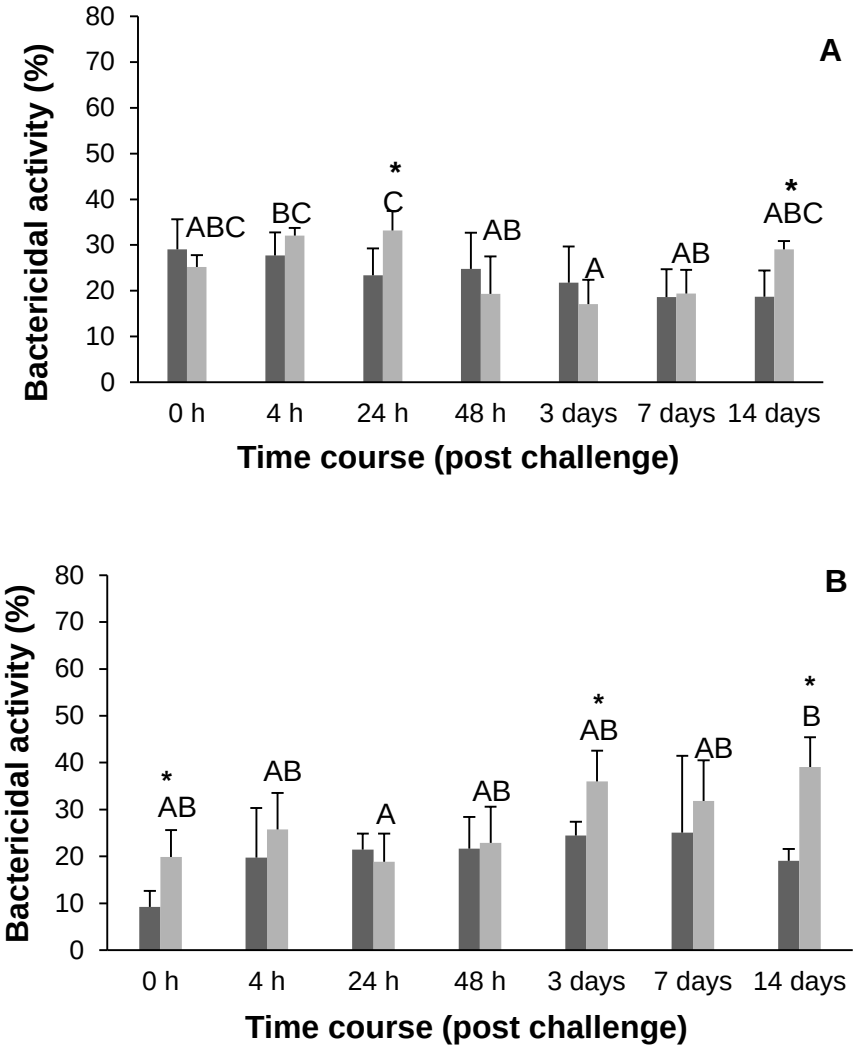
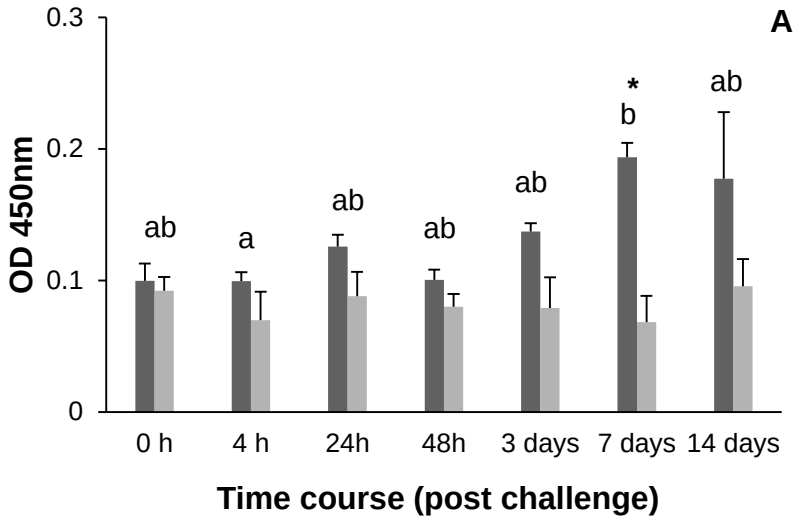


Fig.8. Bactericidal activity (%) against *T. maritimum* (A) and *Photobacterium damsalae* subsp. *piscicida* (B) in Senegalese sole plasma samples collected from control (■) and treated (□) groups challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6). Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

3.3.7. Glycosylation of skin mucus carbohydrates

All the terminal sugar residues were present in the evaluated skin mucus of either the challenged fish or the control one with significant differences in respect to time course and lectins used**.** The following lectins bound to the isolated glycoproteins as evidenced by the OD readings: ConA, SNA, WFA, UEA and LEA (specificity for α-D-mannose, α-D-glucose, N-acetylneuraminic acid, N-acetyl-D-galactosamine, α-L-fucose and N-acetyl-β-D-glucosamine, respectively).

Regardless the time, a decrease in the glycosylation pattern of N-acetyl-β-D-glucosamine and N-acetyl-D-galactosamine was observed in the fish group challenged with *T. maritimum* (Figs. 9A and 9B). Moreover, no significant variation in α-D-mannose, α-D-glucose and N-acetylneuraminic acid glycosylation patterns between the control and the challenged fish were observed (Figs. 9C and 9D, respectively). However, a considerable increase in N-acetylneuraminic acid glycosylation pattern was observed in challenged groups at 3 days post challenge. A decrease in the α-L-Fucose glycosylation pattern was observed in challenged groups at 48 h following bath challenge, whereas this pattern appears to be inverted at 14 days post challenge (Fig. 9E).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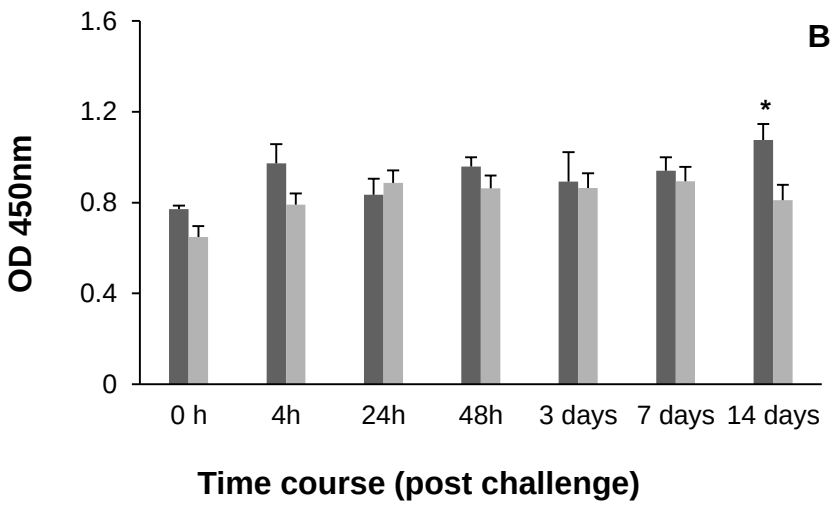
Time course (post challenge)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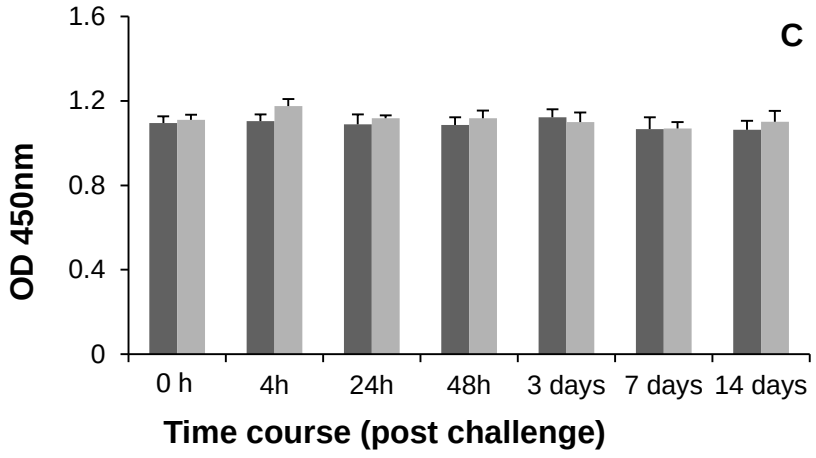
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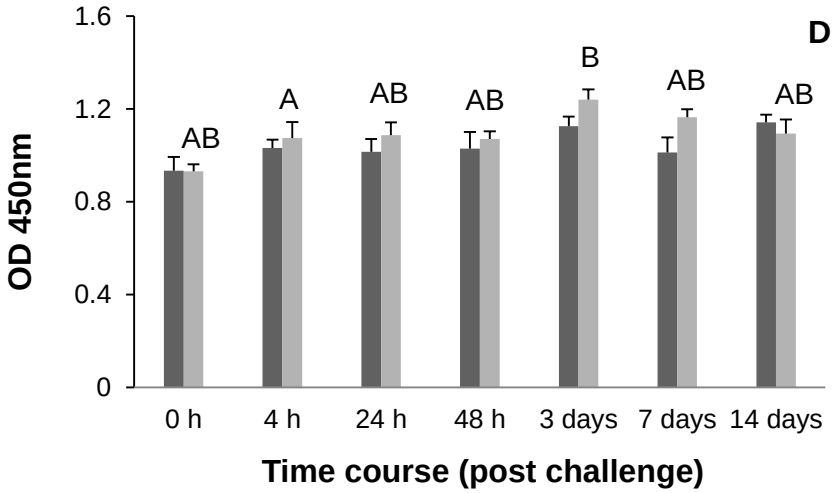
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Time course (post challenge)

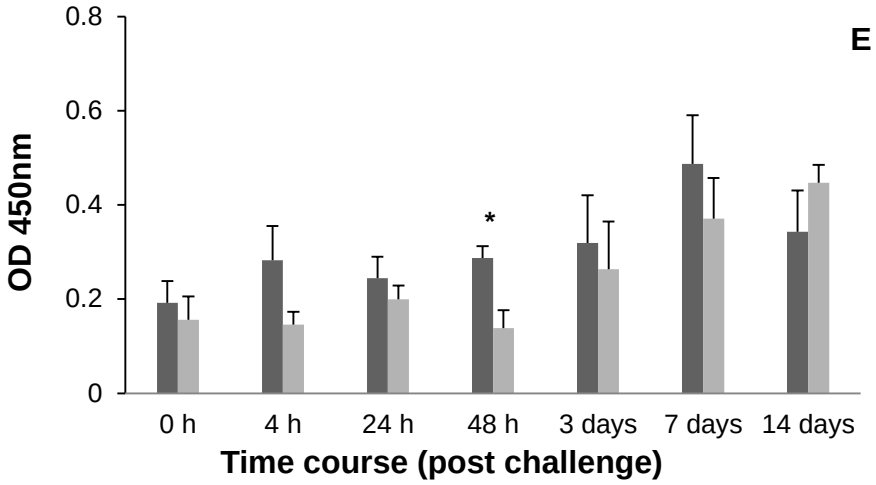

Fig.9. Lectin binding (OD 450 nm) to N-acetyl-β-D-glucosamine (A), N-acetyl-D galactosamine (B), α-D-mannose, α-D-glucose (C), N-acetylneuraminic acid (D) and α-L-fucose (E) carbohydrates present in Senegalese sole skin mucus from control group (■) and treated group (▢) challenged with 2.7 x 10^5 CFU mL^{-1} *T. maritimum* during 14 days. Data are presented as means ± SD (n = 6).Capital letters indicate significant differences among challenged groups regarding time-course post challenge, while single asterisk denotes significant differences between control and challenged groups for a given time (two-way ANOVA; $P \leq 0.05$).

4. Discussion

Fish are constantly exposed to several waterborne pathogens. Therefore, skin mucus is considered the first line of defense against an expansive range of pathogens (Magnadóttir 2010, Rombout *et al*. 2011). For instance, elimination of skin mucus resulted in higher mortalities following challenge with bacterial infection (Lemaître *et al*. 1996). In addition, the persistent shedding of mucus secretion was essential for flushing out of invading bacteria (Smirnotva *et al*. 2003). It is known that fish mucosal barriers contain a wide assortment of innate immune compounds including complement proteins, lysozyme, proteases, esterase, AMPs, C-reactive protein and trypsin-like enzymes (Esteban 2012, Alexander & Ingram 1992). Most of the invading pathogens use sugar-binding proteins as lectins to recognize and bind to host terminal carbohydrates (Imberty & Varro 2008). The effect of endotoxin (Neuhaus *et al*. 2007) and motile aeromonads (Schroers *et al*. 2009) was found to alter the intestinal carbohydrate profiles particularly the high molecular weight glycoproteins (HMGs) in carp. Furthermore, oral administration of endotoxin (LPS, lipopolysaccharide from E. coli O55:B5) leads to a change in intestinal mucus glycoprotein composition (Neuhaus e*t al.* 2007).

To the best of our knowledge, this is the first structured study in Senegalese sole presenting both systemic and mucosal innate immune responses against *T. maritimum*. The presence of the carbohydrate residues assessed in the present study has also been reported in fish mucosal surfaces of skin, digestive tract and gills (Redondo & Alvarez-Pellitero 2010, Estensoro *et al*. 2013). The strong binding affinity of Con A, SNA and WFA lectins to the Senegalese sole skin mucus suggest that the terminal carbohydrates abundance are α-D-mannose, α-D-glucose, N-acetylneuraminic acid and N-acetyl-D-galactosamine. Furthermore, a pronounced change in carbohydrates was also observed following bath challenge. The decrease in N-acetyl-D-galactosamine glycosylation during disease progression could either be attributed to the inhibitory effect or to the degenerative effect of the pathogen on mucin secreting cell. However, those results are contrary to that obtained by Schroers *et al*. (2009) who observed a marked increase in N-acetyl-β-galactosamine after 6 days of infection with *Aeromonas hydrophila*. Results from the present study showed an increase in SNA (specific to N-acetylneuraminic acid) in skin mucus of infected fish at 3 days post challenge. N-acetylneuraminic acid seemed to play a vital role in fish immunity as it provides a negative charge to the mucin molecules to minimize bacterial colonization (Wiggins *et al* .2001). An increased amount of neuraminic acid in secreted skin mucus was also reported in several studies after an irritation of the intestinal mucosa (Enss *et al*. 1992), and following bacterial infection in common carp (Schroers *et al*. 2009). Therefore, it is hypothesized that *T. maritimum* induce some degree of irritation on the Senegalese sole skin mucus which could be time dependent.

In mammals, fucose as the main constituent of intestinal and anal mucosa has a fundamental role in viscoelasticity of the mucus (Tsukise *et al*. 2000). Higher production of fucose was essential for bacterial flushing out and elimination. Its role in bacterial trapping was briefly explained by Lu & Walker (2001). In the present study, the observed decrease in fucose glycosylation pattern at 48 h post challenge could be interpreted as an evading strategy implemented by the pathogen to withstand mucosal immunity. Our result is inconsistent with that obtained by Schroers *et al*. (2009) who detected a considerable increase in fucose glycosylation in common carp intestinal mucosa at one and 3 days post infection with *A. hydrophila*. Similar alterations were observed by Neuhaus *et al*. (2007) after LPS stimulation.

T. maritimum has the ability to produce a substantial amount of extracellular polymers or 'slime', permitting them to adhere more firmly to host skin (Burchard *et al*. 1990). In the present study, skin mucus protease and anti-protease activity increased at 4 h and 14 days in infected fish compared to controls, suggesting both early and late host responses against successful bacterial colonization. Anti-proteases were involved in acute phase reactions (Bayne & Gerwick 2001), including as defense strategy against proteolytic

secreting pathogens (Zuo & Woo 1997). It is also known that the skin mucus of many fish species can display trypsin-like activity which has the ability to destroy most of invading pathogens (Braun *et al*. 1990). The capacity of rainbow trout mucosal protease activity to lyse dead *V. anguillarum,* emphasized its role in innate immune mechanisms (Hjelmeland *et al*. 1983). Proteases could act directly on the pathogen or prevent its invasion by modifying the mucus consistency which in term increased the mucosal sloughing and fascinated the pathogen removal from the body surfaces (Aranishi *et al*. 1998). In the present study, the delayed proteases and antiproteases activities observed at 14 days post challenge may suggest an indication about successful bacterial proliferation. The persistent localization of the bacteria within the mucus layer suggests that *T. maritimum* could be part of the autochthonous populations of the fish skin and therefore, can remain in the aquatic environment for a long time, utilizing fish mucus as a reservoir in line to that reported by Avendaño-Herrera (2005). *T. maritimum*, regardless of its origin and virulence degree, adhered strongly to the skin mucus of three fish species and resisted its bactericidal properties (Magariños *et al*. 1995). This hypothesis could be further supported by the delayed mucosal lysozyme and peroxidase activities.

Complement acts both directly, via the formation of a membrane attack complex, and synergistically, by opsonization of phagocytic cells. The ACP is activated by a variety of microorganisms including bacteria. The current study revealed a significant increase in the ACP at 14 days post challenge, which could be related to *T. maritimum* evading strategies and continuous cell proliferation. Likewise, the downregulation of immune-related genes during the early time points following challenge with the related pathogen *F. columnare* could support our hypothesis (Ren *et al*. 2015). It is known that LPS are responsible for some of the characteristic signs of disease in infections due to Gram-negative bacteria. The composition analysis of *T. maritimum* LPS revealed an O-chain composed of a disaccharide that contained an unusual linkage ([*R*]-2-hydroxyglutaric acid residue), which seems to be unique for this bacterium and suggested to have a role in biofilm formation within the host tissues (Vinagradov *et al.* 2003). Cells within the biofilm could evade complement factor recognition, and thus subsequent white blood cell recruitment and killing (Geesey *et al.* 1977).

Data available concerning the antimicrobial function of epidermal mucus is limited to some fish species and still not fully elucidated. Therefore, the current study also investigated the antimicrobial activity of Senegalese sole skin mucus following experimental infection with *T. maritimum*. Indeed, it was observed that the skin mucus of both control and challenged fish has a stronger bactericidal activity than that from plasma, reinforcing the fundamental role of fish skin mucus in preventing bacterial colonization (Mozumder 2005, Subramanian *et al*. 2008). Moreover, the delayed mucosal bactericidal activity was congruent with the

increase in peroxidase, lysozyme and ACP activities at the same time, suggesting that late response is mainly due to phagocytes recruitment to mucosal surfaces against *T. maritimum*. Moreover, the increase of peripheral lymphocytes was observed at 14 days post challenge, suggesting the delayed bactericidal activity could partially be related to an increase in immunoglobulins production, which still requires further investigation.

Regarding systemic immune responses from the present study, plasma anti-proteases as well as circulating monocytes and neutrophils increased already at time 0 h following bath challenge and suggested an early insight into the dynamics of leucocytes migration. Moreover, peripheral phagocytes numbers as well as lysozyme and peroxidase activities increased during the course of the infection. The observed neutrophilia correlated well with plasma peroxidase and lysozyme activity: $y= 78.706x-2.7$; $R^2= 0.87$; $P = 0.020$ and $y= 51.07x-4.4$; $R^2= 0.89$; $P = 0.004$. Similar results were reported by Costas *et al*. (2013) who stated that the blood neutrophilia following *P. damselae* subsp. *piscicida* was congruent with the increased Senegalese sole plasma peroxidase activity. Moreover, Balfry *et al*. (1997) attributed the lack of serum lysozyme activity in Nile tilapia (*Oreochromis niloticus*) following challenge with *Vibrio parahaemolyticus* to the low proliferative number of neutrophils. Neutrophils secrete lysozyme and increases in serum lysozyme activity have been associated with increases in their numbers (Muona & Soivio 1992).

To the best of our knowledge, this is the first study in Senegalese sole illustrating the peripheral blood leucocyte responses following *T. maritimum* infection. Peripheral blood neutrophils increased at times 0 h, 48 h and 3 days following bath challenge. Moreover, the surge of neutrophils migration is usually associated with monocytes recruitment in several fish species (Neumann *et al*. 2001). The current study proved this assumption since monocytosis was recorded at times 0 h and 24 h. Cases of blood neutrophilia and monocytosis was recorded in several fish species following challenge with different bacterial pathogens (Lamas *et al*. 1994, Ranzani-Paiva *et al*. 2004, Sebastião *et al*. 2011, Costas *et al*. 2013). In the present study, an increase in circulating lymphocytes was only observed in challenged fish at the end of the trial. Moreover, a tenuous lymphopenia was observed following bacterial infection in most of sampling points, which could be attributed to the migration of lymphocyte to the tissues (Lamas e*t al*. 1994). The reduction in the number of blood lymphocytes has been also reported in fish after bacterial insults (Lamas *et al*. 1994, Balfry *et al*. 1997, Garcia *et al*. 2007, Costas *et al*. 2013).

5. Conclusion

T. maritimum appears to modulate the glycoprotein composition and secretion of Senegalese sole skin mucus concurrently with the disease progression. The skin mucosal

system seems to provide a versatile eliminating mechanism against invading pathogens via secretion of mucin with modified glycosylation patterns. *T. maritimum* as a virulent strain could withstand and overcome skin mucus immunity using different evading strategies such as passive changing the glycosylation pattern. Furthermore, *T. maritimum* seemed to labor a clear positive effect on the *Senegalese sole* immune status by improving the recruitment of some peripheral leucocytes mainly neutrophils and macrophages, followed by activation of mucosal and plasma innate immunity. Our results collectively, supported the hypothesis of cell migration toward the inflammatory foci. The delayed mucosal immunity concur with the incipient plasma peroxidase, lysozyme and antiprotease activities suggests the role of bacterial extracellular toxins or enzymes in their evading strategy and, gave an indication about systemic infection. The opsonizing activities of complement together with its synergistic action with lysozyme were considered a withering arm against pathogen invasion. Finally, the bactericidal activity of the skin mucus against evaluated bacterial strains reflects its role as a powerful physical insulator against invading pathogens.

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Chapter 6

General discussion, conclusions and future research

M. Mabrok

6. General discussion

6.1. Development of an effective infection model for tenacibaculosis induction in the Senegalese sole

T. maritimum like other filamentous bacteria, presented many obstacles to laboratory work, resulting in a tendency for aggregation, fluctuation and adhesion to all kind of substrates, thus revealing difficulties in detection and recognition of its colonies (Yamamoto *et al*. 2010). Therefore, several attempts for cultivation and standardization of the bacterial growth curve have been done. In **Chapter 2**, it was investigated that using cellulase hydrolysis, detergents and non-ionic surfactants as a substrate for bacterial cultivation at almost any of the concentrations tested revealed aggregation, fluctuation, and negatively affected bacterial growth. In contrast, inoculating the bacteria directly into marine broth at 25 °C for 48 h with vigorous shaking, which induced strong aeration, prevented bacterial aggregation and provided a representative bacterial inoculum for an experimental bath challenge. The role of agitation and strong aeration in preventing bacterial aggregation and slime formation in other filamentous bacteria was briefly discussed by Burchard *&* Schwarz (1989) and Sorongon *et al*. (1991).

Knowledge of the pathogenicity of an organism is essential for understanding the disease it causes. Therefore, **Chapter 2** also aimed to establish a model of infection for Senegalese sole. It was observed that incubation of Senegalese sole with *T. maritimum* for 18 h under normal aeration showed no mortalities, and the fish did not show symptoms of the pathology. In contrast, a prolonged bath for 24 h together with high water temperature (23 ± 1 ºC) and strong aeration appear to be an effective challenge model without any need for skin or gill scarification, suggesting a critical role of those parameters in *T. maritimum* disease induction during Senegalese sole experimental infection. The role of temperature as a promoting factor in disease induction was previously mentioned in many other studies (Holt *et al*. 1975, Decostere *et al*. 1999, Yamamoto *et al*. 2010). Moreover, the detection of *T. maritimum* in different host tissues by the nested PCR suggests that the body surfaces including skins and gills can be the main route of entry for *T. maritimum*, which gives rise to ulcerative skin as previously reported by other authors (Toranzo *&* Barja 1993**,** Magariños *et al*. 1995). The ability of the pathogen to gain access into host tissues is due to its ability to proliferate and produce enzymes once it reaches the dermis (Vilar *et al*. 2012, Faílde *et al*. 2013).

6.2. Mucus and plasma bactericidal capacity against *T. maritimum* strains

Fish skin mucus has several immune substances that provide the first line of defense against a broad spectrum of pathogens although they are poorly studied to date. The immunological or protective function of the mucus is the result of its mechanical and

biochemical properties. Once the superficial barriers are breached, an inflammatory reaction is prompted with a definitive inundation of leukocytes proliferation and humoral components activation. In order to understand how Senegalese sole mucus or plasma interact with *T. maritimum* during disease progression, the present Thesis evaluated the bactericidal capacity of naïve fish mucus and plasma (**Chapter 2**), or activated fish mucus and plasma (**Chapter 5**) against different *T. maritime* strains.

Both mucosal and systemic responses from unstimulated fish presented a relatively low bactericidal capacity, suggesting that Senegalese sole does not contain adequate compounds, either local (i.e. mucus) or systemically (i.e. plasma), with potent bactericidal activity to kill *T. maritimum*. The observed changes in Senegalese sole mucus glycosylation pattern, together with the delayed plasma and mucosal immune responses following challenge with *T. maritimum* from Chapter 5, gave some insights into the role of pathogen on the modulation of the host immune response. Indeed, the capacity of *T. maritimum* to withstand host mucosal immunity is not novel and was already reported in many other species including turbot, *Scophthalmus maximus* (L.), seabream, *Sparus aurata* (L.), and seabass (Magariños *et al.* 1995). Furthermore, the experimental studies in red and black seabream (Baxa *et al***.** 1987) concluded that the pathogenicity of this pathogen can be attributed to the synergistic interaction of the toxins and enzymes present in the extracellular products (ECPs), which in turn facilitate the host tissue erosion, invasion and colonization.

Although both heat-inactivated and untreated plasma samples from unstimulated fish presented week bactericidal activities (**Chapter 2**), higher activities were recorded after 3 days following bath challenge and extended up to 14 days (chapter 5). Moreover, a higher mucosal and plasma bactericidal activity was congruent with higher lysozyme, complement and anti-protease activities. Still, increases in those immune parameters seemed to be not strongly enough to fully destroy that pathogen particularly during the early stage of infection, as it could be observed in **Chapter 2** during bacterial challenge. The data reported here further suggest that the alternative pathway of the complement system has no major role against *T. maritimum*, during the early stage of infection. The comparative analysis of the mucosal glycosylation patterns and the humoral immunity of Senegalese sole mucus and plasma before and after infection with *T. maritimum* could be of assistance to understand host immune responses against this particular pathogen as well as bacterial evading strategies.

6.3. *In vitro* assessment of Senegalese sole cellular immune response against *T. maritimum* strains

To the best of our knowledge, there are currently few data regarding the interactions between *T. maritimum* and host cells, and none study has assessed the activity of *T. maritimum* ECP and LPS in Senegalese sole. Therefore, the current Thesis focused on functional (e.g. ROS and NO production) and molecular immune responses of Senegalese sole head-kidney leukocytes (HKL) following stimulation with live and UV killed bacteria as well as ECPs and LPS from different *T. maritimum* strains (**Chapters 3 and 4**). HKL poorly increased ROS production following exposure to live and UV killed bacteria, ECPs and LPS. Although values obtained following exposure to live strains were higher than those observed following exposure to the UV killed strains, those levels were too far below ROS production by HKL stimulated with PMA. These data suggest that *T. maritimum* appears to present the ability to evade host phagocyte responses. Although one evading strategy could be linked to the elimination of reactive radicals through antioxidant enzymes such as catalase, the present study also suggests that this evasion strategy may not require viable bacteria. Therefore, it is hypothesized that *T. maritimum* cell wall components may redirect the host immune response through distinct TLR signalling pathways from those desired for proper pathogen elimination. Although the expression profile of IL1β after stimulation with UV killed strains was extremely high during the early sampling points (**Chapter 3**), they failed to induce ROS production, suggesting that the IL1β-Myd88 axis and NADPH oxidase-mediated ROS signaling are two independent pathways that differentially regulate neutrophil migration during inflammation. A similar result was obtained in a Zebrafish after induction of inflammation (Yan *et al*. 2014). Moreover, Senegalese sole HKL increased NO production following exposure to UV killed strains while failed in the case of live strains, ECPs and LPS. These data appear to suggest: i) on the one hand, Senegalese sole HKL are not responsive to low LPS concentrations based on ROS and NO production; and ii) on the other hand, *T. maritimum* ability to avoid host cells NO responses seems to require live bacteria and could be mediated through ECPs. A similar result was recorded by Billings (2006) who stated that the NO production was not affected following treatment of channel catfish kidney cells with live *F. columnare*. Similarly to that observed in **Chapter 4**, Billings (2006) stated that *F. columnare* ECPs failed to increase nitric oxide production following stimulation. Likewise, Villamil *et al*. (2003) observed a reduction in NO production following *in vitro* stimulation of turbot head-kidney macrophages with higher doses of ECPs from *Vibrio pelagius*. In **Chapter 3**, low expression levels were observed in most of the evaluated genes after stimulation with live strains, providing some insights into the pathogen evading strategy, and suggesting the role of ECPs in host immune suppression.

Regarding cytotoxicity induced by bacteria, it was observed in **Chapter 3** that both live and UV killed strains present cytotoxic effects against Senegalese sole HKL in a different manner, as indicated by the production of LDH. The increased survival from strain ACC6.1 could be related to its higher virulence (according to **Chapter 2**), and seems to be related to the increased LDH activity observed in cells inoculated with live ACC6.1 after 4 h (**Chapter 3**). Releasing of LDH suggests pore formation in the host cell membrane (Pei & Ficht 2004), and emphasizes the bacterial virulence which could be mediated by ECPS. This assumption was confirmed in **Chapter 4**, since an increase in ECPs concentration induced a greater cytotoxic activity and cell morphological changes. Thus, a synergistic action of the toxins and enzymes present in the ECPs may present a main role in *T. maritimum* virulence. The role of ECP from *T. maritimum* in host cell death and bacterial pathogenicity was previously mentioned by Baxa *et al*. (1987), Pazos (1997), and Rahman *et al.* (2014).

6.4. *In vivo* assessment of Senegalese sole cellular immune response against *T. maritimum* strains

The synergistic action between host leucocytes results in the activation of both innate and adaptive immunity (Secombes 1996). **Chapter 5** presented for the first time the peripheral blood leucocyte responses of Senegalese sole to inflammation following bath challenge with *T. maritimum*. The surge of neutrophils migration was usually associated with monocytes recruitment in several fish species (Neumann *et al*. 2001, Sebastião *et al*. 2011, Costas *et al*. 2013). In fact, neutrophils are quickly attracted to infectious loci by microbial products and chemotactic substances released by host cells, including macrophages (do Vale *et al*. 2002). Moreover, a significant lymphocytosis was only observed in the challenged group after 14 days post challenge. The delayed response gave a further indication about bacterial colonization, systemic infection and adaptive immune system activation. Based on the time course, a tenuous lymphopenia was observed following bacterial infection in most of sampling points, which could be attributed to the migration of lymphocyte to the tissues. A marked lymphopenia was recorded in several fish species following bacterial infection (Lamas *et al*. 1994, Balfry *et al*. 1997, Garcia *et al*. 2007, Costas *et al*. 2013).

6.5. Further research

The present Thesis not only proposes the possibility of *T. maritimum* disease induction in Senegalese sole by a prolonged bath method, but also follows up the host cellular and humoral immune responses against this pathogen. Although several issues regarding *T. maritimum* virulence have been uncovered in this Thesis, gaps concerning *T. maritimum*

evading strategies as well as the route of entry into the host still need to be elucidated. This future work will help to understand the mechanism of *T. maritimum* infection and assist to increase vaccine efficiency.

In **Chapter 3**, it was observed that UV killed *T. maritimum* strains enhanced Senegalese sole immune responses via activation of NO production and upregulation of pro and anti-inflammatory genes. In this way, UV killed strains could be included in vaccination Senegalese sole studies, which are now possible since an effective bath challenge was developed in **Chapter 2**.

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