U. PORTO

DOUTORAMENTO EM CIENCIA ANIMAL
MORFOLOGIA E FISIOLOGIA

Tenacibaculum maritimum pathogenesis: crosstalk between host and pathogen and beyond

Inês Almeida Ferreira

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2024

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

U.PORTO

3º CICLO
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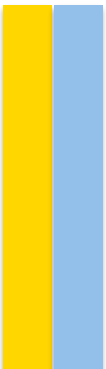

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Thesis for applying to a doctoral degree in Animal Science, Specialization in Morphology and Physiology, submitted to the School of Medicine and Biomedical Sciences (ICBAS) of the University of Porto

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Declaração de honra

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

Inês de Almeida Ferreira

(Inês de Almeida Ferreira)

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Para ti avó

Estás aqui

Que bom é rir contigo, mais uma história tens para contar! No teu olhar guardas a perspicácia usual e perscrutas com ironia o espírito dos que te rodeiam… Tantos são os cenários que gostas de imaginar. Percorres o Passado e Presente, como a peregrina destemida que foste. De repente, vejo o teu rosto a transformar-se… as rugas nascidas do tempo e das emoções adensam-se, aprofundam-se como os veios e canais que levam a água aos campos sedentos, os olhos azuis que absorveram o reflexo do mar, a boca que ditou palavras de poesia, cerram sobre si, desaparecem num único ponto. São sugados para dentro de ti própria. Onde vais?! Tento agarrar-te com as minhas mãos, mas fluis por entre os meus dedos, como se de areia fosses feita… tento impedir que vás, de novo, e de novo, e de novo, mas não cedes. Condensas-te nesse ponto, e ao solo cai uma semente. Desapareceste?! Agarro naquilo que foste e levo-a ao peito. Escuto o teu silêncio com cuidado. Afinal, enganei-me, estás aqui… Dentro de mim. Sempre.

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DISSEMINATION

This thesis includes two scientific papers published and one in preparation for submission in international peer-review journals originating from part of the results obtained in the experimental work, referenced to as:

Ferreira, I. A., Peixoto, D., Losada, A. P., Quiroga, M. I., do Vale, A., Costas, B. (2023). Early innate immune responses in European sea bass (*Dicentrarchus labrax* L.) following *Tenacibaculum maritimum* infection. *Frontiers in Immunology*, 14, 1254677. https://doi.org/10.3389/fimmu.2023.1254677

Ferreira, I. A., Santos, P., Moxó, J. S., Teixeira, C., do Vale, A., Costas, B. (2024). *Tenacibaculum maritimum* can boost inflammation in *Dicentrarchus labrax* upon peritoneal injection but cannot trigger tenacibaculosis disease. *Frontiers in Immunology*, 15, 1478241. https://doi.org/10.3389/fimmu.2024.1478241

Ferreira, I. A., Carvalho, I., Robledo, D., Barreiro Felpeto, A., do Vale, A., Costas, B. Mucosal transcriptome and proteome analyses in European sea bass (*Dicentrarchus labrax*) following *Tenacibaculum maritimum* bath challenge. In preparation for submission to Aquaculture.

ABSTRACT

Aquaculture is a worldwide fast-growing food sector driven by increased global food demand. However, it has been severely affected by bacterial fish pathogens that compromise the health status of reared species and the profitability of the sector. To increase yields and productivity, high rearing densities are commonly used in fish production units, which potentiates the transmission of pathogenic microorganisms. The high stocking densities and other intensive farming practices cause significant strain on the fish's innate immune defences, ultimately jeopardising a swift and effective host response. Nevertheless, several strategies may be applied to avoid potentially disastrous infection outbreaks. Treatment therapies (e.g., antibiotics) and prevention measures (e.g., vaccination) have been successfully used in aquaculture to diminish pathogen loads and prevent some infectious diseases. However, for several established and emergent bacterial infections, appropriate disease control and prophylactic measures are still lacking and can only be achieved through multidisciplinary studies focusing on the characteristics of the pathogens, the immune response of the host species, and the environmental factors affecting both the pathogen and the host.

Several bacterial diseases (mainly caused by Gram-negative bacteria) have been reported to affect important commercial fish species, and they are, in fact, a major cause of morbidity and mortality in aquaculture. One of the most devastating bacterial diseases of wild and farmed marine fishes is tenacibaculosis, whose causative agent is *Tenacibaculum maritimum*. This pathogen has a wide range of host species and a worldwide geographical distribution and poses a significant threat to the global marine aquaculture industry. Tenacibaculosis is characterized by ulcerative lesions, mainly on the host skin and fins, frequently leading to systemic infection. Due to the high mortalities and economic losses associated with *T. maritimum*, it is relevant to gather more knowledge regarding its virulence mechanisms to develop effective methods to avoid the disease spreading.

Therefore, the main goal of this PhD thesis is to contribute to a deeper understanding of the interactions between *T. maritimum* and European sea bass (*Dicentrarchus labrax*), a species with commercial interest in the Mediterranean aquaculture sector and severely affected by tenacibaculosis. To achieve this, the present study focused mainly on *in vivo* approaches to gather new insights about the innate immune response of the host, both at a systemic and mucosal level. It has been proposed that the extracellular products (ECPs) secreted by *T. maritimum* are among its main virulence mechanisms to invade and colonise the host, but few studies have focused on identifying the proteins that constitute the

proteolytic cocktail of *T. maritimum* ECPs. Therefore, in this PhD thesis, the characterisation of the protein composition of the ECPs produced *in vitro* and *in vivo* by the virulent strain ACC13.1 was performed (**Chapters 5**). Proteomic analysis (NanoLC-MS/MS) of *in vitro* produced ECPs revealed the presence of several proteins, including a sialidase, metalloproteases, collagenases, outer membrane proteins (e.g., TonB-dependent receptors), lipoproteins and type IX secretion system (T9SS)-related proteins. The presence of a C-terminal sorting domain suggests that these proteins are actively secreted by the pathogen. Most of these proteins are predicted virulence factors for *T. maritimum*, suggesting an essential role during tenacibaculosis infection. The lack of knowledge also extends to the mechanisms behind the host's innate immune response and inflammatory process activated by tenacibaculosis. Despite some studies using different host species and different challenge models to assess the effectiveness of inducing tenacibaculosis, there is no consensus regarding the best experimental model to study this pathology in different fish species. Here, an initial *in vivo* trial involving different inoculation routes (intraperitoneal - i.p - injection and bath challenge - known to induce mortality) was used to access these models to induce tenacibaculosis (i.e., using the same *T. maritimum* inoculum), as well as to evaluate the short-term immune response of European sea bass. Additionally, the host response against i.p. injection of only ECPs was also studied (**Chapter 2**). As a control, a group of fish was bath-challenged with the same bacterial inoculum. Since no mortality was recorded for the fish challenged by i.p., occurring only in bath-challenged fish, it was concluded that i.p. inoculation is not a suitable route to induce tenacibaculosis in European sea bass. This is supported by the fact that bacteria were isolated from the blood and peritoneal exudates of i.p. challenged fish at 3 and 6 h post-challenge, but from 24 h onwards, no bacterial growth was observed, which suggests a low capacity of *T. maritimum* for invasion and colonisation following i.p. inoculation. However, the i.p. injection of the *T. maritimum* cells and ECPs resulted in a typical local inflammatory response, with an increase in leukocyte populations in the peritoneal cavity and a simultaneous abrupt drop in total leukocytes, lymphocytes, and thrombocytes in the bloodstream. It is likely that the complex protein profile of *T. maritimum* ECPs, as exhibited in the *in vitro* study, contributes to a strong chemotactic effect that results in the recruitment of immune cells to the peritoneal cavity. Supporting a solid inflammatory and systemic response is the accentuated expression of molecular markers related to an acute inflammatory response (i.e., *il1β*, *il6*, *il8*, and *hamp1*) in the head-kidney of fish challenged with *T. maritimum* plus ECPs. Interestingly, a similar inflammatory response at the head-kidney was seen in the group i.p injected with ECPs alone, suggesting that ECPs trigger a subtle (non-significant) cellular response locally (i.e., at the peritoneal cavity) but activate several pro-inflammatory genes at a systemic level. To further investigate the host immune response against *T. maritimum*,

another *in vivo* trial was performed, using the bath-challenge method to evaluate the response at mucosal level (**Chapter 3**). The collection of mucosal tissues (i.e., gills, skin, and posterior intestine) allowed to study gene expression kinetics associated with tenacibaculosis infection in such organs. An increased expression of *il1β*, *il8*, *mmp9*, and *hamp1* was observed in all mucosal tissues of challenged fish, similar to what occurred in the head-kidney of i.p. challenged fish (**Chapter 2**), which, again, suggests a pro-inflammatory response transversal to all organs. Cell counts and analysis of humoral parameters of bath-challenged fish revealed an increase in peripheral leucocytes, lysozyme, and bactericidal activities, indicating a possible systemic response after the local mucosal one. The faster kinetics seen for the gills may suggest that *T. maritimum* can use gill mucosa as a route of entry into the fish.

To obtain a broader perspective of the molecular effectors and pathways behind the host mucosal immune response against *T. maritimum*, a combined transcriptomic and proteomic analysis was used to evaluate the local immune response in the skin and skin mucus of European sea bass challenged by bath (**Chapter 4**). Following the challenge, a response to the bacteria was seen both in the skin and in its mucus protective layer. In the skin, it was observed stimulation of the genes involved in eicosanoids metabolism, acute phase response, iron-withholding mechanisms and tissue remodelling. These results are in agreement with the results of the previous study presented in **Chapter 4** since genes related to the acute-phase response (i.e., *il1β* and *hamp1*) were among the most up-regulated in infected fish. Moreover, infected fish also showed a down-regulation of several genes related to wound healing, especially collagens and other extracellular matrix structural (ECM) components. This modulation was paralleled by an increased expression of genes coding for proteinases responsible for ECM degradation. The proteins present in the skin mucus corroborated the existence of a pro-inflammatory response. Contrary to what was observed in the skin, proteins that participate in wound healing were upregulated in the mucus, maybe due to the host's mechanisms of regulation and homeostasis (i.e., negative feedback mechanism). The obtained results showed a complex mucosal response against *T. maritimum*, which includes triggering skin inflammation and modulation of the host's wound healing and remodelling processes.

This work contributes to increased knowledge concerning the development of tenacibaculosis, which is needed to improve future strategies to prevent and treat this infection in aquaculture fish species.

Keywords: Tenacibaculosis; Innate immunity; Mucosal immunity; Inflammatory response; Pathogen-host interaction;

RESUMO

A aquacultura é um setor alimentar global em rápido crescimento, impulsionado pelo aumento da procura global de alimentos. No entanto, tem sido gravemente afetado por agentes patogénicos bacterianos de peixes que comprometem e o estado de saúde das espécies em cultivo e a rentabilidade do sector. Para alcançar altos rendimentos e produtividade, são comumente utilizadas na indústria altas densidades de cultivo, o que inadvertidamente potencia a transmissão de microrganismos patogénicos. As elevadas densidades utilizadas, bem como outras práticas intensivas da indústria aquícola causam uma pressão significativa na defesa inata imunitária dos peixes, eventualmente comprometendo, uma resposta rápida e eficaz do hospedeiro. No entanto, os surtos potencialmente desastrosos que advêm destas práticas podem ser evitados. Tratamentos terapêuticos (p. ex., antibióticos) e profiláticos (p. ex., vacinação) são frequentemente utilizadas em aquacultura para diminuir a carga de agentes patogénicos e prevenir algumas doenças infeciosas. No entanto, diversas infeções bacterianas, estabelecidas e emergentes, ainda carecem de estratégias profiláticas adequadas, que só podem ser alcançadas através de estudos multidisciplinares centrados nas características dos agentes patogénicos, na resposta imunitária das espécies hospedeiras e nos fatores ambientais globais que podem afetar todos estes outros componentes. Relatórios e estudos têm demonstrado que diversas doenças bacterianas (principalmente causadas por bactérias Gram-negativas) afetam importantes espécies de peixes comerciais e são uma das principais causas de morbilidade e mortalidade em aquacultura. Uma das doenças bacterianas mais devastadoras dos peixes marinhos selvagens e criados em aquacultura é a tenacibaculose, cujo agente causador é a bactéria *Tenacibaculum maritimum*. Este agente patogénico tem ampla gama de espécies hospedeiras e distribuição geográfica mundial, pelo que representa uma ameaça significativa para a indústria global da aquacultura. A tenacibaculose é caracterizada por lesões ulcerativas, principalmente na pele e barbatanas do hospedeiro, e frequentemente culmina numa infeção sistémica. Devido às elevadas mortalidades e perdas económicas associadas à bactéria *T. maritimum*, é relevante adquirir mais conhecimento sobre os seus mecanismos de virulência com o fim de desenvolver métodos eficazes para evitar a propagação desta doença.

Deste modo, o principal objetivo desta tese de doutoramento é contribuir para uma compreensão mais profunda das as interações entre a bactéria *T. maritimum* e o robalo europeu (*Dicentrarchus labrax*), uma espécie com interesse comercial no setor da

aquacultura mediterrânica e que é gravemente afetada pela tenacibaculose. Para atingir tal objetivo, o presente estudo concentrou-se principalmente em abordagens *in vivo* para reunir novos conhecimentos sobre a resposta imune inata do hospedeiro, tanto a nível sistémico, quanto ao nível da mucosa. Foi proposto que os produtos extracelulares (ECPs) secretados pela bactéria *T. maritimum* encontram-se entre os principais mecanismos de virulência para invadir e colonizar o hospedeiro, mas poucos estudos se têm focado na identificação das proteínas que constituem o *cocktail* proteolítico dos ECPs desta bactéria. Portanto, nesta tese de doutoramento foi realizada a caracterização da composição proteica dos ECPs produzidos *in vitro* e *in vivo* pela estirpe virulenta ACC13.1 (**Capítulo 5**). A análise proteómica (NanoLC-MS/MS) dos ECPs produzidos *in vitro* revelou a presença de diversas proteínas, incluindo uma sialidase, metaloproteases, proteínas de membrana externa (p. ex., recetores dependentes de TonB), lipoproteínas e proteínas relacionadas com o sistema de secreção tipo IX (T9SS). A presença de um sinal *C-terminal* sugere que estas proteínas são, de facto, secretadas por *T. maritimum*. A maioria destas proteínas são fatores de virulência previstos deste agente patogénico, pelo que poderão desempenhar um papel essencial durante a tenacibaculose. A falta de conhecimento também se estende aos mecanismos subjacentes à resposta imune inata do hospedeiro e ao processo inflamatório ativado durante esta patologia. Apesar de alguns estudos utilizarem diferentes espécies hospedeiras e diferentes modelos de infeção para avaliar a eficácia da indução da tenacibaculose, não há consenso quanto ao melhor modelo experimental para estudar esta patologia em diferentes espécies de peixes.

Um ensaio inicial *in vivo* que envolveu diferentes métodos de infeção (intraperitoneal - injeção i.p - e infeção por banho – capaz de induzir mortalidade) foi usado para determinar a eficácia destes métodos para induzir tenacibaculose (utilizando o mesmo inóculo de *T. maritimum*), bem como avaliar a resposta imunológica de curto prazo do robalo europeu. Além disso, a resposta do hospedeiro contra injeção i.p. de apenas ECPs foi também estudada (**Capítulo 2**). Como controlo, um grupo de peixes foi infetado por banho com o mesmo inóculo bacteriano. Dado que não foi registada mortalidade para os peixes inoculados por injeção i.p., ocorrendo apenas em peixes infetados por banho, concluiu-se que a inoculação pela via i.p. não permite induzir a tenacibaculose no robalo europeu. Esta conclusão é apoiada pelo facto de se terem detetado bactérias viáveis no sangue e exsudados peritoneais dos peixes infetados por injeção i.p às 3 e 6 horas pós-infeção, mas não a partir das 24 h, o que sugere uma baixa capacidade da *T. maritimum* para invasão e colonização após administração por injeção i.p. No entanto, a injeção i.p de células de *T. maritimum* e ECPs resultou numa resposta inflamatória local típica, com um aumento do número de leucócitos na cavidade peritoneal e uma queda abrupta simultânea na contagem total de leucócitos, linfócitos e trombócitos na corrente sanguínea.

É provável que o perfil proteico complexo dos ECPs da *T. maritimum*, conforme exibido no estudo *in vitro*, contribua para um forte efeito quimiotático que resulta no recrutamento de células imunes para a cavidade peritoneal. Apoiando uma resposta inflamatória e sistémica sólida está a expressão acentuada de marcadores moleculares relacionados a uma resposta inflamatória aguda (como, *il1β*, *il6*, *il8* e *hamp1*) no rim de peixes infetados com *T. maritimum* juntamente com os ECPs. Curiosamente, foi observada uma resposta inflamatória semelhante no rim no grupo injetado i.p apenas com ECPs, sugerindo que os ECPs desencadeiam uma resposta celular subtil (não significativa) localmente (ou seja, na cavidade peritoneal), mas ativam vários genes pró-inflamatórios a nível sistémico. Para investigar de modo mais profundo a resposta imunitária do hospedeiro contra *T. maritimum*, foi realizado outro ensaio *in vivo*, utilizando o banho como método de infeção, para avaliar a resposta ao nível da mucosa (**Capítulo 3**). A recolha de tecidos mucosos (como brânquias, pele e intestino posterior) permitiu estudar a cinética de expressão génica associada à infeção por tenacibaculose nestes órgãos. Uma expressão aumentada de *il-1β*, *il8*, *mmp9* e *hamp1* foi observada em todos os tecidos da mucosa dos peixes infetados, semelhante ao que ocorreu no rim dos peixes infetados por injeção i.p. (**Capítulo 2**), o que, mais uma vez, sugere uma resposta pró-inflamatória transversal a todos os órgãos. A contagem celular e a análise dos parâmetros humorais dos peixes infetados por banho revelaram um aumento nos leucócitos periféricos e atividade bactericida, indicando uma possível resposta sistémica após a resposta local da mucosa. A cinética mais rápida observada nas brânquias parece sugerir que a *T. maritimum* pode utilizar a mucosa branquial como via de entrada no hospedeiro. Para obter uma perspetiva mais ampla dos efetores moleculares e das vias responsáveis pela resposta imune da mucosa do hospedeiro contra a *T. maritimum*, uma análise combinada de transcriptómica e proteómica foi utilizada para avaliar a resposta imune local na pele e no muco da pele do robalo europeu infetado por banho (**Capítulo 4**). Após infeção, foi observada uma resposta à bactéria tanto na pele quanto na sua camada protetora de muco. Na pele foi observada estimulação dos genes envolvidos no metabolismo dos eicosanóides, resposta de fase aguda, mecanismos de retenção de ferro e remodelação de tecidos. Estes resultados encontram-se de acordo com os apresentados no estudo anterior (**Capítulo 3**), uma vez que os genes relacionados com a resposta de fase aguda (como, *il1β* e *hamp1*) encontram-se entre os que apesentaram maior regulação positiva em peixes infetados. Além disso, os peixes infetados também mostraram uma regulação negativa de vários genes relacionados com a cicatrização de feridas, como alguns tipos de colagénio e outros componentes estruturais da matriz extracelular (MEC). Esta modulação foi acompanhada por uma expressão aumentada de genes que codificam proteinases responsáveis pela degradação da MEC. As proteínas presentes no muco da pele corroboraram a existência de uma

resposta pró-inflamatória. Os resultados obtidos mostraram uma resposta complexa da mucosa contra *T. maritimum*, que inclui o desencadeamento de um processo inflamatório na pele e modulação dos processos de cicatrização e remodelação de feridas do hospedeiro.

Este trabalho contribuiu para aumentar o conhecimento sobre o desenvolvimento da tenacibaculose, necessário para melhorar futuras estratégias de prevenção e tratamento desta infeção em espécies de peixes de aquacultura.

Palavras-chave: Tenacibaculose; Imunidade inata; Imunidade das mucosas; Resposta inflamatória; Interação agente patogénico-hospedeiro;

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LIST OF ABBREVIATIONS

LPS	Lipopolysaccharides
ECPs	Extracellular Products
SpeB	Streptopain
S-ECPs	Soluble ECPS
OMVs	Outer Membrane Vesicles
Ais	Autoinducers
QS	Quorum-Sensing
AHLs	Acyl-Homoserine Lactones
i.p.	Intraperitoneally
FKCs	Formalin-Killed Cells
ECM	Extracellular Matrix
RPS	Relative Percentage Survival
PAMPs	Pathogen-Associated Molecular Patterns
TCR	T Cell Receptors
MHC	Major Histocompatibility Complex
PRRs	Pattern Recognition Receptors
LTA	Lipoteichoic Acid
NK	Natural Killer
MCs	Mast Cells
DCs	Dendritic Cells
DAMPs	Damage-Associated Molecular Patterns
TLRs	Toll-like Receptors
NLRs	NOD-like Receptors
PGRPs	Peptidoglycans Recognition Proteins
NF	Nuclear Factor
AP-1	Activator Protein-1
IFR3	Interferon Regulatory Factor 3
AMPs	Antimicrobial Peptides
ROS	Reactive Oxygen Species
NO	Nitric Oxide
NETs	Extracellular Traps
APCs	Antigen-Presenting Cells
CLRs	C-type Lectin Receptors
DCs	Dendritic Cells
NCCs	Non-Specific Cytotoxic Cells

nkef	NK Cell Enhancement Factor (nkef)
TNF-α	Tumor Necrosis Factor-α
IL1β	Interleukin-1 Beta
IFN	Interferon
TGF-β	Transforming Growth Factor-β
C3	Complement 3
CRP	C-Reactive Protein
SAA	Serum Amyloid A
CTLs	Cytotoxic T Cells
Th	Helper T Cells
BCR	B-Cell Receptors
MHC	Major Histocompatibility Complex
MALT	Mucosal-Associated Lymphoid Tissue
GIALT	Gill-Associated Lymphoid Tissue
GALT	Gut-Associated Lymphoid Tissue
SALT	Skin-Associated Lymphoid Tissue
Dpi	Days Post-Infection
Ig	Immunoglobulin
MCs	Mast Cells
SIgs	Secretory Immunoglobulins
WCL38	Carp intestinal T Cells
RAS	Recirculation Aquaculture Systems
EPC	Epithelioma Papulosum Cyprini
MA	Marine Agar
MB	Marine Broth
TCA	Trichloroacetic Acid
WBC	White Blood Cells
RBC	Red Blood Cells
Ht	Haematocrit
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MS	Mass Spectrometry
RT	Room Temperature
MTT	3-(4, 5 dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric Oxide
LPO	Lipid Peroxidation

BHT	2,6-Di-tert-butyl-4-methylphenol
TBARS	Thiobarbituric Acid Reactive Substances
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GR	Glutathione Reductase
DTNB	5,5'-dithiobis-2-nitrobenzoic Acid
Ct	Cycle Thresholds
ef1β	Elongation Factor 1β
40s	Ribosome 40s Subunit
il6	Interleukin 6
il8	Interleukin 8
il10	Interleukin 10
il34	Interleukin 34
casp1	Caspase 1
mmp9	Matrix Metallopeptidase 9
cxcr4	Chemokine CXC Receptor 4
mif	Macrophage Migration Inhibitory Factor
mcsfr	Macrophage Colony-Stimulating Factor 1 Receptor
mhcII	Major Histocompatibility Complex II
hsp70	Heat Shock Protein 70
hamp1	Hepcidin-1
fpn	Ferroportin
PE	Peritoneal Exudates
Hb	Haemoglobin
OD	Optical Density
TMB	3,3’,5,5’- tetramethylbenzidine hydrochloride
SOD	Superoxide Dismutase
GST	Glutathione-S-Transferase
CDNB	1-Chloro-2,4-Dinitrobenzene
GSH	Reduced Glutathione
H&E	Haematoxylin and Eosin
IHC	Immunohistochemistry
PBS	Phosphate-Buffered Saline
nf-κB	Nuclear Factor Kappa B
stat3	Signal transducer and activator of transcription 3
bcl2-like	Apoptosis regulator bcl-2-like
nod1	Nod-like Receptor 1

nod2	Nod-like Receptor 2
tlr2	Toll-like Receptor 2
tlr9	Toll-like receptor 9
ROS	Reactive Oxygen Species
RIN	RNA Integrity Number
DEGs	Differentially Expressed Genes
alox15b	Polyunsaturated fatty acid lipoxygenase ALOX15B-like isoform X1
cd209c	CD209 Antigen-like Protein C
mfap4	Microfibril Associated Protein 4
pinlyp	Phospholipase A2 Inhibitor
noxo1b	NADPH oxidase organizer 1b
cxcr2	C-X-C chemokine receptor type 2-like
il11	Interleukin 11 isoform X1
il17C	Interleukin 17C
m17	IL 6 subfamily cytokine M17
il12b	Interleukin 12B
il11a	Interleukin 11a
irak4	Interleukin 1 receptor-associated kinase 4
ptafr	Platelet-activating factor receptor
c5	Complement component 5
nlrp3	NLR family CARD domain-containing protein 3-like isoform X2
hamp2	Hepcidin-2
PTX3	Pentraxin-Related Protein
PRSS3	Serine Protease 3
tlr5	Toll-like Receptor 5
soul5	Heme-binding Protein Soul5
PAFs	Platelet-activating Factors
LOX	Lipoxygenase
PGE	Prostaglandin E
GAGs	Glycosaminoglycans
GPCRs	G Protein-coupled Receptors
il17	Interleukin 17
FPRs	Formyl Peptide Receptors
MAC	Membrane Attack Complex
C7	Complement 7
OMPs	Outer Membrane Proteins
T9SS	Type IX Secretion System

PPI	Protein-Protein Interaction
MCL	Markov Clustering
CTD	C-terminal Sorting Domain
TJ	Tight Junction

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CHAPTER 5

CHAPTER 1

General introduction

1. General introduction

1.1. Global aquaculture challenges: disease outbreaks

Aquaculture is regarded as one of the fastest-growing food production sectors and, therefore, has the potential to answer to the future demand for animal protein. Global fish production reached an estimated value of 223.2 million tons in 2022, of which 130.9 million tons came from aquaculture production (FAO, 2024) (Fig. 1). Also, according to FAO 2024 report, it was the first time that aquaculture surpassed capture fisheries in aquatic animal production with 94.4 million tons' production (which represents 57% of the world production destined for human consumption). Improving living standards in developing countries, such as China, and the health consciousness of several developed countries have contributed to increased fish consumption (Supartini et al., 2018). The intensification of aquaculture production reflects the need to meet the demands of a fast-growing world population (Ahmed & Thompson, 2019). The particular nature of the applied aquaculture practices enhances the susceptibility of the farmed aquatic organisms to infectious diseases. The introduction and translocation of fish stocks between aquaculture facilities can lead to the dissemination of diseases (Peeler et al., 2011) that are associated with high stocking densities, allowing the thriving of several pathogens (Krkošek, 2010; Salama & Murray, 2011). Since the fitness gains of increased infectiousness augment with the number of susceptible hosts (Bolker et al., 2010; Borovkov et al., 2013; Day & Proulx, 2004), the increasing density at which animals are maintained is considered a risk factor for the emergence of more severe pathologies (Borovkov et al., 2013). Another susceptibility aspect is the low genetic diversity of fish in aquaculture due to selective breeding, founder effects, or inbreeding in broodstock fish, which can contribute to the high prevalence of certain diseases (Janssen et al., 2017; Kennedy et al., 2016).

The previously described factors undoubtedly contribute to the aquaculture industry as an environment prone to disease outbreaks and dissemination of pathogens (Bouwmeester et al., 2021), such as bacteria, viruses, and parasites (Bastos Gomes et al., 2017; Carbone & Faggio, 2016). Among these, infectious bacterial diseases are one of the significant constraints to the global aquaculture industry (Shefat, 2018). Some bacteria can survive in aquatic environments independently of their hosts. Thus, their prevalence in the marine farming environment is high, potentiating the occurrence of outbreaks (Aich et al., 2018; Pridgeon & Klesius, 2012). Additionally, disease monitoring can be complex due to the ability of bacteria to asymptomatically colonize farmed species as an integral component of a "healthy" microbiome (Bayliss et al., 2017; de Bruijn et al., 2018). In an aquaculture

setting, bacterial diseases are often induced by *Aeromonas* sp., *Edwardsiella* sp., *Flavobacterium* sp., *Renibacterium* sp., *Photobacterium* sp., *Streptococcus* sp., *Pseudomonas* sp., *Vibrio* sp. and *Yersinia* sp. (Austin & Austin, 2012; Sudheesh et al., 2012), many of them considered opportunistic pathogens (Derome et al., 2016). The lack of information on the epidemiology and pathogenesis of most of these pathogens underscores the need to study the pathogen itself as well as the host-pathogen interactions occurring during infection.

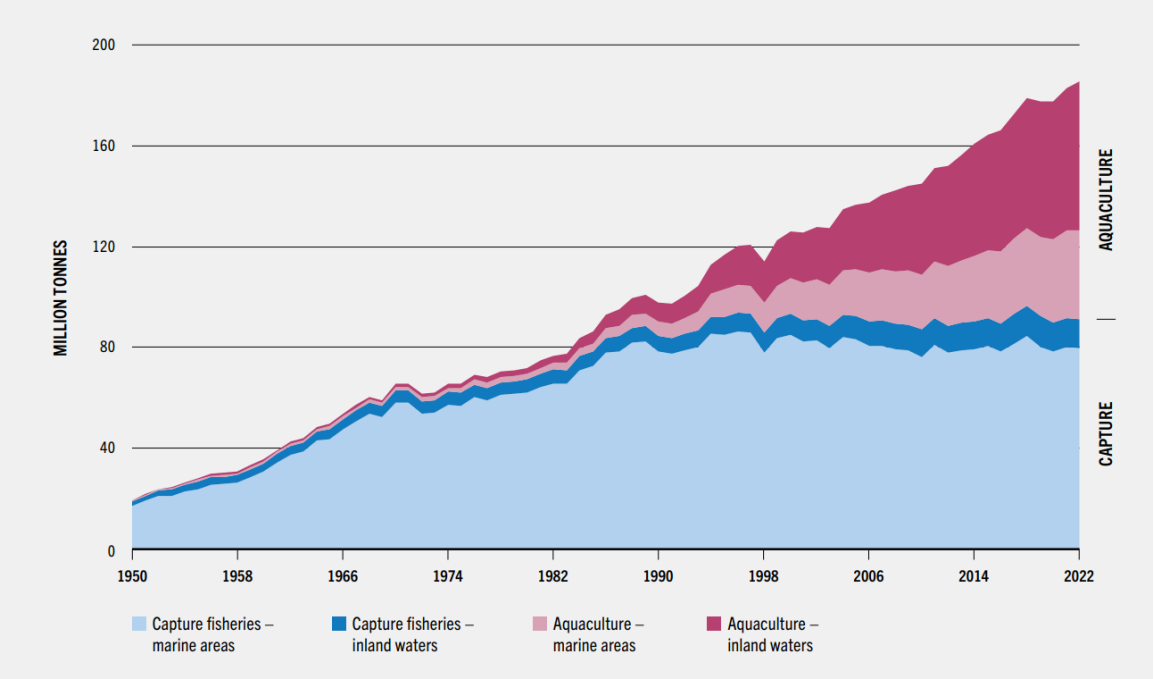

Figure 1: World fisheries and aquaculture production of aquatic animals between 1950-2022 (Source: FAO, 2024).

1.2. Tenacibaculosis: a menace to the aquaculture industry

In recent years, the aquaculture industry witnessed a worldwide emergence that affects several commercially important species, commonly known as tenacibaculosis (Avendaño-Herrera et al., 2020; Carbone & Faggio, 2016; Fernández-Álvarez & Santos, 2018; Flores-Kossack et al., 2020; Mabrok et al., 2023). Tenacibaculosis, formerly known as marine flexibacteriosis, is an ulcerative bacterial disease associated with high mortalities that presents itself as a challenge for the marine aquaculture industry (Avendaño-Herrera et al., 2006b; Toranzo et al., 2005). The economic impact associated with tenacibaculosis is due to the associated high mortalities, but even more with the prominent lesions typically found in infected fish, which lead to loss of commercial value, reduced growth, and costs associated with antibiotic treatment (Jones & Madsen, 2019).

Although current research suggests that several species from the genus Tenacibaculum have the potential to induce tenacibaculosis (e.g., *Tenacibaculum finnmarkense*, *T. dicentrarchi*, *T. solae*), *T. maritimum* has been repeatedly identified in several aquaculture systems worldwide, which demonstrates its significant impact (Bateman et al., 2022; Bridel et al., 2020; López et al., 2010; Mabrok et al., 2023; Nowlan et al., 2020) (Table 1).

Tenacibaculosis was first reported by Masumura and Wakabayashi (1977) as a gliding bacterial infection affecting black sea bream fry (*Acanthopagrus schlegeli*) reared in floating net cages in Japan. Since then, this disease spread and affected many wild and cultured marine fish species in Japan and other neighbouring countries (i.e., Korea), including Japanese flounder (*Paralichthys olivaceous*) and red sea bream (*Pagrus major*) (Baxa et al., 1986; Jang et al., 2009).

After the first reports in Asia, tenacibaculosis outbreaks began to occur in Europe, namely in Scotland, seriously impacting the culture of Dover sole (*Solea solea*) (Bernardet et al., 1990; McVicar & White, 1979). In farmed European sea bass (*Dicentrarchus labrax*), reports of tenacibaculosis were first reported at the French Mediterranean coast (Pepin & Emery, 1993) and later in Mediterranean countries (e.g. Malta, Greece, Turkey and Italy) (Kolygas & Athanasopoulou, 2012; Magi et al., 2007; Salati et al., 2005; Timur & Yardimci, 2015). The diversity of affected fish species increased in southern Europe (Spain and Portugal) where *T. maritimum* was responsible for losses in the culture of gilthead sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), and Wedge sole (*Dicologoglossa cuneate*) (Avendaño-Herrera et al., 2004; Cepeda & Santos, 2002; Devesa et al., 1989; López et al., 2009; Pazos, 1993; Piñeiro-Vidal et al., 2007; Vilar et al., 2012).

T. maritimum was also isolated from white sea bass (*Atractoscion nobilis*), northern anchovy (*Engraulis mordax*), Pacific sardine (*Sardinops sagax*), and Chinook salmon (*Oncorhynchus tschawytscha*) in North America (Chen et al., 1995).

Fish species susceptible to *T. maritimum*, such as orbicular batfish (*Platax orbicularis*) and Chinook salmon, were also identified in Oceania (e.g., French Polynesia and New Zealand), (Brosnahan et al., 2019; Lopez et al., 2022). Also, recently, *T. maritimum* was detected for the first time in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) reared in sea cages in Chile (Apablaza et al., 2017; Valdes et al., 2021), which demonstrates the continuous spreading capacity and adaptability of this pathogen to other environments and new hosts.

Table 1: Marine fish species susceptible to *Tenacibaculum maritimum*.

Geographical distribution	Susceptible species	Country	References
Africa	European sea bass (*Dicentrarchus labrax*)	Egypt	Moustafa et al., 2014
	Black Damselfish (*Neoglyphieodon melas*)	Egypt	Haridy et al., 2015
	Gilthead sea bream (*Sparus aurata*)	Egypt	Moustafa et al., 2015
	Picasso Triggerfish (*Rhinecanthus assasi*)	Egypt	Haridy et al., 2015
America	Pacific sardine (*Sardinops sagax*)	USA	Chen et al., 1995
	White sea bass (*Atractoscion nobilis*)	USA	Chen et al., 1995 Drawbridge et al., 2021
	Atlantic salmon (*Salmo salar*)	Chile	Apablaza et al., 2017
	Rainbow trout (*Oncorhynchus mykiss*)	Chile	Valdes et al., 2021
	Atlantic salmon (*Salmo salar*)	Canada	Ostland et al., 1999 Frisch et al., 2018 Bateman et al., 2022
	Coho salmon (*Oncorhynchus kisutch*)	Canada	Bass et al., 2022
Asia	Black sea bream (*Acanthopagrus schlegeli*)	Japan	Masumura & Wakabayashi, 1977
	Red sea bream (*Pagrus major*)	Japan	Masumura & Wakabayashi, 1977
	Japanese flounder (*Paralichthys olivaceous*)	Japan	Baxa et al., 1986
	Japanese flounder (*Paralichthys olivaceous*)	Korea	Jang et al., 2009
Europe	Dover sole (*Solea solea*)	UK (Scotland)	McVicar & White, 1979 Bernardet et al., 1990
	Turbot (*Scophthalmus maximus*)	Spain	Alsina & Blanch, 1993 Devesa et al., 1989 Pazos et al., 1993 Piñeiro-Vidal et al., 2007
	European sea bass (*Dicentrarchus labrax*)	France, Malta, Italy	Pépin & Emery, 1993 Bernardet et al., 1994 Salati et al., 2005

	Senegalese sole (*Solea senegalensis*)	Portugal, Spain	Cepeda & Santos, 2002 Piñeiro-Vidal et al., 2007 Vilar et al., 2012
	Gilthead sea bream (*Sparus aurata*)	Spain	Avendaño-Herrera et al., 2004
	Tub gurnard (*Chelidonichthys lucerna*)	Italy	Magi et al., 2007
	Wedge sole (*Dicologoglossa cuneate*)	Spain	López et al., 2009
	Gilthead sea bream (*Sparus aurata*)	Greece	Kolygas & Athanasopoulou, 2012
	European sea bass (*Dicentrarchus labrax*)	Malta	Yardimci & Timur, 2015
	Sand tiger shark (*Carcharias taurus*)	Italy	Florio et al., 2016
	Lumpsucker (*Cyclopterus lumpus*)	Norway	Småge et al., 2016
	European sea bass (*Dicentrarchus labrax*)	Turkey	Yardimci & Timur, 2016
	Atlantic salmon (*Salmo salar*)	Ireland	Downes et al., 2018
Oceania	Atlantic salmon (*Salmo salar*)	Australia	Handlinger et al., 1997
	Greenback flounder (*Rhombosolea tapirine*)	Australia	Handlinger et al., 1997
	Yellow eye mullet (*Aldrichetta forsteri*)	Australia	Handlinger et al., 1997
	Black bream (*Acanthopagrus butcheri*)	Australia	Handlinger et al., 1997
	Rainbow trout (*Oncorhynchus mykiss*)	Australia	Handlinger et al., 1997
	Orbicular Batfish (*Platax orbicularis*)	Tahiti	Lopez et al., 2022
	Salmon (*Oncorhynchus tschawytscha*)	New Zealand	Kumanan et al., 2024

1.3. *Tenacibaculum maritimum*

1.3.1. Phenotypic and serological characterization

T. maritimum is a Gram-negative filamentous bacterium belonging to the Phylum Bacteroidetes, Family Flavobacteriaceae. *T. maritimum* strains can be recognized by several phenotypic characteristics, which indicates that this is a relatively homogeneous species (Hikida et al., 1979; Pazos, 1993; Rahman, 2014; Wakabayashi et al., 1986). Throughout the years, many authors have described the morphological, physiological, and biochemical characteristics of this pathogen (Alsina & Blanch, 1993; Avendaño-Herrera et al., 2004; Baxa et al., 1987; Bernardet et al., 1990; Chen et al., 1995; Pazos, 1993; Soltani & Burke, 1994; Wakabayashi et al., 1986). Morphologically, it presents long and slender rods, 0.5 µm wide by 2 to 30 µm long, although cells up to 100 µm in length were previously described (Rahman, 2014). This mesophilic pathogen grows at temperatures ranging from 15 to 34 °C with an optimum growth temperature of 30 °C (Avendaño-Herrera et al., 2006b). Numerous studies reported an increased prevalence of tenacibaculosis at higher temperatures (above 15 °C) and salinities (30 to 35‰) (Avendaño-Herrera et al., 2006b; Downes et al., 2018; Handlinger et al., 1997; López et al., 2009; Nowlan et al., 2021; Timur & Yardimci, 2015). Like most species from the Flavobacteriaceae family, *T. maritimum* moves over wet surfaces by gliding motility (Pérez-Pascual et al., 2017). This is an active process that relies on a complex protein motility machinery, and therefore, this bacterium does not depend on pili or flagella for motion (Pérez-Pascual et al., 2017). Initially, results of slide agglutination assays developed by Wakabayashi et al. (1984) and Pazos (1993) suggested that *T. maritimum* was an antigenic homogenous species. However, further studies conducted by Ostland et al. (1999) revealed antigenic differences between *T. maritimum* isolates collected from Atlantic salmon in British Columbia (Ostland et al., 1999). Immunodiffusion and immunoblot techniques detected differences in the cell wall composition among British Columbian isolates and between these and the used *T. maritimum* reference strains (Ostland et al., 1999). The same conclusions were reached in a later study where the antigenic characteristics of the membrane proteins of several *T. maritimum* strains were evaluated through slide agglutination test, dot-blot assay, and immunoblotting of lipopolysaccharides (LPS) (Avendaño-Herrera et al., 2004). Several studies disclosed the existence of antigenic heterogeneity in *T. maritimum*, with four serotypes being identified (O1-O4), together with eight subtypes (Avendaño-Herrera et al., 2004; Avendaño-Herrera et al., 2005b; Fernández-Álvarez et al., 2018; Lopez et al., 2022; Piñeiro-Vidal et al., 2007). Most of the reported outbreaks are caused by serotypes O1, O2, and O3, with serotype O1 being the dominant one regardless of the host fish species or geographic location (Fernández-Álvarez et al., 2018). Nevertheless, in recent years,

serotype O4 has been identified more frequently (Escribano et al., 2024; Santos, 2022). The reasons underlying the variations in the incidence and prevalence of these serotypes have not been disclosed.

1.3.2. Clinical signs and pathology

The clinical manifestation of *T. maritimum*'s infection starts with small lesions, upraised spots, scale loss, and some disintegration of the epidermis in the host's body surface, namely in the head, skin, or fins (Haridy et al., 2015; Lopez et al., 2021; Van Gelderen et al., 2011). The ulcers can present flaps of necrotic epidermis with exposed and considerably shortened spines (Vilar et al., 2012). In juveniles, it is frequent to observe signs of hemorrhagic stomatitis or "mouth rot" that can be covered in a layer of pale yellow mucus (Frisch et al., 2018; Gourzioti et al., 2016; Ostland et al., 1999). This pale yellow coloration is explained by the abundance of bacteria in the infected epidermal tissues (Gourzioti et al., 2016). Necrosis and lamellar hyperplasia of the gills and increased mucus production have also been reported, sometimes affecting the eyes of some fish species (Powell et al., 2004; Småge et al., 2016; Van Gelderen et al., 2011). The progression of the disease leads to augmented and hemorrhagic lesions. These open wounds can be a gateway for other opportunist and pathogenic bacteria or parasites, leading to mixed infections (Kimura & Kusuda, 1983; López et al., 2009; McVicar & White, 1979). Juveniles and adult fish can display the same clinical signs, but younger fish seem more susceptible and can develop a more severe form of tenacibaculosis (Toranzo et al., 2005). A plethora of environmental and host-related factors, such as higher water temperatures, skin surface condition, and stress, can determine the development and progression of the disease (Avendaño-Herrera et al., 2006b; Magariños et al., 1995; Rahman et al., 2015; Toranzo et al., 2005). Similarly, strain virulence is another possible factor to be considered since acute or chronic disease can be triggered depending on the pathogenicity of the specific strain (Gourzioti et al., 2016; Mabrok, 2016).

Since *T. maritimum* is endowed with the ability to develop profuse biofilms within a fast kinetic, Levipan et al. (2019) suggested that aquaculture settings exposed to this pathogen can hold these cellular accretions, acting as temporary reservoirs that can contribute to its prevalence in the aquaculture industry.

1.3.3. Virulence mechanisms

Under favourable conditions, *T. maritimum* invades and colonizes the host tissues, and a systemic disease affecting different internal organs can become prevalent (Alsina & Blanch, 1993; Avendaño-Herrera et al., 2004; Cepeda & Santos, 2002). Besides the favourable conditions for thriving, the adherence ability of bacterial pathogens is another key factor that

may determine the successful invasion of a host (Stones & Krachler, 2016). As demonstrated by Burchard et al. (1990), various gliding bacteria, including *T. maritimum*, adhered tenaciously to hydrophobic surfaces when compared to hydrophilic ones, which suggests that bacterial components that make surface contact are, as well, hydrophobic. Later, Magariños et al. (1995) demonstrated that *T. maritimum* firmly adheres to fish mucus, which is unable to inhibit its growth and proliferation in the skin, regardless of the strain's origin and virulence degree (Magariños et al., 1995). This solid adherence to fish tissues is directly dependent on the ability of *T. maritimum* to evade and counteract the innate immune response of its host, namely the mucus bactericidal activity, and its capacity to gather and accumulate the nutrients necessary for its growth and proliferation (Avendaño-Herrera et al., 2006b; Magariños et al., 1995). The genome sequencing of *T. maritimum* led to the discovery of several genes encoding proteins involved in the biosynthesis of exopolysaccharides, various adhesins, and proteins with lectin or carbohydrate-binding motifs that can be involved in its sophisticated adhesion mechanisms that allow this pathogen to adhere to various surfaces, such as agar, plastic, and glass (Pérez-Pascual et al., 2017). The several extracellular polymers that *T. maritimum* produces enable the formation of surface-attached biofilms, granting an advantage for this pathogen (Avendaño-Herrera et al., 2006b; Levipan et al., 2019). In Levipan et al. (2019), it was shown that the bacterial attachment to polystyrene surfaces was fast and effective, leading to the appearance of tiny clusters of bacteria (i.e., microcolonies) or multi-layered-like cell aggregates within the first 24 h of incubation. Based on the fast kinetics of this virulence mechanism, it has been proposed that it may allow the formation of reservoirs of virulent strains in aquaculture settings that can potentiate the occurrence of tenacibaculosis outbreaks (Di Bonaventura et al., 2008; Levipan et al., 2019; Min et al., 2006). This ability to easily adhere to different hydrophobic surfaces can be explained by nutrient availability, growth conditions, properties of the adherent substratum, and incubation temperature, which induce modifications in the pathogen surface-exposed proteins (Sorongon et al., 1991). This may indicate that the initial bacterial hydrophobicity is not an adequate predictor for *T. maritimum* biofilm formation ability (Levipan et al., 2019).

Additionally, *T. maritimum* has been described as a pathogen able to agglutinate a broad spectrum of erythrocytes (Pazos, 1993). This activity is likely related to the presence of cell-surface proteins, such as adhesins and agglutinins, which are known to mediate interactions that are essential for the success of pathogenic microorganisms in the host (Lewis et al., 2022).

Another crucial strategy for *T. maritimum*'s ability to colonize the host is the expression of high-affinity iron-uptake mechanisms, which can compete directly with the host's iron-binding proteins. Iron is necessary for a diverse number of enzymatic reactions, and its

availability is crucial to allow the proliferation of the pathogen during infection (Martínez et al., 1990). However, iron is a limiting nutrient to many organisms, only existing at low levels as free ferric ions (Avendaño-Herrera et al., 2005b; Martínez et al., 1990). Therefore, successful pathogens must possess high-affinity iron acquisition strategies to acquire iron in the host. One of those strategies is the release of siderophores, which chelate iron and transfer it to the bacteria's iron-regulated outer membrane proteins that work as receptors for iron-siderophore complexes (Khasheii et al., 2021; Kramer et al., 2020). Avendaño-Herrera et al. (2005b) demonstrated that different *T. maritimum* strains have at least two different iron-uptake mechanisms, one related to the synthesis of siderophores and the other involving the use of heme groups as iron sources (Avendaño-Herrera, et al., 2005b). Indeed, the genome of *T. maritimum* encodes a siderophore biosynthesis gene cluster, which is accepted to be related to the production of the macrocyclic hydroxamate class bisucaberin siderophore (Pérez-Pascual et al., 2017). Alongside this cluster, *T. maritimum* encodes a TonB-dependent outer membrane receptor possibly corresponding to a bisucaberin siderophore-iron transporter (Pérez-Pascual et al., 2017). This variety of iron-related genes suggests that *T. maritimum* uses efficient iron-uptake systems to overcome the iron-limited conditions faced in the host during infection (Pérez-Pascual et al., 2017), although a detailed functional characterization of such systems at the molecular level is still lacking.

T. maritimum is also able to secrete extracellular products (ECPs) with important biological roles, including proteases with gelatinase, amylase, caseinase, and nuclease activities that can cause damage to host tissues (Pazos, 1993), hence allowing the bacteria to obtain nutrients. Furthermore, the genome of *T. maritimum* encodes several proteins homologous to proteins that in other bacteria act as toxins and virulence factors, such as sphingomyelinase and ceramidase (Pérez-Pascual et al., 2017). Sphingomyelinase belongs to a group of esterases that hydrolyze sphingomyelin (Flores-Díaz et al., 2016) and have potent hemolytic activity, inducing cytotoxicity in host cells (Oda et al., 2010). Studies in *Pseudomonas aeruginosa* and *Photobacterium damselae* subsp. *damselae* have already demonstrated that these bacterial enzymes are important since they can contribute to tissue colonization, infection establishment, and progression, or immune evasion (Hinkel & Wargo, 2020; Vasil et al., 2009). Ceramidases are enzymes that hydrolyze the N-acyl linkage between the sphingoid base and fatty acid of ceramide (Ito et al., 2014) and can function as an exotoxin or activator of exotoxin (Ito et al., 2014; Okino et al., 2010). Chondroitin sulfate lyase is another notable enzyme encoded in *T. maritimum*'s genome that was shown to degrade chondroitin sulfate A and C *in vitro* (Pérez-Pascual et al., 2017) and may have a virulence role *in vivo*. Another gene putatively associated with virulence in *T. maritimum* encodes is a multi-domain C10 family peptidase, very similar to streptopain (SpeB), an

important virulence factor of *Streptococcus pyogenes* that is reported to participate in bacterial colonization, invasion, inhibition of wound healing and is potentially able to inhibit migration of monocytes/macrophages (Nelson et al., 2011). Besides the demonstration of *in vitro* activity of *T. maritimum* sphingomyelinase and chondroitin AC lyase (Pérez-Pascual et al., 2017), no other proteins from this pathogen were functionally characterized *in vivo* or *in vitro*, despite the various clues that gene homology offers. The existent gap in knowledge regarding the roles of *T. maritimum* virulence factors in infection reveals how much remains to be explored about these bacteria.

Several studies investigated the biological activities of *T. maritimum*'s ECPs. Baxa et al. (1988) assessed the consequences induced by the i.p injection of *T. maritimum*'s ECPs in black sea bream and red sea bream fry (Baxa et al., 1988), showing that in both species, the i.p. injected ECPs registered the lowest LD50, followed by i.p. injection of hemolysin, sonicated cell-free supernatant, and lyophilized culture filtrate (Baxa et al., 1988). This preliminary study was already able to reveal the toxic abilities of *T. maritimum* ECPs, which included petechial haemorrhages in visceral fats and intestines and severely damaging the liver (Baxa et al., 1988). Thus, it was proposed that the pathogenicity of *T. maritimum* is related to the toxic effects of ECPs, and a synergetic effect can exist between the secreted proteases and other virulent factors, leading to high mortality (Baxa et al., 1988). Later, Van Gelderen et al. (2009b) described the pathology and mortality in Atlantic salmon i.p. injected with *T. maritimum* ECPs (Van Gelderen et al., 2009b). Cell necrosis in the internal organs was observed, leading the authors to propose that this pathogen might use the ECPs to facilitate cellular breakdown. To delve deeper into the ECPs properties, Escribano et al. (2023) analysed the protein content of total ECPs of *T. maritimum* by a high-throughput proteomic approach. Moreover, the total ECPs were divided into soluble (S-ECPs) and insoluble fractions, the latter containing outer membrane vesicles (OMVs) shed by the bacteria. The protein content of each fraction (S-ECPs and OMVs) was also analysed by a high-throughput proteomic approach, and each fraction was used to perform *in vitro* and *in vivo* trials in Senegalese sole fingerlings by i.p. injection. Interestingly, among the most abundant proteins in total ECPs, there were diverse hydrolytic enzymes, including chondroitinase, sialidase, sphingomyelinase, ceramidase, and collagenase, previously predicted as virulence factors by Pérez-Pascual et al. (2017). Sole fingerlings were i.p. injected with total ECPs and one of the obtained fractions (S-ECPs and OMVs). Total ECPs displayed higher toxic effects, causing ulcerative and hemorrhagic lesions between 12 and 24 h after injection, whereas less toxic effects were observed with S-ECPs or OMVs (Escribano et al., 2023), suggesting that S-ECPs and OMVs may play different roles in virulence, being both needed for maximal pathogenic effects in fish (Escribano et al., 2023). The main proposed virulence mechanisms used by *T. maritimum* are mentioned in Fig. 2.

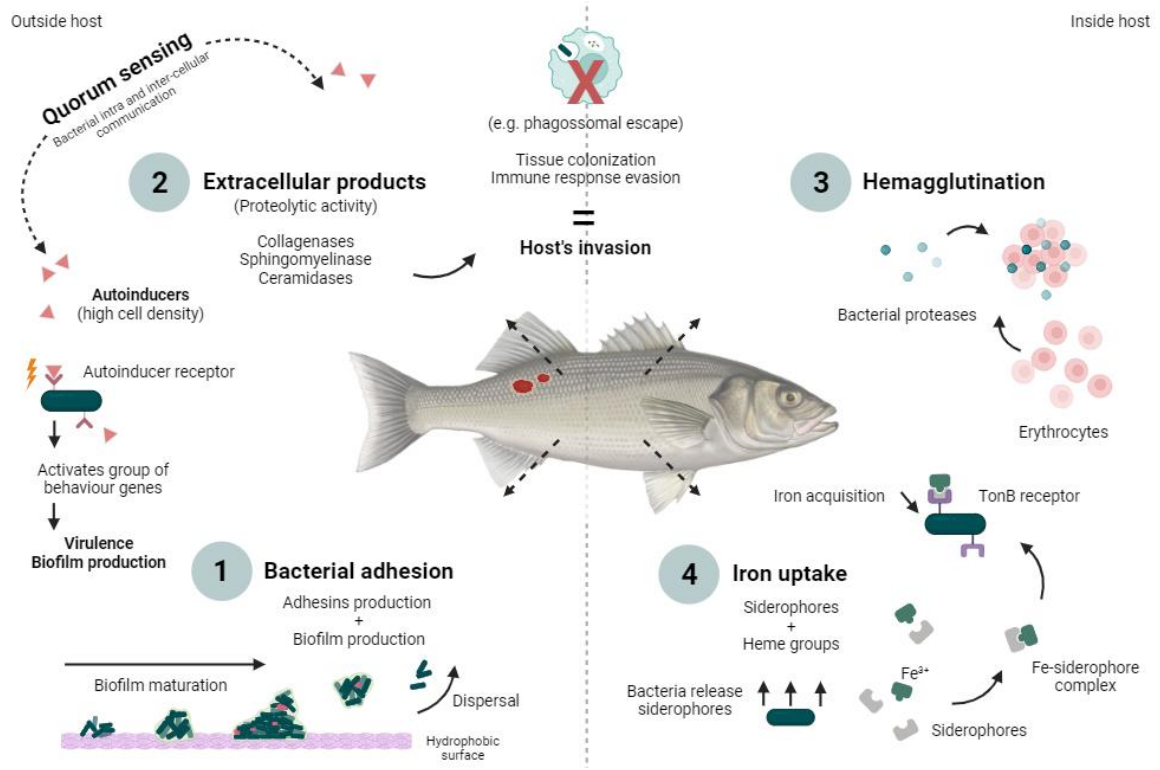

Figure 2: Summary of the proposed *T. maritimum*'s virulence mechanisms. Bacterial adhesion is an essential mechanism used by *T. maritimum* to ensure strong adhesion to the host's surface, such as mucosal tissues, and allow the bacteria to establish infection. Biofilm production offers protection from the host's immune defenses and contributes to the difficulty of eradicating this pathogen in an aquaculture environment. It is speculated that these bacteria are also able to use quorum-sensing as a means to communicate and coordinate their behavior (e.g., production of biofilms and secretion of virulence factors). The extracellular products (ECPs) secreted by *T. maritimum* comprise a proteolytic cocktail that degrades the host tissues, which leads to tissue necrosis and allows access to deeper tissues; this provides essential nutrients for bacterial growth while compromising host barriers. It is speculated that these ECPs may intervene in the evasion of host defense mechanisms. *T. maritimum* possesses an arsenal of toxins, named hemolysins, that target and disrupt blood cells; siderophores are also employed to chelate iron and transfer it to iron-regulated outer membrane proteins that work as receptors for iron-siderophore complexes.

Bacteria use strategies to communicate and coordinate their behaviour to be more effective in the proliferation and evasion/fight against the host's immune response. Quorum-sensing (QS) is an essential process for bacterial intra and inter-cellular communication that involves signalling molecules, such as autoinducers (AIs) (Rutherford & Bassler, 2012). Higher densities of the bacterial population will induce an increase in the concentration of AIs, giving information to each cell to coordinate the expression of specific genes, thereby synchronizing their behaviour (Mukherjee & Bassler, 2019). Among the processes controlled by QS are the production of protective biofilms and the secretion of virulence

factors (De Kievit, 2009; Gahan et al., 2021; Rutherford & Bassler, 2012; Whiteley et al., 2017). The production of AIs in several strains of Bacteroidetes isolated from marine biofilms has already been demonstrated, although the process behind the control of the QS mechanism has not been fully clarified (Huang et al., 2008). In Gram-negative bacteria, the most common class of AIs are acyl-homoserine lactones (AHLs), which are bound by specific receptors that reside either in the inner membrane or in the cytoplasm (Garg et al., 2014; Miller & Bassler, 2001; Papenfort & Bassler, 2016). Short-type AHL activity, similar to the one produced by *Vibrio* sp., was detected in cultures of *T. maritimum* through the use of different biosensor strains (Romero et al., 2010). Surprisingly, no homologous genes for the biosynthesis of AHLs were detected in *T. maritimum*'s genome (Pérez-Pascual et al., 2017).

Despite the above-described attempts to identify crucial virulence factors of *T. maritimum*, the detailed mechanisms underlying its strong virulence have yet to be fully clarified.

1.4. Experimental models of tenacibaculosis

In recent years, different experimental methods of infection with *T. maritimum* resulted in distinct mortality rates (Frisch et al., 2018; Mabrok, 2016; Nishioka et al., 2009; Yamamoto et al., 2010), which constitutes an obstacle to identifying the preferred route of infection for this pathogen. Therefore, an effort has been made to find the most effective ways to reproduce tenacibaculosis in an experimental setting and, thus, an effective standardized challenge method. The initial studies in European sea bass demonstrated that abrasion/scarification of fish skin, followed by smearing these induced lesions with pure broth culture, was the most effective way to reproduce tenacibaculosis experimentally (Bernardet et al., 1994). The abrasion method was also applied in Atlantic salmon gills to enhance the progression of tenacibaculosis, resulting in increasing mortalities (Powell et al., 2004). Then, ensuing studies in turbot tested a prolonged immersion challenge for 18 h at 18 to 20°C. It effectively reproduced this pathology without skin/gills abrasion (Avendaño-Herrera et al., 2006a). Faílde et al. (2013) intraperitoneally (i.p.) infected turbots with a high dose of bacterial suspension (10^9 CFU/fish), and while fish developed septicaemia, no typical clinical signs were observed. Furthermore, a high dose of bacteria was required to cause damage in the analysed internal organs compared to other infection methods, such as subcutaneous injection (Faílde et al., 2013). More recently, Mabrok (2016) used a more extended infection bath of 24 h at 23°C to challenge Senegalese sole with several strains of *T. maritimum*, being successful in obtaining high mortality rates (50-100%, depending on the *T. maritimum*'s strain), although not referring if clinical signs were displayed by the infected fish. Thus, immersion seems a reliable and effective experimental challenge

method, being able to induce satisfactory mortality rates and reproducing the characteristic clinical signs of tenacibaculosis.

1.5. Disease management

Although the environmental conditions that can increase the incidence of tenacibaculosis in industry settings are still unclear, high stocking densities, augmented stress, and poor water quality (i.e., amount of organic material) play an important role in emerging outbreaks and proliferation of *T. maritimum* (Escribano et al., 2020; Mabrok et al., 2023; Van Gelderen et al., 2011; Yamamoto et al., 2010). Thus, avoiding overfeeding, controlling fish densities, and manipulating the temperature and salinity can help to reduce tenacibaculosis outbreaks and, consequently, fish mortality (Soltani et al., 1996; Soltani & Burke, 1994). Additionally, the use of several chemotherapeutics, immunomodulatory agents, and vaccines has been studied in recent years, but tenacibaculosis prevention with available tools is far from being efficient. *In vitro*, studies showed that different strains of *T. maritimum* are susceptible to amoxicillin, ampicillin, erythromycin, nitrofurantoin, florfenicol, chloramphenicol, oxytetracycline, and trimethoprim-sulphamethoxazole (Avendaño-Herrera et al., 2008; Avendaño-Herrera et al., 2005a; Baxa et al., 1988; Cepeda & Santos, 2002; Soltani et al., 1995). The main obstacle relies on differences between *in vitro* and field efficacies of those chemotherapeutics, which cannot prevent fish mortalities (Cepeda & Santos, 2002; McVicar & White, 1979). Moreover, antibiotics are associated with several environmental and human health issues, which raises concerns regarding their frequent use in aquaculture (Ahmad et al., 2021; Lulijwa et al., 2020; Santos & Ramos, 2018; Shao et al., 2021; Zhao et al., 2020). Other chemicals, such as hydrogen peroxide (H_2O_2) and formalin, were tested against tenacibaculosis, but these treatments have some restrictions on their applicability *in vivo* (Aly et al., 2020; Avendaño-Herrera et al., 2006a; García-Magaña et al., 2019). The first i.p. vaccine (FM 95) against tenacibaculosis (formalin-inactivated bacterial cells of a strain of *T. maritimum* isolated from turbot, deposited in the Spanish Collection of Type Cultures under the reference 5045) was patented by the University of Santiago de Compostela (Spain) and remains the only commercially available vaccine for effective prevention of tenacibaculosis in turbot (Santos et al., 1999). This vaccine can be applied by bath when the fish is 1 to 2 g, followed by an i.p. boost to achieve 85% protection (Toranzo et al., 2004). The consistent application of this vaccine allowed to reduce the incidence of tenacibaculosis in turbot farms (Avendaño-Herrera et al., 2006b). Still, the serological diversity among *T. maritimum* strains remains a major obstacle that may compromise the efficacy of this vaccine for preventing infections caused by different *T. maritimum* serotypes (Avendaño-Herrera et al., 2006b). Other trials focused on the development of vaccines containing LPS, ECPs, and formalin-

killed cells (FKCs) for protection against tenacibaculosis. In European sea bass, FKCs and LPS preparations increased agglutinating antibody titer right after the first i.p. injection when compared to the control fish (Salati et al., 2005). After a second i.p. injection, all the preparations (LPS, ECPs, and FKCs) displayed a booster response (Salati et al., 2005). In the *in vitro* trials, all preparations stimulated phagocytosis in the total blood when compared to the controls, however, higher phagocytic activity was recorded for blood cells from LPS immunized sea bass (Salati et al., 2005). As with other fish vaccines, the LPS seems to be promising as the main protective antigen (Salati et al., 2005). Formalin-killed bacterial cells were also used to produce a vaccine that was i.p. injected into Atlantic salmon (Van Gelderen et al., 2009a). After a challenge with *T. maritimum*, the relative percentage survival (RPS) obtained for the vaccine with adjuvant was 79.6% when compared with the control fish (sterile PBS) (Van Gelderen et al., 2009a). Kato et al. (2007) developed an immersion vaccine with FKCs that induced significant protection of red sea bream against experimental tenacibaculosis (Kato et al., 2007). More recently, Escribano et al. (2024) developed an encapsulated multi-antigen vaccine made of *T. maritimum* OMVs from strain SP9.1, which was able to offer significant protection (RPS = 70%) against *T. maritimum* bath challenge. Immunization increased anti-Tm antibody titres in the blood plasma of juvenile turbot in a dose-dependent manner; moreover, this immunization with OMVs from strain SP9.1 (serotype O4) induced high antibody levels against all *T. maritimum* serotypes, suggesting cross-protection (Escribano et al., 2024).

Another alternative and promising solution to cope with tenacibaculosis outbreaks is the use of therapeutic bacteriophages (Ramos-Vivas et al., 2021). However, a better understanding of their biology and genomes is required to achieve efficient protection against bacteria, namely *T. maritimum*. Recently, two phages (PTm1 and PTm5) of *T. maritimum* were isolated from seawater around a fish aquaculture field in Japan (Kawato et al., 2020), but despite harbouring lytic activity, the host ranges of PTm1 and PTm5 are too narrow for use as therapeutic agents for tenacibaculosis.

1.6. Teleost immune system

The studies focusing on the relationship between a pathogen and its host are on the rise due to the urgent demand for understanding the mechanisms behind infection and host susceptibility. Fish reared in aquaculture facilities constantly face challenges, such as global climate change, emergent pathogens, limited genetic diversity, and inadequate biosecurity, which require a swift immune system response. The fish immune system is similar to that of higher vertebrates (Mokhtar et al., 2023) and comprises two main components: the innate and the adaptive immune systems. Initially, pathogens and infections/diseases are

addressed by innate immunity. This involves the participation of physical barriers (i.e., mucus, scales, mucosal epithelia) and humoral and cellular components (Riera Romo et al., 2016), which react by identifying pathogen-associated molecular patterns (PAMPs) present in pathogens (Smith et al., 2019). If the pathogen persists, the host counteracts with the adaptive immune response, which takes longer to develop but involves specificity and memory, acting through antigen-specific receptors. The adaptive response comprises immunoglobulins (Igs), T cell receptors (TCR), and major histocompatibility complex (MHC) and provides long-lasting pathogen memory (Smith et al., 2019).

Both components, innate and adaptive, are deeply interconnected, with innate response providing the first line of defense and simultaneously instructing and enhancing adaptive immune response. In contrast, the adaptive system refines and improves innate immunity during and after an infection.

1.6.1. Innate immune response

In contrast to mammals, fish heavily depend on innate immune response and its components since the first embryonic stages of life (in several fish species, most of those components are functional on the first day of embryogenesis) (Buchmann et al., 2024). The teleost innate immune system is generally divided into three components: physical, humoral, and cellular (Mokhtar et al., 2023). Such components can respond to pathogens through non-opsonic receptors (pattern recognition receptors, PRRs) (Zhu & Su, 2022). These can recognize PAMPs, like LPS and lipoteichoic acid (LTA), which are not expressed in the host cells (Dalmo & Bøgwald, 2022; Smith et al., 2019). Pathogen recognition is generally followed by an orchestrated response that induces the transcription of pro-inflammatory and immunomodulatory cytokines (i.e., interleukins 1, IL1, and -6, IL6) and chemokines (i.e., interleukin-8, IL-8) (Sakai et al., 2021). Moreover, through the activation of complement cascades, neutrophils and macrophages are attracted, facilitating the phagocytosis and destruction of pathogens by these cells (Vandendriessche et al., 2021; W. Zhu & Su, 2022). Innate immunity is the first line of defense against pathogens in fish, and although it does not guarantee long-standing protection, it presents a swift response against invading microorganisms (You et al., 2024).

1.6.1.1. Physical barriers

The physical barriers include the mucus layer, scales, and the epithelia of the mucosal surfaces (i.e., gills, skin, and alimentary tract), all contributing to infection resistance (Salinas et al., 2022). Besides preventing the entry of foreign materials and pathogens, the epithelial layer is constantly populated by immune cells (i.e., macrophages, lymphocytes, and eosinophilic granular cells) that rapidly respond to pathogen invasion (Mokhtar et al.,

2023). The mucus layer that covers the epithelial layer of physical barriers is a reactive and synergetic cocktail constituted by microbicide substances like lectins, pentraxins, lysozymes, complement proteins, and proteases, among other essential molecules, with protective and regulatory capacities (Santoso et al., 2020). Moreover, mucus is constantly being produced, allowing it to be sloughed off frequently, avoiding the adherence of the entrapped pathogens and invasion of host cells (Arasu et al., 2013). The integrity of both cells and the mucus layer is essential in preserving the osmotic balance and eradicating microorganisms. More particularly, the skin mucus of European sea bass presents bactericidal activity against several pathogenic bacteria, such as *Aeromonas hydrophila*, *Pseudomonas anguilliseptica*, *Vibrio fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum* (Baba, 2021; Gabriella et al., 2021; Sanahuja et al., 2019).

1.6.1.2. Cellular response in innate immunity

The cellular response in innate immunity is driven by several immune cells that are able to recognize and eliminate pathogens without the need for prior exposure. Similar to mammals, fish possess cells like macrophages, neutrophils, eosinophils, natural killer (NK)-cells, mast cells (MCs), and dendritic cells (DCs) that collaborate to destroy pathogens while activating and mediating the broader immune response (Mokhtar et al., 2023). Besides these, fish also have rodlet cells and melanomacrophage centers (Sayyaf Dezfuli et al., 2022; Steinel & Bolnick, 2017). All these offer a fast response, providing immediate defense of the host and providing the basis for more specific and long-term protection by the adaptive immune system.

PRRs are germline-encoded receptors that can sense PAMPs conserved in many microorganisms or endogenous damage-associated molecular patterns (DAMPs) (Liao & Su, 2021). There are several groups of PRRs, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors, and peptidoglycans recognition proteins (PGRPs), already identified in several fish species; nevertheless, the immunoregulatory mechanisms that regulate PRRs actions remain relatively unknown (Chuphal et al., 2022; Liao & Su, 2021; Sahoo, 2020; Zhang et al., 2019). These PRRs are expressed in innate immune cells (e.g., neutrophils and macrophages) (Boltaña et al., 2011; Buchmann, 2022). Each group of PRRs is characterized by its different structure and specificity but also by its tissue-specific expression and localization (which can include the plasma membrane, endosomes, lysosomes, and cytosol) (Iwasaki & Medzhitov, 2015). Upon recognition of PAMPs, such as cell wall molecules (e.g., LPS, lipopeptides, peptidoglycan), flagellin, bacterial DNA, and other molecules (Cavaillon, 2017), PRRs trigger signalling cascades, which include nuclear factor-(NF-)κB, activator protein-1 (AP-1), and interferon regulatory factor 3 (IFR3) pathways (Liao & Su, 2021; Wills-Karp, 2010). This orchestrated response

induces the transcription of cytokines, chemokines, and growth factors, as well as various pro-survival and anti-apoptotic genes (D. Li & Wu, 2021). The simultaneous activation of several PRRs by the same pathogen allows a combination of signals specially adapted for a particular group of invading organisms, resulting in tailored host cellular and humoral responses by the host. Despite the many PRRs identified and characterized in teleost fish (Chuphal et al., 2022; X. Li et al., 2020; Mushtaq et al., 2024; Wang et al., 2021), PPRs ligands and immunoregulatory mechanisms remain largely unknown.

As mentioned above, similarly to what occurs in mammals, the cellular response in fish innate immunity is carried out by different immune cells that recognize and eliminate the invading pathogens. Neutrophils are present in the blood, peritoneal cavity, and lymphoid organs and can phagocytose cells and foreign particles and produce superoxide anions with an antibacterial effect (Havixbeck & Barreda, 2015). In fish, neutrophils are essential for the inflammatory response against bacterial, viral, and fungal pathogens (Havixbeck et al., 2016, 2017; Secombes & Wang, 2012; Zhao et al., 2017). They are the first cells to arrive at the infection site, followed by macrophages, both recruited by chemotactic signals generated from the injury (Smith et al., 2019). Recruited neutrophils phagocyte and destroy microorganisms, releasing AMPs, proteolytic enzymes, reactive oxygen species (ROS), and nitric oxide (NO) with antimicrobial effects (Havixbeck & Barreda, 2015; Soliman & Barreda, 2023). Moreover, neutrophils can release extracellular traps (NETs) to further entrap and inactivate pathogens (Chen et al., 2021; Zhao et al., 2022). Blood monocytes can migrate into the connective tissue during the inflammatory phase and convert to macrophages or DCs (Hodgkinson et al., 2015). This differentiation is determined by the inflammatory milieu and pattern recognition receptors on the cells (Murray, 2018). Macrophages exert antimicrobial defense through phagocytosis and release of antimicrobial mediators (e.g., ROS and NO) (Fig. 3), also serving as professional antigen-presenting cells (APCs) to activate the adaptive immune system (T and B cells) (Joerink et al., 2006). These express various receptors on their cell surface, like TLRs, PRRs, and C-type lectin receptors (CLRs), in addition to complement and scavenger receptors (Mokhtar et al., 2023). Macrophages also contribute as a source of chemokines and cytokines, mediating the immune system (Sinha, 2022). In teleosts, monocytes/macrophages have been recognized in various fish species, including zebrafish (*Danio rerio*), rainbow trout, and goldfish (*Carassius auratus*) (Hodgkinson et al., 2017; Leal et al., 2017; Yu et al., 2017). DCs are antigen-presenting cells already found in the gills, skin, and gut of fish (Alesci et al., 2022; Mokhtar et al., 2023). These cells can deliver processed antigens, initiating the cell-mediated adaptive response and modulating tolerance mechanisms towards self-antigens (Soleto et al., 2019). With high expression levels of PRRs, DCs can swiftly detect pathogens, internalize them, and present the antigens in the MHC I (intracellular antigens)

or MHC II (extracellular antigens) to T lymphocytes (Mellman & Steinman, 2001). Teleost fish display two types of NK cell homologs important in innate immunity: non-specific cytotoxic cells (NCCs) and NK-like cells (Yoshida et al., 1995). NK-like cells can destroy allogeneic and virus-infected cells (Yoshida et al., 1995). The gene encoding the NK cell marker, NK cell enhancement factor (*nkef*) has been reported in fish and its expression was induced in the gills and skin after a bacterial infection (Huang et al., 2021). The NCCs can target virus-infected cells, tumour cells, and protozoan parasites (Mokhtar *et al*., 2023) and can also participate in antibacterial immunity (Huang et al., 2024; Y. Huang et al., 2020, 2021; Jaso-Friedmann et al., 2001). Eosinophils are granulocytic leukocytes mainly involved in defense against parasites and modulation of diverse immune responses (Ni et al., 2024; Sfacteria et al., 2015). These are distributed in the connective tissue, especially in the bloodstream, gills, and gastrointestinal tract (Mokhtar et al., 2023). Molecular analysis has shown that zebrafish eosinophils can express genes that are important for the activities performed by mammalian eosinophils (Balla et al., 2010). Moreover, when zebrafish are infected with the pathogenic nematode *Pseudocapillaria tomentosa*, there is a marked increase in eosinophil numbers in the intestine (Balla et al., 2010).

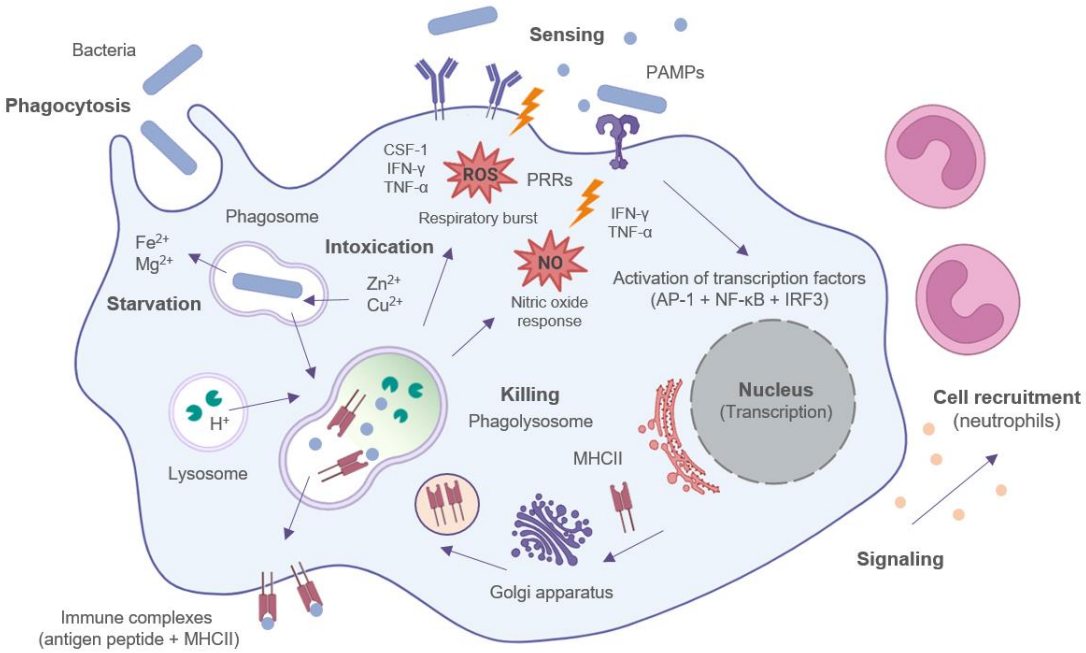

Figure 3: Phagocytosis and pathogen processing by macrophages in teleost fish (adapted from W. Zhu & Su, 2022). Pathogens are sensed through the recognition of Pathogen-Associated Molecular Patterns (PAMPs) mediated by Pattern Recognition Receptors (PRRs), such as Toll-Like receptors (TLRs), present in the phagocytic cells. The targets are then engulfed in the phagosome, which then fuses with the lysosome, forming the phagolysosome, which contains potent antimicrobial compounds, including degradative enzymes (e.g., proteases, nucleases, lipases). In addition, respiratory burst (ROS) and nitric oxide (NO) responses produce a variety of anti-microbial

compounds, which help in the elimination of phagocytosed pathogens. Simultaneously, MHC II molecules are produced by the endoplasmic reticulum and processed in the Golgi apparatus, fusing with phagolysosomes. The formed immune complexes (antigen plus MHC II) are later presented on the cell surface.

MCs are located near the blood vessels of the gills and intestinal mucosa of teleost fish and fulfil essential roles in host defence (Sayyaf Dezfuli et al., 2023). It was demonstrated that in stress conditions (e.g., chronic inflammation, exposure to chemical contaminants, parasitic infections), there is an increase in mast cells in the affected tissues (Lauriano et al., 2012; Sayyaf Dezfuli et al., 2021). Mast cells can release at the site of inflammation a wide range of inflammatory mediators, like cytokines, proteolytic enzymes, and piscidins (Corrales et al., 2010; Salger et al., 2016; Silphaduang & Noga, 2001).

1.6.1.3. Humoral response in innate immunity

Humoral innate immunity consists of a plethora of cell receptors, soluble substances, and other non-specific defence molecules (e.g., antimicrobial peptides, lysozyme, lectins, natural antibodies, cytokines, and complement components) able to inhibit the growth of microorganisms and neutralize the enzymes on which pathogens depend (Kordon et al., 2018; Mokhtar et al., 2023). Many of these humoral factors (and their lytic, pro-inflammatory, chemotactic, and opsonization properties) are tightly intertwined with the action of the previously mentioned cellular components of the fish immune system. A broad spectrum of activity, without the need for previous exposure, makes the humoral component an important branch of fish immunity.

1.6.1.3.1. Cytokines and chemokines

Cytokines are proteins produced and secreted by different types of cells, including leukocytes, endothelial cells, fibroblasts, and stromal cells (Cao et al., 2023). They regulate and integrate innate and adaptive immune responses by actively participating in inflammatory, chemotactic, and recruited-cell growth and differentiation processes (Alejo & Tafalla, 2011; Soliman & Barreda, 2023). Through binding to specific receptors in the cell membrane, cytokines provide a cascade-enhancing induction that triggers or suppresses the inflammatory response (Mokhtar et al., 2023). Many cytokines were already identified in fish, including tumor necrosis factor-α (TNF-α) (Nascimento et al., 2007), interleukin-1 beta (IL-1β) (Zou, 1999), interferon (IFN) (Altmann et al., 2003), transforming growth factor-β (TGF-β) (Haddad et al., 2008), and other types of chemokines (Baoprasertkul et al., 2004; Laing et al., 2002; Peatman & Liu, 2006).

IL-1β was one of the first cytokines to be identified in teleosts (Zou, 1999). This cytokine is produced by several types of cells (Pelegrín et al., 2004; Zou & Secombes, 2016) after stimulation by PAMPs or damage-associated molecular patterns (DAMPs) (Mokhtar et al., 2023). Similarly, to what occurs in mammals, in teleosts, IL-1β is produced as a precursor protein that is processed, secreted, and participates in host innate immunity (J. Y. Li et al., 2020). In primary leucocytes and macrophages, mature IL-1β can induce the expression of pro-inflammatory genes like IL8, IL6, IL17C, and TNF-α (Hong et al., 2013). In European sea bass, pro-IL1β is known to be processed by caspase-1 at aspartate D^{100} to originate the 18 kDa mature IL1β (Reis et al., 2012). A recombinant European sea bass IL1β starting at alanine A^{86} was demonstrated to increase yeast phagocytosis by sea bass head kidney leukocytes *in vitro* (Buonocore et al., 2005). Moreover, when i.p. injected in European sea bass, it increased the expression of *il1β* and of a cyclooxygenase-2 homolog (COX-2) in head-kidney leukocytes (Buonocore et al., 2005).

The TNF superfamily, which includes TNF-α, mainly comprises type II transmembrane proteins that can be cleaved and release a soluble cytokine (Mokhtar et al., 2023). In teleost fish, TNF-α expression was demonstrated in T cells and macrophages (Mingyue Huang et al., 2020; Yang et al., 2023). TNF-α is up-regulated in the head-kidney of European sea bass after i.p. injection of UV-killed *P. damselae* subsp. *piscicida* (Nascimento et al., 2007). Many other studies in fish have been investigating the pro-inflammatory properties of TNF-α, suggesting an important role in the activation of the antibacterial immune response (Cui et al., 2020; Kong et al., 2021; Nguyen et al., 2017; Ren et al., 2020).

IL8, a cytokine closely related to IL1β, is a pro-inflammatory mediator belonging to the CXC chemokine family (Soliman & Barreda, 2023). Some preliminary studies in fish recognized its potent chemotactic activity for neutrophils and macrophages (Omaima Harun et al., 2008; Zhonghua et al., 2008). Likewise, IL8 is also able to trigger antimicrobial mechanisms of peripheral blood leukocytes (e.g., increased expression of immune genes, resistance to bacterial infection, respiratory burst, acid phosphatase, chemotactic, and phagocytic activity) in fish (Zhang et al., 2023). The properties of IL-8 upon bacterial infections have been studied in several fish species (de Oliveira et al., 2013; Soliman et al., 2021; Zhao et al., 2024).

IL-6 is a pleiotropic cytokine with critical functions in innate and adaptive immune responses. Since its first description in fish (Bird et al., 2005), IL6 has been identified in several teleost species like Japanese flounder (Nam et al., 2007), gilthead sea bream (Castellana et al., 2008), large yellow croaker (*Larimichthys crocea*) (Zhu et al., 2016) and rainbow trout (Iliev et al., 2007), and more recently in Siberian sturgeon (*Acipenser baeri*) (Wang et al., 2020) and channel catfish (*Ictalurus punctatus*) (Zhu et al., 2023). Studies demonstrated that this cytokine is involved in lymphocyte differentiation and stimulation of

antimicrobial peptides (Chen et al., 2012; Costa et al., 2011). Moreover, co-administration of IL6 is a possible alternative to potentiate the immune response (i.e., inducing antibody production) to prevent or treat bacterial diseases in fish (Huang et al., 2019; Lin et al., 2022). In recent years, the chemokine research (i.e., identification and clarification of biological function) in fish species with commercial interest in the aquaculture industry has increased, with the purpose of better understanding the role of cytokines in immune function and during pathogenesis.

1.6.1.3.2. Lytic factors (lysozyme, complement factors, and AMPs)

Lysozymes are non-specific innate immune molecules involved in host protection against bacterial invasion (L. Li et al., 2021). The activity of such molecules is increased by infection and is modulated by several factors, including stress and nutrition (Gao et al., 2016; Ghafarifarsani et al., 2022; Khansari et al., 2019). Lysozymes can hydrolyse a major structural component of bacterial cell walls, the peptidoglycan layer, through cleaving beta-(1,4)-glycosidic bonds in peptidoglycan (Ferraboschi et al., 2021). They are involved in defence mechanisms, like bacteriolysis and antiviral and antineoplastic activities (Saurabh & Sahoo, 2008). The antibacterial activity of lysozyme has been described for some fish species, including Japanese flounder, Asian sea bass (*Lates calcarifer*), cod (*Gadus morhua*), orange-spotted grouper (*Epinephelus coioides*), and European sea bass (Buonocore et al., 2014; Fu et al., 2013; Minagawa et al., 2001; Seppola et al., 2016; Wei et al., 2014). The complement is another vital element of innate immunity, comprising a complex network of more than 30 proteins in vertebrates (Bai et al., 2022). This network consists of plasma proteins produced by the liver that may also act as an effector and signalling mechanism for adaptive immunity (Najafpour et al., 2020). Through antigen-antibody complex and other effector proteins, carbohydrates (e.g., mannans), or hydrolysis of complement 3 (C3) the complement is activated (Merle et al., 2015; Najafpour et al., 2020; Petersen et al., 2000). After activation, the complement system induces chemotaxis and cell activation, mediating inflammation through phagocytosis, degranulation, and cell lysis promotion (Bai et al., 2022). These functions assist host defence against pathogens, eliminating immune complexes, and triggering adaptive immune response (Li & Zhang, 2022). In teleosts, including in sea bass, the complement factors have been described as pivotal in the response against bacterial pathogens (Du et al., 2019; Li & Hu, 2016; Mauri et al., 2011; Wang et al., 2017; Wu et al., 2022). Antimicrobial peptides (AMPs) are small peptides (18-46 amino acids) that present a broad-spectrum antimicrobial activity towards bacteria, viruses, parasites, and fungi (Katzenback, 2015). In response to pathogens, AMPs can regulate the activity of immune cells, and stimulate the secretion of inflammatory cytokines via the activation of NF-κB and MAPK signalling pathways (Lyu et al., 2023). The

AMPs identified in fish can be divided into five families according to their structure: β-defensins, cathelicidins, hepcidins, histone-derived peptides, and piscidins (exclusive from fish) (Katzenback, 2015). AMPs have activity against a wide range of Gram-positive and Gram-negative bacteria through pore-forming and disruption of cell membranes (Shabir et al., 2018). The antibacterial activity of AMPs was already proven in fish (Bae et al., 2016; Hirono et al., 2007; Ke et al., 2015; Ruangsri et al., 2012), namely in sea bass (Meloni et al., 2015; Neves et al., 2011).

1.6.1.3.3. C-reactive protein (CRP) and other acute-phase proteins

Acute phase proteins (e.g., C-reactive protein - CRP -, serum amyloid A - SAA -, α2-macroglobulin, pentraxins, ferritin, etc.) are mainly synthesized by hepatocytes in the liver in response to tissue damage (Ehlting et al., 2021). The acute phase proteins are involved in host defence, including in the inactivation of proteolytic enzymes, destruction of pathogens or modification of cell targets, and wound healing (Roy et al., 2016). CRP is a well-known acute-phase protein that plays a role in systemic inflammation and pathogen clearance. In Nile tilapia (*Oreochromis niloticus*), CRP participated in antibacterial immune response (i.e., agglutination and regulation of phagocytosis and inflammation) triggered by i.p. challenge with *S. agalactiae* and *A. hydrophila* (Li et al., 2022). In what concerns SAA, it has been shown that it can modulate inflammation and serve as a chemoattractant for neutrophils and macrophages in fish bacterial infections (e.g., *A. hydrophila*) (Castellano et al., 2020; Kovacevic & Belosevic, 2015). Additionally, the acute-phase protein α2-macroglobulin was upregulated in rainbow trout upon i.p. challenge with *A. salmonicida* (Causey et al., 2018). This protein was described as a potentially important infection-resistant biomarker in farmed brown-marbled grouper (*Epinephelus fuscoguttatus*), readily detectable in the plasma of resistant individuals (Ibrahim et al., 2022). Equally important roles in resistance against bacterial infections were described for pentraxins (e.g., enhancer of antibacterial activity and phagocytosis) (Hou et al., 2023; Qiu et al., 2023) and ferritin (e.g., regulation of iron metabolism) (Ding et al., 2017; Xiong et al., 2022).

1.6.2. Adaptive immune response

If the innate immune response is not enough to clear the infection, the adaptive immune response is activated by non-specific immunity (e.g., complement system), resulting in the activation of mechanisms that provide a specific response and long-lasting protection against the pathogen (Kordon et al., 2021b). The adaptive immune response comprises specialized cells (T and B lymphocytes) and proteins that eliminate pathogenic microorganisms or limit their growth (Stosik et al., 2021). This response mainly depends on the highly diverse antigen-specific receptors expressed on T and B cells (Kordon et al.,

2021b). Another key characteristic of adaptive immunity is immunological memory, a specific reaction developed when the host comes in contact with an antigen for the first time (Zinkernagel, 2018) that leads to a stronger and faster response upon a second encounter with the same antigen. Similarly, to what is described for mammals, the adaptive immune response in teleosts encompasses both cellular and humoral responses. The cellular component consists of T cells, which can differentiate into effector cells, like cytotoxic T (CTLs) cells or helper T (Th) cells (Kordon et al., 2021b; Nakanishi et al., 2015). The humoral component is constituted by B cells, which express B-cell receptors (BCR) (antibodies in membrane-bound form) and can differentiate into plasma cells that produce immunoglobulins (antibodies in soluble form) (Smith et al., 2019). T and B cells orchestrate a coordinated immune response through the involvement of cytokines, transcription factors, and antibodies that allow the host to develop long-lasting and pathogen-specific immunity.

1.6.2.1. Antigen processing and presentation

Antigen processing and presentation are essential to adaptive immune response. At the core of this process are the variable genes encoding the major histocompatibility complex (MHC) (Monos & Winchester, 2019). The peptides presented to MHC proteins result from the processing of proteins that are produced intracellularly (endogenous antigens) or extracellularly (exogenous antigens) (Johnstone & Chaves-Pozo, 2022). In mammals, it was demonstrated that MHC class I specifically interacts with the TCR of $CD8^+$ T lymphocytes, while MHC class II interacts with the TCR of $CD4^+$ T lymphocytes (Wu et al., 2021). The antigen processing and presenting process was demonstrated in teleost fish in studies using channel catfish, which indicated that MHC-like molecules regulated antigen processing and presentation similarly to mammals (Vallejo et al., 1992). Evidence showed that exogenous antigens were endocytosed by APCs with induction of lymphocyte proliferation (Vallejo et al., 1991). The sophisticated antigen-presenting system also allows the recognition and elimination of pathogens and host cells infected by pathogens through autophagy (Johnstone & Chaves-Pozo, 2022). During self-degradation (lysosomal degradation of cytoplasmic material), antigens can be processed and presented by the MHC to immune effector cells, activating T lymphocytes (Johnstone & Chaves-Pozo, 2022; Münz, 2021). This process has also been investigated in various teleost fish organs and cells during infection by viruses or bacteria (Li et al., 2019; Lü et al., 2012; Muñoz-Sánchez et al., 2020).

1.6.2.2. Cell-mediated response in adaptive immunity

T cells are fundamental in cell-mediated adaptive immunity responses through regulating leukocyte functions or directly killing infected or abnormal host cells (Kordon et al., 2021b).

T cells are developed in the thymus, and when mature, they are also present in other lymphoid tissues of teleost fish, including kidney, spleen, and mucosa-associated lymphoid tissues of the gills, skin, and intestine (Nakanishi et al., 2015). All T cells express T cell receptors (TCRs) that allow recognition of the peptides presented by MHC (Nakanishi et al., 2015). Vertebrates and teleosts can express two types of TCRs: TCRα and TCRβc expressed in αβ-T cells and TCRγ and TCRδ which are expressed in the surface of γδ-T cells (Scapigliati et al., 2018). While αβ-T cells are more abundant in the circulatory system and lymphoid tissues, it is hypothesized that γδ-T cells mainly reside in the mucosal tissues (Luo et al., 2019). Depending on their function, αβ-T cells can be classified as helper Th cells (expressing CD4 molecules that interact with MHCII) or CTLs (that express CD8 molecules that interact with MHCI) (Cao et al., 2023). Many studies have demonstrated the importance of T cells. For example, $CD4^+$ Th cells can stimulate macrophages and B cells to produce antibodies and enhance cell-mediated immunity, resulting in increased microbicidal activity in teleost (Robertsen, 2006). Moreover, $CD4^+$ and $CD8\alpha^+$ cells also effectively kill bacteria (i.e., *Lactococcus garvieae* and *Edwardsiella tarda*) in ginbuna carp cells (Nayak & Nakanishi, 2013). An increased expression of MHC class II and CD4 genes was correlated with an enhanced adaptive immune response against the *Edwardsiella ictaluri* live attenuated vaccine in channel catfish lymphoid organs (Kordon et al., 2019; Kordon et al., 2021a).

1.6.2.3. Humoral response in adaptive immunity

B cells, another important component of cell-mediated adaptive immunity, mature in response to antigenic stimulation (Cao et al., 2023). These can later proliferate and differentiate into plasma cells (that can secrete Igs) and partially transform into memory B cells that remain dormant to respond to recurrent pathogen invasion (Cao et al., 2023). Furthermore, B cells can also phagocyte pathogens and exhibit bactericidal capacities in teleost (Wu et al., 2020). Three major subsets of B cells were identified in teleost fish: IgM^+ B cells, IgM^-/IgD^+ B cells, and IgM^-/IgT^+ B cells (that secrete the three Ig isotopes, IgM, IgD, and IgT) (Cao et al., 2023; Y. Yu et al., 2020; Zhang et al., 2010). Antibodies (soluble or membrane-bound forms) can induce neutralization, internalization, and elimination of pathogens, or antibody-dependent cellular cytotoxicity (Kordon et al., 2021b). These can also activate a complement cascade and phagocytosis of complement-coated pathogens through effector cells (Mashoof & Criscitiello, 2016). In the case of IgM^+/IgD^+ B cells, they secrete IgM in response to bacterial, viral, and parasitic infections (Abós et al., 2015; Wu et al., 2024; X.-T. Zhang et al., 2021). These also display innate immune functions like phagocytosis and microbicidal activities (Wu et al., 2024). The immune role of IgM^-/IgD^+ B cells remains to be fully elucidated. The subset of IgM^-/IgT^+ B cells seems to be a mucosa-

associated B lymphocyte, specialized in mucosal immunity (Zhang et al., 2010), being also essential against bacteria (Dong et al., 2020) and viruses (Díaz-Rosales et al., 2019).

1.7. Mucosal immune response in fish

The mucosal tissues of the fish gills, skin, and intestine are closely associated with the external environment and play a crucial role in protection against pathogens. The mucosal-associated lymphoid tissue (MALT) is equipped with cells and molecules from both innate and adaptive immune systems able to coordinate a broad-range immune response and generate immune tolerance to allow beneficial symbiont colonization and homeostasis maintenance (Fig. 4) (Gomez et al., 2013; Liang et al., 2022). In teleost, the best-described MALTs are the gill-associated lymphoid tissue (GIALT), the skin-associated lymphoid tissue (SALT), and the gut-associated lymphoid tissue (GALT) (Fig. 4) (Salinas, 2015).

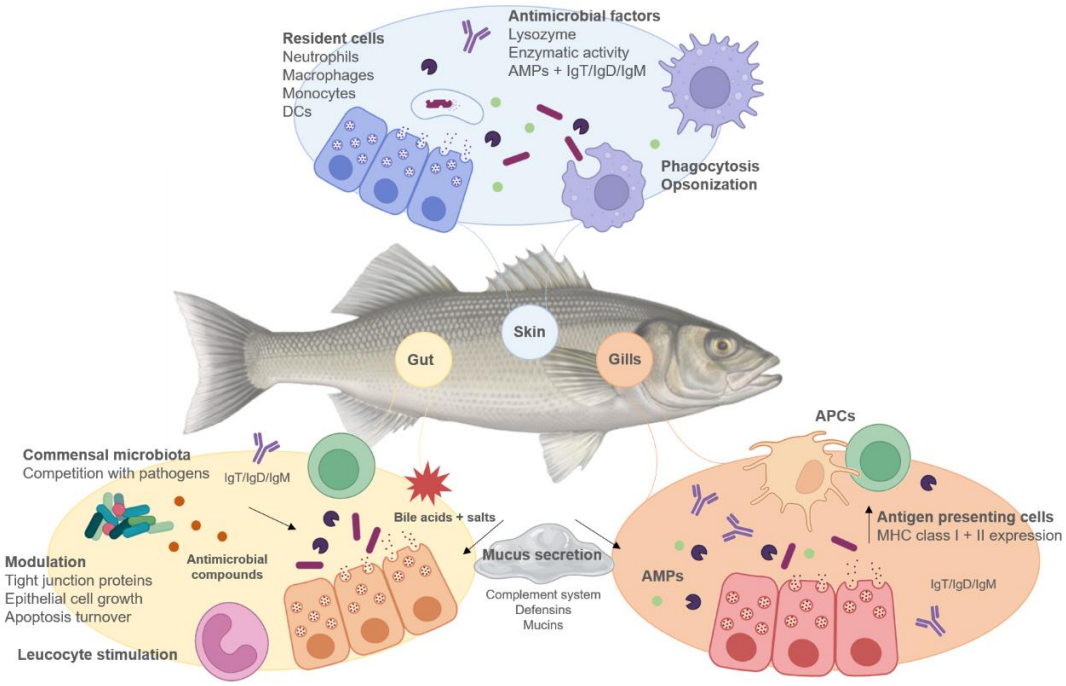

Figure 4: Summary of some of the innate immune mechanisms present in fish mucosal tissues (gills, skin, and gut) (adapted from Firmino et al., 2021). The mucosal-associated lymphoid tissue (MALT) presents several common characteristics between its different compartments (gills, skin, and gut-associated lymphoid tissues), such as mucus production (a protective layer of mucus covers the epithelial layer, containing antimicrobial peptides - AMPs -, several enzymes - e.g., lysozyme and complement system, among others - and immunoglobulins - Igs -, which help neutralize pathogens) and resident immune cells that offer constant surveillance (including neutrophils, macrophages, lymphocytes, and dendritic cells, responsible for identifying and responding to invading pathogens). In these compartments, macrophages and dendritic cells can act as antigen-presenting cells (APCs), capturing antigens while presenting them to T cells and B cells to initiate an immune response. In MALT, the role of secretory IgT is highlighted, being especially important against parasitic infections

in the gut. The MALT also comprises commensal microbiota that helps maintain homeostasis of the mucosal environment by targeting harmful bacteria. In addition, microbiota is responsible for the modulation of the mucosal environment (e.g., strengthening of tight junctions in the gut).

1.7.1. Gill-associated lymphoid tissue (GIALT)

The gills are covered in a mucus layer (a common characteristic among mucosal surfaces) that presents similarities with the epidermal mucus, containing several biologically active molecules such as lysozymes, AMPs, and immunoglobulins (Chen et al., 2023). The challenge of Atlantic salmon with *Yersinia ruckeri* leads to a significant increase in lysozyme concentration in gill mucus, suggesting that this response might be essential to protect the fish against yersiniosis (Costa et al., 2011). Moreover, 52 related to inflammation, such as C-reactive protein, apolipoprotein 1, granulin, cathepsin, and angiogenin-1 was found to be differentially expressed in the gill mucus of Atlantic salmon infected with pathogenic protozoan parasite *Neoparamoeba perurans* (Valdenegro-Vega et al., 2014), highlighting the importance of such a protective matrix in mucosal immunity. The teleost gills are also diffusively populated by immune cells, such as B and T-cells, NK-like cells, monocytes, macrophages, dendritic-like cells, neutrophils, eosinophilic granule cells, rodlet cells, thrombocytes, and melanin-containing cells (Salinas et al., 2021). All these cells act orchestrated and can dominate acute and chronic infections in the gills (Buchmann, 2022). There are indications that neutrophils released chemokines/cytokines, AMPs, complement factors, and cathelicidins to attract other immune cells to the gills following bacterial infection (Buchmann, 2022). In agreement with this, in the gills of rainbow trout exposed to *V. anguillarum*, there was an increased expression of IL-8, lysozyme, complement factors, and cathelicidin1 and 2 (Karami et al., 2020). The role of the gills in antibacterial defence has been studied in other fish species, including Atlantic salmon, rainbow trout, and Japanese flounder (Marcos-López & Rodger, 2020; Tongsri et al., 2020; Rebl et al., 2014). In fish infected with *Flavobacterium columnare* (which belongs to the Phylum Bacteroidetes and is phylogenetically close to *T. maritimum*), the secretory IgT was the predominant bacteria-specific Ig in gill mucosa, while negligible or no IgM or IgD-specific titres were detected in the gill mucus (Tongsri et al., 2020). In this same study, a 2 to 4-fold increase in IgT and IgM transcripts was detected at 2 days post-infection (dpi), and their maximum expression (45 to 50-fold increase) was observed in survival fish at 75 dpi (Tongsri et al., 2020). The accumulation of IgT^+ B cells in the gill lamellae of infected fish (28 dpi) and survivor fish (75 dpi) suggests that IgT and IgT^+ B cells play a relevant role in the adaptive immune responses against bacteria in fish gill mucosa (Tongsri et al., 2020). Other studies focused more on the transcriptional modulation in the gills upon bacterial challenge. For example, the expression of IgT was increased in the gills (and other examined tissue like skin, spleen,

head kidney, liver, hindgut, stomach, and muscle) in Japanese flounder following *E. tarda* challenge (Du et al., 2016). Nevertheless, the induction levels were influenced by the route of infection, with immersion challenge leading to higher up-regulation of IgT transcripts in gill, skin, hindgut, and stomach when compared to i.p. injection (Du et al., 2016). Likewise, ayu fish (*Plecoglossus altivelis*) displayed increased levels of IgT and IgM transcripts in gills 10 and 20 dpi after *V. anguillarum* bacterin immunization, whereas IgD transcript levels remained unaffected (Kato et al., 2015). However, in other fish species, Rohu (*Labeo rohita*), IgD transcripts were increased in the gills at 24 h after i.p. injection of *A. hydrophila* (Basu et al., 2016).

In European sea bass, there is still a lack of knowledge regarding mucus humoral parameters and immune-related genes associated with immune responses modulated by pathogens in gills' mucosa. A study focused on the molecular characterization and antibacterial activity of a g-type lysozyme showed that the basal expression of lysozyme transcripts was highest in gills compared to the head-kidney and peripheral blood leukocytes (Buonocore et al., 2014). This may suggest a connection of this molecule to immune responses in this mucosal tissue. Also, it was observed a dominance of the IgT^+ B cell population in European sea bass gills (Picchietti et al., 2017). Furthermore, IgT transcripts increased in gills after infection with nodavirus, highlighting the importance of IgT in antiviral responses. Quite recently, a chimeric IgT/IgD gene was found to be expressed in sea bass, and increased transcription levels were observed in gills (Buonocore et al., 2020).

1.7.2. Skin-associated lymphoid tissue (SALT)

In general, skin from adult teleost fish presents a cuticle or mucus layer that includes the microbiota layer forming bacteria, the epidermis (a non-keratinized squamous stratified epithelium with goblet cells) and the dermis (consisting of two layers, the hypodermis - site of development of infectious processes - and the stratum compactum) (Esteban, 2012; Parra et al., 2015).

As the gills, the skin relies on a mucus layer, secreted by epidermal cells, mostly goblet and club cells (Esteban, 2012; Magnadóttir, 2010) for protection. The secretions of different types of mucus cells create a reactive and synergetic cocktail that gives rise to mucus itself, with protective and regulatory capacities. Mucus is mainly constituted by highly glycosylated glycoproteins named mucins, which grant its adhesive, viscoelastic, and rheological properties (Esteban & Cerezuela, 2015). Skin mucus is constantly being produced, allowing it to be sloughed off frequently, which helps avoid the adherence and invasion of host cells by the entrapped pathogens (Arasu et al., 2013). Nevertheless, fish skin reacts uniquely to different pathogen adhesion and colonization strategies. Since epidermal mucus is a

dynamic matrix, fish can shift its amount, viscosity, or chemical composition to adapt the immune response to the pathogen.

Skin mucus composition comprises essential biologically active compounds, such as high-molecular-weight glycoproteins, lysozyme, alkaline phosphatase, Igs, complement proteins, lectins, agglutinin, vitellogenin, proteolytic enzymes, and other types of proteases (e.g., trypsin), metalloproteases and cathepsin, and AMPs (Abolfathi et al., 2020; Dash et al., 2018; Lallès, 2019; Salinas et al., 2022; Vibhute et al., 2022) that promote actions as opsonization, phagocytosis, inflammatory response and direct bactericidal effects of mucus (Santoso et al., 2020). Besides these, epidermal mucus also contains crinotoxins (Dash et al., 2018; Reverter et al., 2018), cytokines, hemolysin, acute phase and heat-shock proteins, and superoxide dismutase (Brinchmann, 2016). The amount and composition of epidermal fish mucus change in response to an infection, where the activity of some enzymes (e.g., proteases) can increase. Upon recognition of a pathogen, protease secretion is triggered, acting directly on the pathogen (by proteolysis) or indirectly by modification of mucus consistency/sloughing (Esteban & Cerezuela, 2015). Additionally, proteases can activate and enhance the production of other innate immune components, such as complement, Igs, or AMPs (Fernández-Montero et al., 2021). Several AMPs (e.g., hepcidin, defensins-like peptides, piscidin, cathelicidins, and apolipoproteins) found in skin mucus (Díaz-Puertas et al., 2023; Mori et al., 2021), often display selective properties against bacteria and other pathogens, operating in chemotaxis, immunomodulation, iron metabolism, and wound repair (Esteban, 2012; Masso-Silva & Diamond, 2014; Rajanbabu & Chen, 2011). Cathelicidin and hepcidin exhibited a significant upregulation in the skin of fish challenged with *E. tarda* (Chen et al., 2019). Lysozyme exhibits lytic activity against Gram-positive and Gram-negative bacteria, triggering the complement system and phagocytic processes (Saurabh & Sahoo, 2008). When challenged with *Streptococcus parauberis*, starry flounder (*Platichthys stellate*) exhibited higher lysozyme activity in the skin mucus as early as 4 h post-challenge, suggesting that lysozyme partakes in acute phase responses against bacterial infection (Kim & Nam, 2015). Another component, lectins, can interact with pathogenic surface structures, blocking pathogen attachment and resulting in amplified phagocytosis (opsonization) by resident cells (Esteban & Cerezuela, 2015). Several types of lectins were linked to essential functions against the pathogenesis of bacteria (Beck et al., 2012; Tasumi et al., 2004; Tsutsui et al., 2011).

Along with the mucus layer, teleost skin contains resident cells, including plasma cells, macrophages, granulocytes, and Langerhans-like cells (Xu et al., 2013). It also contains MCs, which are in close association with other cells and actively participate in immune defence (Sfacteria et al., 2015) through the release of cytokines and other functional proteins (Esteban, 2024). Skin also resorts to sentinel cells like resident macrophages and

granulocytes to ensure homeostatic and wound healing processes, elimination/clearance of invading pathogens and resolution of the inflammatory response (Speirs et al., 2024; Sveen et al., 2020).

In early studies, specific Igs were detected in fish skin mucus after vaccination with bacterins (i.e., *Flavobacterium psychrophilum*) and after challenge with the parasite *Ichthyophtirius multifiliis*, which supported the hypothesis that specific Igs could present an important role in skin immune response (Makesh et al., 2015; Xu et al., 2013). In the study developed by Makesh et al. (2015), the levels of IgM in the skin mucus of rainbow trout increased significantly 28 days after i.p. immunization with *F. psychrophilum* bacterins, together with a late expression of IgT and IgD in the skin. *I. multifiliis* infection in rainbow trout induced parasite-specific IgT in the skin mucus, but insignificant IgM responses; moreover, the parasite was overwhelmingly coated by specific IgT in the skin of infected fish, while negligible IgM coating was detected (Xu et al., 2013). Following infection, significant accumulations of IgT^+ B cells were detected, representing the major B-cell subset in the skin epidermis. At the same time, IgT was mainly present in a polymeric form in the skin mucus (Xu et al., 2013). A recent study found that after infection with *F. columnare*, the skin mucus of surviving rainbow trout had significant IgT^- but not IgM- or IgD-specific titres against the bacteria (X.-T. Zhang et al., 2021). This sustains the idea that IgT is the key Ig isotype induced in the skin during an immune response. Some studies have suggested the same important role of IgT in the skin mucosa (Gallardi et al., 2019; Velázquez et al., 2018; Zhi et al., 2020). Nevertheless, other studies revealed an increase of IgT transcripts in the spleen of Atlantic salmon after 15 dpi with sea lice, presenting higher values than IgM, whereas IgM transcripts were more up-regulated in the skin than IgT transcripts at 5 and 15 dpi (Tadiso et al., 2011). Therefore, further research is needed to unravel the role of IgT, IgD, and IgM in the skin mucosa.

1.7.3. Gut-associated lymphoid tissue (GALT)

The gastrointestinal tract is in direct contact with the environment, allowing the entry of several pathogens (Yu et al., 2020). It is constituted by a hollow muscular tube that links several alimentary organs, from the buccal cavity to the rectum (Picchietti et al., 2021b). The gastrointestinal tract is a multifunctional system that participates in digestion and nutrient absorption, water and electrolyte balance, hormone secretion, and establishment of immunity (Lee et al., 2021). Connected to this last-mentioned function is the ability to uptake and process antigens, already described in several previous studies (Amthauer et al., 2000; Fuglem et al., 2010; Løkka & Koppang, 2016). This underlies the importance of the gut in inducing tolerance, immune response, or infection and highlights its potential in the context of oral immunoprophylaxis in fish (Løkka & Koppang, 2016).

According to the literature, the gastrointestinal tract is generally separated into three segments: anterior (or proximal), mid, and posterior (or distal) intestine (Lee et al., 2021); nevertheless, this division and terminology can vary among fish species due to morphological slightly different traits. For example, in a recent study, the intestine of European sea bass was characterized thoroughly by analysing four segments (i.e., anterior, mid, posterior, and rectum) (Ferreira et al., 2023). The anterior segment displayed characteristics related to absorption (e.g., with the largest absorptive area, the longest villi, and the higher number of neutral goblet cells, whereas the posterior segment and rectum seemed to have an essential role in immunity due to the high count of acid GC and expression of immune-related genes (Ferreira et al., 2023).

Covering the fish's gastrointestinal tract is a mucus layer, with similar characteristics and functions to the one present in the gills and skin, that acts as a barrier, helps in osmoregulation and lubrication (Salinas & Parra, 2015), and participates in nutrient uptake and digestion (Bakke et al., 2010). Gut mucus is mainly constituted of glycoproteins (mucins) and other components such as lysozyme, proteases, complement factors, lectins, cytokines, AMPs (e.g., defensins and piscidins), and secretory immunoglobulins (sIgs) (Firmino et al., 2021; Salinas & Parra, 2015). Several of these molecules were already described in the gut of some fish species, like Asian sea bass, grass carp (*Ctenopharyngodon idella*), Atlantic cod, and European sea bass (Fu et al., 2013; Lokesh et al., 2012; Picchietti et al., 2017; Shen et al., 2012; Xia et al., 2013). Nevertheless, the studies focusing on the presence and levels of these immune factors are pretty limited due to the existence of proteases secreted by luminal bacteria (i.e., which may result in degradation and cleavage of these immune factors) (Salinas & Parra, 2015). Gut mucus also includes bile acids and salts, enzymes, bicarbonate, and surface-active phospholipid materials, creating a hostile environment for potential pathogens (Sayyaf Dezfuli et al., 2016).

The intestinal epithelium of fish consists of a monolayer of absorptive epithelial cells (also known as enterocytes), mucus-producing goblet cells, neuroendocrine and immune cells (e.g., neutrophils, macrophages, lymphocytes, NK-like, dendritic and rodlet cells) (Minghetti et al., 2017; Mokhtar et al., 2023; Soleto et al., 2019). The epithelial cells of the intestine can express PRRs for sensing components of the microbiota, which include lectins, NLRs, and TLRs (Lauriano et al., 2016; Liu et al., 2022). Studies have already validated the presence of many TLRs in the intestines of fish, with several demonstrating increased expression upon bacterial challenge (K. Chen et al., 2021; Liu et al., 2022; Liu et al., 2023; J. Yu et al., 2021).

The GALT is mainly composed of two major leukocyte populations: intraepithelial lymphocytes (IELs) (i.e., adaptive lymphoid cells that reside in the epithelial layer) and

lamina propria leukocytes (lymphocytes, macrophages, granulocytes, and dendritic-like cells) (Lee et al., 2021).

Once a pathogen adheres and translocates through intestinal epithelium, the damaged or infected cells initiate signalling cascades that culminate in the mucosal inflammatory response (Salinas & Parra, 2015). The gut resident macrophages respond with enhanced phagocytic activity and antigen uptake (Salinas & Parra, 2015). MCs and granulocytes, also present in the gut of teleost fish as resident populations, can increase their numbers, migrate, and degranulation, producing mediators of inflammation in response to parasites (Lee et al., 2021). In a scenario of infection with bacterial pathogens, the expression of pro-inflammatory cytokines (i.e., IL1β, IL8, TNF-α, IFN-γ, interleukin 22) is induced in the intestine (C. H. Li et al., 2020; Mar et al., 2023; Xin et al., 2020). The percentage of B cells in fish GALT is lower compared to the spleen or head kidney; nevertheless, these cells can migrate through the intestinal epithelium upon parasitic infection (Zhang et al., 2010) or vaccination (Ballesteros et al., 2013, 2014), increasing in numbers. Fish B cells can generally differentiate into antibody-secreting cells, producing specific Igs against pathogens (Parra et al., 2015; Picchietti et al., 2021b). Some studies have already reported gut B cells as important mediators in immune response against parasites, bacteria, or viruses in fish (Parra et al., 2016), not only because of their capacity to secrete antibodies but also for being able to phagocyte and kill bacteria (Li et al., 2006; Øverland et al., 2010; Zhang et al., 2010). IgM and IgT are involved in the immune response against bacterial pathogens in the gut, as shown by the upregulation of the expression of these Igs in rainbow trout subjected to immersion challenge with *Y. ruckeri* (Evenhuis & Cleveland, 2012). On the other hand, in fish infected i.p. with *F. psychrophilum*, IgM but not IgT expression was modulated (Parra et al., 2016). Intriguingly, these contrasting responses can be a product of the route of infection since the immersion challenge can trigger a mucosal response leading to IgT upregulation (Parra et al., 2016). In the sea bass intestine, there are two major subsets of B cells, IgM^+ B and IgT^+ B cells (Picchietti et al., 2017). Moreover, the sea bass GALT is dominated by IgT^+ B cells (~34%), outnumbering IgM+ B cells (~2–10%) (Buonocore et al., 2017; Picchietti et al., 2017; Picchietti et al., 2021b). These also corroborate other mucosa studies highlighting IgT^+ B cells as an essential B cell lineage specialized for mucosa immunity (Magadán-Mompó et al., 2011; Zhang et al., 2010).

The presence of T cells in the gut mucosa was initially suggested by Rombout et al. (1998), who used a monoclonal antibody against carp intestinal T cells (WCL38) to identify these in the intestinal epithelium and lamina propria. The existence of TCRγδ, TCRαβ, Th1, Th2, Th17, Treg, and Tc lymphocytes in fish (i.e., European sea bass, rainbow trout, Atlantic salmon, and common carp) is recognized (Tafalla et al., 2016). For rainbow trout, the total number of gut-associated T-IELs is equivalent to the total number of T cells in the remaining

lymphoid organs, emphasizing the importance of the intestine in the T cell responses (Tafalla et al., 2016). $CD8^+$ and $CD4^+$ cells (that belong to the T-IEL subpopulation of lymphocytes) displayed bactericidal activity against both intracellular and extracellular bacteria in ginbuna crucian carp (*Carassius auratus langsdorfii*) (Nayak & Nakanishi, 2013). Moreover, T cells interact with mucosal bacteria, inducing tolerance or immune responses to commensal microbiota to preserve mucosal homeostasis (Y. Y. Yu et al., 2021). In zebrafish, it has been described that intestinal T cells may modulate the composition of commensal bacteria by regulating Vibrio species diversity (Brugman et al., 2014). This process is probably regulated through the induction of inflammatory responses by T lymphocytes (e.g., increased secretion of IFNγ) (Brugman et al., 2014).

In European sea bass, the presence of T cells in the whole intestine was confirmed by detecting the expression of T-cell specific markers (e.g., CD4, CD8-α, CD3ε, rag-1, TCR-β, and TCR-γ) (Boschi et al., 2011; Picchietti et al., 2021a). The sea bass gut mucosa houses a large proportion of the body's T lymphocytes (Picchietti et al., 2021a). In agreement with this, high basal expression levels of the TCR gene were detected in the intestine (Boschi et al., 2011).

1.8. European sea bass as an experimental model

European sea bass is a marine finfish with a significant economic impact on the Mediterranean aquaculture industry and cultural importance in Europe. Thus, alongside gilthead sea bream, sea bass is one of the species that occupy the second rank in the European Union (EU) aquaculture sector in value terms, after the Atlantic salmon (Llorente et al., 2020). The sea bass production industry has demonstrated high growth in the last decades due to increased EU production and imports from third countries, such as Turkey (Llorente et al., 2020). Although this fish species is currently well adapted to a wide range of rearing conditions, including cages, ponds, and recirculation aquaculture systems (RAS), sea bass remains a sensitive species to stress factors and, consequently, to disease outbreaks (Rigos et al., 2021; Rosado et al., 2019; Q. Zhang et al., 2021). Several reports of aquaculture facilities affected by tenacibaculosis have shown how susceptible European sea bass is to *T. maritimum* (Bernardet et al., 1994; Pepin & Emery, 1993; Timur & Yardimci, 2015). Therefore, due to the commercial interest regarding this fish species and the devastating economic effects of tenacibaculosis in its rearing industry, it is imperative to gather more information regarding the pathogen-host interaction during tenacibaculosis. Moreover, there is a considerable lack of knowledge regarding the main key points for the management of this disease, such as transmission pathways, virulence mechanisms, and

natural reservoirs and vectors; thus, the successful prevention and control of tenacibaculosis is a distant reality.

Besides being an economically important species, the use of European sea bass as a model species offers several advantages. Important molecular and biochemical tools (i.e., available genome, full-length coding sequences for targeted immune-related genes, monoclonal antibodies, etc.) allow investigation of innate and acquired immune responses against a particular stimulus in this species (Miccoli et al., 2024). The amount of knowledge gathered about European sea bass validates it as a reference teleost model in the fish immunology field.

The absence of information concerning the efficacy of therapeutic practices in European sea bass - when compared with salmonids or terrestrial vertebrates - turns into few authorized and implemented therapeutics in most European countries (Rigos et al., 2021) Therefore, an emerging need to develop strategies and establish good practices is arising to offer better disease management of tenacibaculosis in the aquaculture industry context, namely in European sea bass rearing farms.

1.9. Thesis main objectives

The main objective of this work is to offer more insights into the virulence mechanisms of *T. maritimum* and the innate immune mechanisms triggered in European sea bass challenged with this pathogen. More specifically, it is intended to:

1. Identify possible virulence factors among *T. maritimum*'s ECPs;
2. Assess changes in systemic immune response in European sea bass challenged with *T. maritimum*;
3. Assess changes in innate immune parameters in mucosal organs of European sea bass challenged with *T. maritimum*;
4. Unravel the sea bass skin-localized innate immune response against *T. maritimum* through transcriptomic and proteomic analyses.

1.10. References

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CHAPTER 2

Tenacibaculum maritimum can boost inflammation in *Dicentrarchus labrax* upon peritoneal injection but cannot trigger tenacibaculosis disease

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Tenacibaculum maritimum can boost inflammation in *Dicentrarchus labrax* upon peritoneal injection but cannot trigger tenacibaculosis disease

Abstract

Despite being a bacterial pathogen with devastating consequences, *Tenacibaculum maritimum*'s pathogenesis is not fully understood. The aim of the present study was to elucidate if different inoculation routes (intraperitoneal - i.p - injection and bath challenge - known to induce mortality) can induce tenacibaculosis (i.e., using the same *T. maritimum* inoculum), as well as evaluate the short-term immune response of European sea bass (*D. labrax*). Additionally, the host response against i.p. injection of extracellular products (ECPs) was also studied. Fish were i.p. challenged with 5.5×10^5 CFU mL^{-1} of *T. maritimum* cells with or without ECPs (BECPs and BWO, respectively), ECPs alone or marine broth (mock). Another group of fish was bath-challenged with 5.5×10^5 CFU mL^{-1} to confirm the virulence of the bacterial inoculum. Undisturbed specimens were used as controls. The severity of both challenges was determined by following percentage survival. Blood, liver, and head-kidney samples were collected at 0, 3, 6, 24, and 48 h post-challenge for assessing immune parameters, oxidative stress, and gene expression. Total and differential peritoneal cell counts were performed. The presence of viable bacteria in the blood and peritoneal cavity was studied. Symptoms of tenacibaculosis, such as skin/fin abrasions, were only observed in the bath-challenged fish, where 0% survival was recorded, whereas 100% survival was observed after i.p. injection of the same bacterial inoculum. An increase in total leukocyte numbers in the peritoneal cavity was observed 3 h post-injection of BECPs when compared to the other treatments. Blood total leukocytes, lymphocytes, and thrombocyte numbers dropped after the challenge, mainly in fish challenged with BECPs. At 48 h post-challenge, bactericidal activity in the plasma increased in fish injected with bacteria (with and without ECPs). The same tendency was seen for some of the oxidative stress parameters. The increased expression of *il1β*, *il*6, *il8*, and *hamp1* in fish challenged with ECPs and BECPs suggests a more exacerbated pro-inflammatory response in the head-kidney against these inocula. The infection trial and the observed immune responses showed that the infection route is a determinant factor regarding *T. maritimum*-induced pathogenesis in European sea bass.

Keywords: Aquaculture; Tenacibaculosis; Infection route; Innate immunity; Gene expression

Highlights

- Intraperitoneal injection of *T. maritimum* plus ECPs induced a significant and fast increase in peritoneal cells' numbers, whereas no changes occurred after injection of ECPs;
- Intraperitoneal injection of *T. maritimum* plus ECPs or ECPs led to increased expression of the inflammatory mediators *il1β*, *il6*, *il8*, *tnfα* and *hamp1* in the head-kidney;
- The infection route is a determinant factor for *T. maritimum* pathogenesis development;

2.1. Introduction

Bacterial diseases are one of the significant constraints to the global aquaculture industry (Shefat, 2018). In aquaculture sites, the prevalence of bacteria is high (Aich et al., 2018), and disease monitoring can become rather complex due to the ability of opportunistic pathogens to asymptomatically colonize farmed species as an integral component of a "healthy" microbiome (Bayliss et al., 2017; de Bruijn et al., 2018).

Tenacibaculum maritimum (Family Flavobacteriaceae, Phylum Bacteroidetes) is pathogen that threatens the production of many economically important fish species, such as European seabass (*D. labrax*) (Moustafa et al., 2014), Gilthead seabream (*Sparus aurata*) (Moustafa et al., 2015), turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*) (Piñeiro-Vidal et al., 2007), and Atlantic salmon (*Salmo salar*) (Apablaza et al., 2017). It induces small lesions, upraised spots, scale loss, and some disintegration of the epidermis in the fish body surface, namely in the head, skin, or fins (Haridy et al., 2014; Lopez et al., 2022; Mabrok et al., 2023; Van Gelderen et al., 2011). The extensive skin lesions, together with the gill abrasions, offer a matchless chance for other opportunistic pathogens to enter the host, leading to secondary infections, some of which can culminate in systemic infections (Avendaño-Herrera et al., 2006b; Mabrok et al., 2023). Due to mortality rates and tenacibaculosis symptomatology, the global economic losses associated with this pathology are considerable (Mabrok et al., 2023; Nowlan et al., 2020).

Although several studies have been carried out to understand better *T. maritimum*'s pathogenicity, its transmission, route of infection and the dynamics established between the pathogen and host are not fully disclosed. Over the last few years, different experimental infection methods and inocula resulted in distinctive rates of mortality (Frisch et al., 2018; Mabrok et al., 2016, 2023; Nishioka et al., 2009; Powell et al., 2004). Among the different types of inocula, several studies have used *T. maritimum* extracellular products (ECPs) to

ascertain their potential immunogenic effects (Avendaño-Herrera et al., 2006b; Salati et al., 2005; Van Gelderen et al., 2009). The ECPs have been described as a virulence mechanisms used by *T. maritimum* to facilitate erosion, colonization and invasion of the host's tissues (Avendaño-Herrera et al., 2006b; Mabrok et al., 2023; Pérez-Pascual et al., 2017). Indeed, ECPs contain toxins with high proteolytic and cytotoxic activities *in vitro* and high toxicity *in vivo* (Mabrok, 2016; Avendaño-Herrera et al., 2006b; Van Gelderen et al., 2009). Escribano et al. (2023) performed *in vitro* trials using an epithelioma papulosum cyprini (EPC) cell line to define the cytotoxicity of different doses of ECPs extracts: total ECPs, OMVs, and soluble ECPs (at the same protein concentration, 0.5 mg ml^{-1}). All extracts displayed dose-dependent cytotoxic effects. However, the cytotoxicity effect was higher for total ECPs than for OMV or S-ECP fractions (Escribano et al., 2023). Moreover, qualitative effects in sole fingerlings subcutaneously injected with total ECPs, OMVs, or S-ECPs confirmed the higher toxicity of total ECPs, which induced ulcerative and hemorrhagic lesions between 12 and 24 h after the challenge (Escribano et al., 2023). Although these studies support the active role of ECPs in *T. maritimum* virulence, their effects on the host have not been fully explored.

The first experimental studies of tenacibaculosis were conducted in the 1990s, and they focused on commercial fish species and different infection methods. Initially, abrasion/scarification of fish skin, followed by smearing these lesions with pure broth culture, was used to experimentally induce tenacibaculosis (Bernardet et al., 1994). Later on, Powel et al. (2004) used the abrasion method to directly inoculate high concentrations of *T. maritimum* (4×10^{11} cells per fish) on the gills of Atlantic salmon smolts to induce and enhance necrotic branchitis of the gill epithelium successfully. Other methods, such as subcutaneous injection (Baxa et al., 1987), cohabitation (Frisch et al., 2018), and prolonged immersion (Avendaño-Herrera et al., 2006a; Mabrok et al., 2016) were also tested, leading to different degrees of mortality and symptomatology. More recently, Faílde et al. (2013) experimentally infected turbot using the subcutaneous and intraperitoneal (i.p.) routes, demonstrating that they are both able to cause bacteremia in fish. For the group subcutaneously challenged with *T. maritimum*, extensive areas of necrosis were observed in the muscles, with an inflammatory response in the inoculation site; degeneration of muscle fibers was also detected with scattered inflammatory cells in these necrotic areas (Faílde et al., 2013). For the i.p. challenged group, no lesions were observed in the skin or muscle throughout the study; however, the organs in the coelomic cavity exhibited inflammatory response and necrosis in the spleen, kidney, liver, and gastrointestinal tract (Faílde et al., 2013). Finally, Avendaño-Herrera et al. (2006a) reported a sole isolate that could not induce mortality in turbot fry challenged by both immersion and i.p. routes. This inability to consistently induce tenacibaculosis under experimental conditions underscores

the importance of disclosing the factors behind its pathogenesis, disease development, and host immune response. The aim of the present study was two-fold. On the one hand, it was intended to elucidate if different inoculation routes (intraperitoneal injection and bath challenge - known to induce mortality) can induce tenacibaculosis (i.e. using the same inoculum), whereas on the other hand, it also aimed to evaluate the short-term innate immune response of European sea bass when challenged through these two different challenge models. As a further step, the host response against i.p. injection of ECPs was also studied.

2.2. Material and Methods

2.2.1. Bacterial culture and inoculum preparation

This study used a *T. maritimum* strain (ACC13.1, serotype O3) that was previously isolated from Senegalese sole (*Solea senegalensis*) during a farm outbreak (Avendaño-Herrera et al., 2005). Preceding studies with this strain involving bath challenge as an infection model confirmed the pathogenicity of this isolate (Ferreira et al., 2023; Mabrok et al., 2016). The strain was supplied by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain). Recovery from the frozen stocks at −80 °C was done using marine agar (MA; Laboratories CONDA, Spain) at 25 °C for 48 h.

For preparing the inoculum, 50 mL of marine broth (MB; Laboratories CONDA, Spain) was inoculated with bacteria in a 500 mL Erlenmeyer, grown at 25 °C, with continuous shaking (180 rpm) for 48 h. Turbidity was measured at 600 nm (Spectrophotometer, UV-1600PC, VWR), and exponentially growing bacteria (OD = 0.613) were collected. For the bath challenge, bacteria were collected by centrifugation at 3,000 × *g* for 10 min, resuspended in MB, and adjusted to a 5.5×10^5 CFU mL^{-1}. For the i.p. challenge using whole cells without *T. maritimum*'s ECP, bacteria were centrifuged at 3,000 × *g* for 10 min, the obtained pellet was resuspended in MB and adjusted to a 5.5×10^6 CFU mL^{-1} (treatment designated by BWO). For the i.p. challenge using cells with *T. maritimum*'s plus ECP, bacteria were adjusted to 5.5×10^6 CFU mL^{-1} without any washing procedure (treatment designated by BECPs). The bacterial concentration was adjusted with a predetermined growth curve for this strain: $y = 2 \times 10^8 x + 4 \times 10^7$, where the x corresponds to turbidity at 600 nm (OD) and y to the bacterial concentration (CFU mL^{-1}).

2.2.2. Preparation of extracellular products

To obtain *T. maritimum*'s ECPs, bacteria were cultured as previously described until OD600 = 0.646 (exponential phase) and centrifuged at 4,000 × *g* for 30 min at 4 °C. Culture supernatant was filtered using a 0.2 µm pore-size Vacuum Filtration System (VWR, USA), concentrated approximately 20-fold using Amicon ultra-15 centrifugal filter units (10 KDa cut-off) (Merck Millipore, Germany) according to the manufacturer´s instructions, aliquoted and stored at -80 °C. The protein concentration concentrated ECPs was determined using the Pierce™ BCA Protein Assay kit (Thermo Fischer Scientific USA), with bovine serum albumin as standard. The protein profile of the ECPs was analysed by SDS-PAGE after trichloroacetic acid (TCA) precipitation. Shortly, proteins from 1 mL aliquots of concentrated ECPs were precipitated with 10% (w/v) TCA for 30 min on ice and recovered by centrifugation (19,800 × *g*, 15 min, 4ºC). The obtained pellets were washed with 10% (w/v) TCA, centrifuged, washed once more with acetone, allowed to dry, and solubilized in a gel loading buffer (50 mM Tris-HCl pH 8.8, 2% SDS, 0.05% bromophenol blue, 10% glycerol, 2 mM EDTA, and 100 mM DTT), at 95 ºC for 5 min. Samples were electrophoresed in a 14% polyacrylamide gel using the Laemmli discontinuous buffer system (Laemmli, 1970), followed by staining with Coomassie Brilliant Blue (0.2% Coomassie R-250, 50 % methanol, 1% acetic acid).

2.2.3. Fish farming and experimental design

The experiments were approved by the CIIMAR Animal Welfare Committee and DGAV (ORBEA-CIIMAR_26_2018) and were carried out under license number 0421/000/000/2020 in a registered facility (N16091.UDER). The current study was conducted under the supervision of researchers accredited in laboratory animal science by the Portuguese Veterinary Authority following FELASA category C recommendations and in agreement with the guidelines on the protection of animals used for scientific purposes according to the European Union directive (2010/63/EU).

European sea bass juveniles (35.6 ± 6.5 g) were obtained from a commercial fish farm (Valencia, Spain) with no record of previous tenacibaculosis outbreaks and were maintained in quarantine for 4 weeks at CIIMAR fish-holding facilities in a recirculated aerated seawater (salinity 32.0 ± 1.8 ‰) system, with 8.6 ± 0.1 mg mL^{-1} dissolved oxygen, and a 12 h light/12 h dark photoperiod. Mechanical and biological filtration was used to maintain the water's quality, and fish were given a commercial diet (Aquasoja, Portugal) consisting of 2% of their body weight divided into two meals per day. Ammonia and nitrite levels were measured daily using commercial kits and kept at 0.7 ± 0.2 and 2.2 ± 1.0 mg L^{-1}, respectively. The

water temperature was maintained at 20.4 ± 0.2 °C until the beginning of the bacterial challenge. At the challenge, the temperature was increased to 25 °C to simulate water temperature conditions at which tenacibaculosis outbreaks occur (Mabrok et al., 2023).
Before the bacterial challenge, fish were randomly distributed into closed recirculating seawater systems (7.4 kg m^{-3} stocking density), one for the mock-challenged fish and another for the challenged fish, each with three aquaria for sampling purposes (three replicates per treatment) and one aquarium per treatment to follow percentage survival. An additional system was used for the bath challenge using *T. maritimum* or MB (two replicates per treatment) to follow mortality after the bath challenge. After transfer to the experimental aquaria, fish were acclimated for another 4 weeks under the conditions specified above.
Fish were challenged through i.p. injection with 100 µL of MB containing 5.5 × 10^5 CFU *T. maritimum* with or without ECPs (BWO and WECPs, respectively) or with 100 µL of concentrated ECPs (150 µg of protein $fish^{-1}$). The mock-challenged fish were i.p. injected with 100 µL sterile MB. For the bath challenge, fish at a stocking density of 18 kg m^{-3} were immersed for 2 h with vigorous aeration in MB containing 5.5 × 10^5 CFU mL^{-1} of *T. maritimum*. Mortality was followed for 10 days, and dead or moribund animals were collected or euthanized (0.7 mL L^{-1} 2-phenoxyethanol (Merck, ref. 807291, Germany), and counted as dead.

2.2.4. Sampling

After euthanizing the fish with 0.7 mL L^{-1} 2-phenoxyethanol, post-mortem samples were taken (Merck, ref. 807291, Germany). Fish were sampled before starting the i.p. challenge (time 0, control) and at 3, 6, 24 and 48 h post-challenge. At each time point, four fish were sampled from each triplicated tank (n = 12 per treatment), and blood was aseptically collected from the caudal vein with heparinised sterile 1 mL syringes. A volume of 10 µL of the collected blood was plated in MA, followed by incubation for 72 h at 25 °C to detect the presence of viable *T. maritimum*. The remaining blood was transferred to heparinized 1.5 mL tubes, and one portion was used for haematological analysis, while the remaining blood was centrifuged for 10 min at 10,000 × *g* at 4 °C for collecting plasma that was stored at -80 °C. Fragments of head-kidney were collected and stored in RNA later (1/10, w/v) at 4 ºC for the first 24 h and then at -80 ºC for molecular biology analysis. Liver samples were also collected for oxidative stress analysis and were directly placed in liquid nitrogen and stored at -80 ºC.

2.2.5. Haematological parameters

According to Machado et al. (2015), the haematological profile was carried out. Total white (WBC) and red (RBC) blood cells were counted using a Neubauer chamber. Haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) were also evaluated, and the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated as previously described (Machado et al., 2015). Blood smears were done with 3 µL of lightly homogenised blood, air-dried, and fixed for 1 min in formol-ethanol (10% of 37% formaldehyde in absolute ethanol). The identification of neutrophils was performed using the peroxidase detection method outlined by Afonso et al. (1998). Blood smears were then stained using Wright's stain (Haemacolor; Merck). Slides were examined under oil immersion 100 × objective (final magnification of 1,000 ×), and 200 leucocytes were counted and categorized, based on their morphology and staining characteristics, as thrombocytes, lymphocytes, monocytes, and neutrophils. The total number of WBCs was multiplied by the percentage of each cell population to calculate the number of cells per µL of blood.

2.2.6. Collection of peritoneal exudates

The peritoneal cells were collected according to the procedure first described for mice by Silva et al. (1989) and posteriorly adapted for fish by Afonso et al. (1997). Briefly, after blood collection from the caudal vein, 2 mL of sterile HBSS supplemented with 30 units of heparin mL^{-1} was injected into the peritoneal cavity. Following that, the peritoneal region was gently massaged to spread the peritoneal cells in the injected HBSS, and the i.p. injected HBSS with the resuspended cells was then collected. A volume of 10 µL was plated in MA, followed by incubation for 72 h at 25 °C to evaluate the presence of viable *T. maritimum*. Total peritoneal cell counts were performed with a haemocytometer. The Cytospin preparations were performed using a THARMAC Cellspin device and were stained, as mentioned before, for blood smears. The peritoneal exudates were differentially counted and identified as lymphocytes, macrophages, and neutrophils. The percentage of each cell type was determined after counting a minimum of 200 cells per slide. The obtained counting values were then used to calculate the number of each leucocyte type per peritoneal cavity.

2.2.7. Bacterial DNA Extraction and PCR analysis

Bacterial colonies grown on MA plates inoculated with blood and peritoneal exudates were re-plated in MB and grown at 25 ºC for 48-72 h. DNA was extracted from the cultures using

NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal), following the manufacturer's instructions, and maintained at –20 °C until use. DNA extracted from a pure culture of the *T. maritimum* strain ACC13.1 and sterile distilled water were used as positive and negative controls, respectively. Then, a PCR was performed according to Avendaño-Herrera et al. (2004) using the species-specific primer set MAR1 (5'-AATGGCATCGTTTTAAA-3') and MAR2 (5'-CGCTCTCTGTTGCCAGA-3') (Toyama et al., 1996) designed against 16S ribosomal gene. The PCR amplification was performed with the NZYTaq II Green Master Mix (NZYTech, Lisbon, Portugal). The PCR reaction was done in a Veriti DX 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The samples were denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 2 min, 45 °C for 90 s, and 72 °C for 2 min. Afterwards, the samples were maintained at 4 °C. The PCR products were analysed by 2% agarose gel electrophoresis for 50 min at 100 V in TAE Buffer, pH 8 (NZYTech, Lisbon, Portugal) using NZYDNA Ladder I (NZYTech, Lisbon, Portugal) as a molecular size marker. DNA bands were visualized with GreenSafe Premium (0.03 µL mL^{-1}) (NZYTech, Lisbon, Portugal) and images were obtained with Gel Doc XR+ Image Lab Software (BioRad).

2.2.8. Proteomic analysis

The ECPs sample used for the i.p. challenge was processed for proteomic analysis following the solid-phase-enhanced sample-preparation (SP3) protocol and enzymatically digested with Trypsin/LysC as previously described (Osório et al., 2021). Protein identification and quantitation were performed by nanoLC-MS/MS equipped with a Field Asymmetric Ion Mobility Spectrometry - FAIMS interface. This equipment is composed of a Vanquish Neo liquid chromatography system coupled to an Eclipse Tribrid Quadrupole, Orbitrap, Ion Trap mass spectrometer (Thermo Scientific, San Jose, CA). Briefly, 250 ng of peptides of each sample were loaded onto a trapping cartridge (PepMap Neo C18, 300 µm × 5 mm i.d., 174500, Thermo Scientific, Bremen, Germany). Next, the trap column was switched in-line to a µPAC Neo 50 cm column (COL-nano050NeoB) coupled to an EASY-Spray nano flow emitter with 10 µm i.d. (ES993, Thermo Scientific, Bremen, Germany). A 130 min separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient at a flow of 750 µL min-1: 0.1 min (1% B to 4% B) and 1.9 min (4% B to 7% B). Next, the flow was reduced to 250 µ L min^{-1} with the following gradient: 0.1 min (7.0 to 7.1% B), 80 min (7.1% B to 22.5% B), 30 min (22.5% B to 40% B), 8 min (40% B to 99% B) and 9.9 min at 99% B. Subsequently, the column was equilibrated with 1% B. Data acquisition was controlled by Xcalibur 4.6 and Tune 4.0.4091 software (Thermo Scientific, Bremen, Germany).

MS results were obtained following a Data Dependent Acquisition - DDA procedure. MS acquisition was performed with the Orbitrap detector at 120 000 resolution in positive mode, quadrupole isolation, scan range (m/z) 375-1500, RF Lens 30%, standard AGC target, maximum injection time was set to auto, 1 microscan, data type profile and without source fragmentation. FAIMS mode: standard resolution, total carrier gas flow: static 4 L min-1, FAIMS CV: -45, -60 and -75 (cycle time, 1 s). Internal Mass calibration: Run-Start Easy-IC. Filters: MIPS, monoisotopic peak determination: peptide, charge state: 2-7, dynamic exclusion 30s, intensity threshold, 5.0e3. MS/MS data acquisition parameters: quadrupole isolation window 1.8 (m/z), activation type: HCD (30% CE), detector: ion trap, IT scan rate: rapid, mass range: normal, scan range mode: auto, normalized AGC target 100%, maximum injection time: 35 ms, data type centroid.

The raw data was processed using the Proteome Discoverer 3.0.1.27 software (Thermo Scientific) and searched against the UniProt database for the *T. maritimum* NCIMB2154 Proteome (2022_03 with 2,844 entries). A common protein contaminant list from MaxQuant was also included in the analysis. The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.5 Da for fragment ions. The maximum allowed missing cleavage sites was set to two. Cysteine carbamidomethylation was defined as constant modification. Methionine oxidation, deamidation of glutamine and asparagine, peptide terminus glutamine to pyroglutamate, and protein N-terminus acetylation, Met-loss, and Met-loss+acetyl were defined as variable modifications. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; target FDR (strict) was set to 0.01, and target FDR (relaxed) was set to 0.05, validation based on *q*-value. Protein label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performed at the consensus step with the following parameters: unique plus razor peptides were considered, precursor abundance based on intensity, and normalization based on total peptide amount.

Raw data hits from the single ECPs sample were filtered using coverage above 30%, unique peptides above 3 and a SEQUEST HT score greater than 100; the obtained hits were automatically assigned the corresponding GO terms using the UniProt tool ID Mapping (https://www.uniprot.org/id-mapping, accessed 8th Feb 2024).

2.2.9. Innate immune parameters

2.2.9.1. Antiprotease and protease activities

The antiprotease activity was calculated using Ellis's (1990) methodology, modified for 96-well microplates. Briefly, 10 µL of plasma was incubated with 10 µL of trypsin solution (5

mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3) (Sigma, USA) for 10 min at 22 °C in microtubes. Following the initial incubation, 125 µL of azocasein (20 mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3) and 100 µL of phosphate buffer (115 mM NaH_2PO_4, pH 7.0) were added. This step was followed by another one-hour incubation at 22 °C in the dark with agitation. Next, 250 µL of 10% cold trichloroacetic acid (TCA) was added to the mixture, incubated for 30 min at 22 °C, and centrifuged at 10,000 × *g* for 5 min at room temperature (RT). Lastly, 100 µL was transferred to a 96-well plate containing 100 µL of 1 N NaOH per well, in duplicate, and the absorbance read at 450 nm in a Synergy HT microplate reader. The absorbance obtained with phosphate buffer, instead of plasma, was used as a reference, and the percentage of trypsin activity was calculated as follows: 100 − ((sample absorbance/reference absorbance) × 100).

To determine protease activity, the same protocol was applied, without the initial incubation with trypsin and the incubation with azocasein and phosphate buffer was done for 24 h instead of 1 h, in constant agitation. Plasma was replaced by trypsin (5 mg mL^{-1}) as a positive control or by phosphate buffer as a negative control. The percentage of trypsin activity compared to the positive control was determined according to (sample absorbance/positive control absorbance) × 100.

2.2.9.2. Peroxidase

Plasma peroxidase activity was assessed using the technique described by Quade & Roth (1997). In triplicates, 5 µL of plasma was diluted in 145 µL of HBSS without Ca^{+2} and Mg^{+2} (Cytiva, USA) in flat-bottom 96-well plates. Next, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma, USA) was added to each well. The reaction was stopped after 2 min by adding 50 µL of 2 M sulphuric acid, and the absorbance was measured at 450 nm (Synergy HT microplate reader). Peroxidase activity (units mL-1 plasma) was calculated by defining one unit of peroxidase as the amount needed to produce an absorbance change of 1 at 450 nm.

2.2.9.3. Lysozyme activity

Lysozyme activity was assessed using a turbidimetric assay mentioned by Costas et al., (2011). Initially, a suspension of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} in 0.05 M sodium phosphate buffer, pH 6.2) was prepared. In triplicates, 15 µL of plasma was added to a microplate and 250 µL of the previous suspension was pipetted to give a final volume of 265 µL. The reaction was carried out at 25 °C, and the absorbance (450 nm) was measured after 0.5 and 5 min in a Synergy HT microplate reader. A standard curve was created using lyophilized hen egg white lysozyme (Sigma, USA) serially diluted in sodium phosphate

buffer (0.05 M, pH 6.2). This standard curve was then used to calculate the amount of lysozyme in each sample.

2.2.9.4. Bactericidal activity

Bacteria (*T. maritimum* ACC13.1) were grown on MA at 25 °C for 48 h and resuspended in MB at a concentration of 1.6 × 108 CFU mL^{-1} by determining the turbidity at 600 nm (Synergy HT microplate reader) and using the previously mentioned growth curve: $y = 2 \times 10^8x + 4 \times 10^7$. The bactericidal activity of plasma was subsequently assessed using a method similar to the one outlined by Graham and Secombes (1988) but with certain adjustments, as described by Machado et al. (2015). In a U-shaped 96-well plate, 20 µL of plasma was added in duplicates, and as a negative control, MB was added to the wells instead of plasma. To each well, 20 µL of bacteria was added to the plate and incubated for 2.5 h at 25 °C. Then, 25 µL of 3-(4, 5 dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg mL-1; Sigma) was added to the wells, and the plate was incubated again for 10 min at 25 °C. Plates were centrifuged at 2,000 × *g* for 10 min, and formazan precipitate was dissolved with 200 µL of dimethyl sulfoxide (Sigma, USA) and quantified by measuring the absorbance at 560 nm (Synergy HT microplate reader). In this method, the difference between the formazan formed in the samples and the negative control (100% viability) enables calculating both viable bacteria and the percentage of non-viable bacteria in each sample.

2.2.9.5. Nitrite concentration

Compounds such as nitrite and nitrate, which are endogenously produced as oxidative metabolites of the messenger molecule NO, are considered indicative of NO production (Saeij et al., 2003). Thus, to indirectly access the nitric oxide (NO) concentration in plasma, a Nitrite/Nitrate colorimetric kit (Roche, 11746081001, Germany) was utilized, according to the manufacturer's instructions. The samples were diluted 1:10 in distilled H_2O, and the concentrations were expressed as µM.

2.2.10. Oxidative stress biomarkers

Liver tissue was homogenized 1/10 (w/v) in potassium phosphate buffer (0.2 M, pH 7.4). For lipid peroxidation (LPO) assessment, 200 µL of the homogenized mixture was transferred to a microtube containing 4 µL of 4% BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol. For the assessment of superoxide dismutase and catalase activities, each volume of tissue homogenate was added to a volume of potassium phosphate buffer (0.2 M, pH 7.4), and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were

collected and maintained at −80 °C. Protein concentration was determined using Pierce™ BCA Protein Assay kit (Thermo Fischer Scientific USA), with bovine serum albumin as standard, according to the manufacturer's guidelines. For superoxide dismutase and catalase activities, the homogenized liver was diluted to reach a final protein concentration of 0.3 mg mL^{-1}.

LPO was calculated using the procedure outlined by Bird & Draper (1984) with some modifications (Peixoto et al., 2021). Therefore, 100 µL of 100% TCA was added to 204 µL of liver homogenate with 4% BHT together with 1 mL of 0.73% thiobarbituric acid solution (in 60 mM Tris–HCl, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)). Samples were centrifuged at 15,000 × *g* for 5 minutes after being incubated for 1 hour at 100 °C in a kiln. Afterward, 200 µL of supernatant was transferred to a 96-well plate in triplicates, and the absorbance was measured at 535 nm. The LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) generated per g of wet tissue.

Catalase activity was assessed by measuring the decrease in absorbance through the consumption of H_2O_2, as defined by Claiborne (1985), but by adapting the technique to microplates, as mentioned by Rodrigues et al. (2017). A sample of 10 µL was put in triplicates onto a UV light microplate along with 150 µL of 30% H_2O_2 and 140 µL of 50 mM potassium phosphate buffer (pH 7.0). The absorbance was measured at 240 nm for 2 min. The catalase activity was quantified using the H_2O_2 molar extinction coefficient at 240 nm of 40 M cm^{-1}, expressed in U per mg of protein.

Using cytochrome C method with xanthine/xanthine oxidase, superoxide dismutase (SOD) activity was quantified in accordance with the methodology described by Almeida et al. (2010). In triplicates, a volume of 50 µL of each sample was transferred to a microplate. Then, 200 µL of a reaction solution, which contained 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM Na-EDTA, 0.7 mM xanthine, and 0.03 mM cytochrome C, was added. Immediately after, 50 µL of 0.03 U mL^{-1} xanthine oxidase with 0.1 mM Na-EDTA was also put onto the microplate. The absorbance was measured at 550 nm (Synergy HT microplate reader) at 20 s intervals for 3 min. Activity is described as units of SOD per mg of protein. One unit of activity was defined as the quantity of enzyme necessary to produce a 50% inhibition of the cytochrome C reduction rate.

The reduced (GSH): oxidized (GSSG) glutathione ratio was quantified using the microplate assay for the GSH/GSSG commercial kit (Oxford Biomedical Research, UK), as previously outlined by Hamre et al. (2014). This method depends on the quantitative determination at 412 nm of the total amount of glutathione (GSH + GSSG) and GSSG (Tietze, 1969). In short, the determination of GSSG is achieved by adding a thiol scavenger (N-ethylmaleimide pyridine derivative solution, Oxford Biomedical Research, UK), that reacts with GSH to form a stable complex, removing the GSH before the quantification of GSSG,

without inhibiting glutathione reductase (GR) activity. Adding glutathione reductase, the available GSSG is reduced to GSH, reacting with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), which allows the quantification of pre-existent GSSG. The reaction rate is proportional to the GSH and GSSG concentration. The GSH/GSSG Ratio is calculated as follows: (GSHt – 2GSSG)/GSSG.

2.2.11. Gene expression analysis

Head-kidney tissue (n = 9 per treatment) was weighted (up to 20 mg of tissue), placed in 200 µL of chilled homogenization buffer and homogenized in Precellys Evolution homogenizer at 6,000 × *g* (2 × 20 s, 4 ºC) using the reagents provided by the Maxwell® RSC simplyRNA Tissue Kit (Promega, USA). After adding 200 µL of lysis buffer to the samples, all total RNA isolations were performed by Maxwell® RSC (Cat. # AS4500).

RNA samples were quantified, and purity was evaluated by spectrophotometry using DeNovix DS-11 FX (Wilmington, DE, USA) with absorbance ratios at 260 nm/280 nm of 2.1–2.2. First-strand cDNA was synthesized, and samples were standardized with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal), which was stored at -80 °C. The Veriti DX 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was utilized for reverse transcription. Real-time Quantitative PCR was performed with CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA) using 4.4 µL of diluted cDNA mixed with 5 µL of iTaq Universal SYBR Green Supermix® (Biorad, Hercules, CA, USA) and 0.3 µL (10 µM) of each primer, resulting in a final volume of 10 µL. Primers were designed with NCBI Primer Blast Tool and IDT OligoAnalyzer ToolTM to amplify European sea bass genes of interest. The known qPCR requirements were taken into account. The template sequences used for the primer's design were obtained from both NCBI and the databases dicLab v1.0c sea bass genome (Kuhl et al., 2010). Using serial 2-fold dilutions of cDNA, the efficiency of each primer pair was assessed by calculating the slope of the regression line of the cycle thresholds (Ct) vs. the relative concentration of cDNA. The respective melting curves were analysed to ensure no amplification of primer dimers. The standard cycling conditions were initial denaturation at 95 ºC for 10 min, followed by 40 cycles of two steps (denaturation at 95 ºC for 15 s followed by primer annealing temperature for 1 min), 95 ºC for 1 min followed by 35 s at the annealing temperature, and 95 ºC for 15 s. The reactions were run in duplicates, and target gene expression was normalized using the geometric mean of elongation factor 1β (*ef1β*) and ribosome 40s subunit (*40s*), calculated according to the Pfaffl method (Pfaffl, 2001).

The accession numbers, primer efficiencies, annealing temperatures, amplicon length, and primer sequences are detailed in Table 1.

2.2.12. Statistical analysis

Data were analysed for normality and homogeneity of variance, and when necessary, outliers were removed. Gene expression data was Log-transformed before being statistically analysed, and peritoneal cells per cavity, differential cell counts, and the GSH/GSSG ratio were Box-Cox transformed. The Student's *t*-test was used to evaluate differences between the control (undisturbed) and each treatment (mock or ECPs) group for each time point. An analysis of variance (Two-way ANOVA) was applied, followed by an LSD test to evaluate statistically significant differences between time points and treatment (mock, BWO and BECPs groups) (interaction between factor time and treatment); to determine differences in time points or treatments (mock, BWO and BECPs groups), an analysis of variance (One-way ANOVA) was applied (no interaction between factor time and treatment), followed by Tukey's *post hoc* test. The Student's *t*-test was also used to evaluate differences between control (undisturbed) and treatment (mock, BWO, and BECPs) groups for each time point.

The significance level was set at 0.05 for all statistical tests. All calculations and statistical analyses were performed under the SPSS 29 program for Windows. Results were presented as the mean ± standard error of the mean (SEM). All graphs were designed with the Graph Pad Prism 8.01 Software.

2.3. Results

2.3.1. Protein composition of ECPs

Analysis of the *T. maritimum*'s ECPs revealed a complex protein profile, with band sizes ranging from 20 to over 250 kDa (Fig. S1, Appendix I).

To identify the proteins, present in the *T. maritimum*'s ECPs, these were analysed by NanoLC-MS/MS. A total of 744 non-redundant proteins were identified in the concentrated ECPs, which would represent approximately 11.62% of the theoretical proteome of *T. maritimum* NCIMB 2154 (DOI:10.6084/m9.figshare.26014573). A list of the filtered hits (coverage above 30%, unique peptides superior to 3 and SEQUEST HT score greater than 100) is presented in Table S1 (Appendix I). For a better interpretation of the results, the hits were classified according to their associated Go Term using the UniProt ID mapping platform (https://www.uniprot.org/id-mapping, accessed on 8th Feb 2024). The obtained protein hits were related to important biological processes, such as proteolysis, cell adhesion and carbohydrate metabolic processes (Table S1, Appendix I). Some of the most abundant proteins were lipoproteins, with several others being predicted proteins secreted

by the T9SS, as described by (Pérez-Pascual et al., 2017), such as multimodular sialidase/sialate O-acetylesterase/sialidase (MARIT_2686) and probable M14 family carboxypeptidase (MARIT_2507), containing a C-terminal secretion signal (Table S1). Other proteins were related to iron acquisition strategies, like iron-regulated protein imelysin family lipoprotein (MARIT_1664) and heme binding lipoprotein HmuY-family (MARIT_2477), or related to mechanisms to face up oxidative stress scenarios, like superoxide dismutase (MARIT_3105), thioredoxin (MARIT_2619) and alkyl hydroperoxide reductase (MARIT_0947) (Table S1). Outer membrane and TonB-related proteins like OmpA family protein (MARIT_2995), TonB-dependent outer membrane receptor SusC/RagA family (MARIT_2376) and TonB-dependent receptor (MARIT_0214) were also identified (Table S1). Components of the gliding motility machinery described for Flavobacterium, by Gorasia et al. (2020), were also identified in *T. maritimum*'s ECPs, such as PorU (MARIT_0895), PorV (MARIT_0894), GldM (MARIT_0756), GldN (MARIT_0757) and SprD (MARIT_1320) (DOI:10.6084/m9.figshare.26014573). Additionally, other proteins related to T9SS were identified, including adhesin SprC (MARIT_1318), SprA (MARIT_2960), SprT (MARIT_0579) and SprF (MARIT_1793), (DOI:10.6084/m9.figshare.26014573).

2.3.2. Percentage survival

A 100% survival was observed in fish challenged i.p. with ECPs or with bacteria with or without ECPs (Fig. 1). However, a percentage survival of 0% was obtained at day 7 for the fish challenged by bath (n = 12 per treatment, X2 < 0.0001) (Fig. 1).

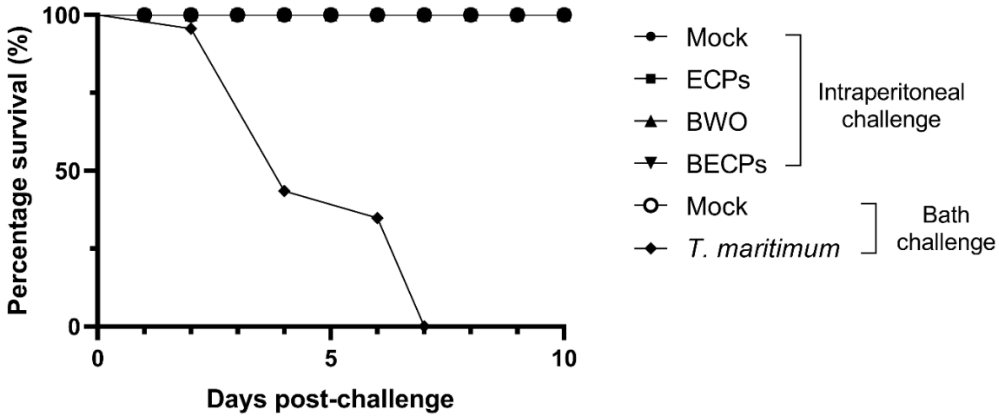

Figure 1: Percentage survival (%) after intraperitoneal injection of 100 µL MB (Mock), 100 µL *T. maritimum*'s ECPs (ECPs), 100 µL MB containing 5.5 x 10^5 CFU *T. maritimum* without ECPs (BWO) or 100 µL MB containing 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs) (n = 21 per group) or after bath challenge with MB (Mock) or 5.5 × 10^5 CFU mL^{-1} *T. maritimum* without ECPs (n = 12 per group).

Table 1: Immune-related genes analysed by Real-time PCR.

Gene	Acronym	Accession ID	Efficiency [a]	Annealing (°C)	Amplicon (bp)	Primer sequence (5'-3')
Elongation factor 1-beta	*ef1b*	AJ866727.1	107.6	60	144	F: AACTTCAACGCCCAGGTCAT R: CTTCTTGCCAGAACGACGGT
40s Ribosomal protein	*40s*	HE978789.1	109.7	60	79	F: TGATTGTGACAGACCCTCGTG R: CACAGAGCAATGGTGGGGAT
Interleukin 1 beta	*Il1β*	AJ269472.1	111.7	60	105	F: AGCGACATGGTGCGATTTCT R: CTCCTCTGCTGTGCTGATGT
Interleukin 6	*Il6*	AM490062.1	102.8	60	81	F: AGGCACAGAGAACACGTCAAA R: AAAAGGGTCAGGGCTGTCG
Interleukin 8	*Il8*	AM490063.1	106.3	60	140	F: CGCTGCATCCAAACAGAGAGCAAAC R: TCGGGGTCCAGGCAAACCTCTT
Interleukin 10	*Il10*	AM268529.1	100.9	55	164	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC
Interleukin 34	*Il34*	DLAgn_00164750	99.8	60	129	F: GGAAATACGCTTCAGGGATG R: GGCACTCTGTCGGGTTCTT
Caspase 1	*casp1*	DQ198377.1	105.8	62	190	F: GTGTTTCAGATGCGGGAGGA R: ATTTAAGTTAACTCACCGGGGG
Tumour necrosis factor-alpha	*tnfα*	DQ070246.1	101.6	60	112	F: AGCCACAGGATCTGGAGCTA R: GTCCGCTTCTGTAGCTGTCC
Matrix metallopeptidase 9	*mmp9*	FN908863.1	105.8	57	166	F: TGTGCCACCACAGACAACTT R: TTCCATCTCCACGTCCCTCA
Chemokine CXC receptor 4	*cxcr4*	FN687464.1	90.9	57	171	F: ACCAGACCTTGTGTTTGCCA R: ATGAAGCCCACCAGGATGTG
Macrophage migration inhibitory factor	*mif*	AY423555.2	97.6	62	88	F: GCTCCCTCCACAGTATTGGCAAGAT R: TTGAGCAGTCCACACAGGAGTTTAGAGT
Macrophage colony-stimulating factor 1 receptor	*mcsfr*	FN582353	104.4	55	76	F: ATGTCCCAACCAGACTTTGC R: GGCTCATCACACACTTCACC
Major histocompatibility complex II	*mhcII*	AM113468.1	108.9	55	81	F: ATCCCTCCATGTTGGTCTGC R: CTTCCTGTCCGTCTCTGAGC
Heat shock protein 70	*hsp70*	AY423555.2	104.9	55	88	F: ACAAAGCAGACCCAGACCTTCACCA R: TGGTCATAGCACGTTCGCCCTCA
Hepcidin	*hamp1*	KJ890396.1	103.1	60	148	F: ACACTCGTGCTCGCCTTTAT R: TGTGATTTGGCATCATCCACG
Ferroportin	*fpn*	KU599935.1	109.7	60	161	F: GCTAGAGTTGGCCTGTGGTC R: GGGTTCGGAGCCAGTATCAC

2.3.3. Re-isolation of *T. maritimum* from blood and peritoneal exudates

All peritoneal exudate samples collected from fish i.p. injected with BWO and BECPs at 3 and 6 h post-challenge presented bacterial growth in MA plates (Table 2). However, only 2/12 and 3/12 blood samples collected at 3 h post-challenge from fish injected with BWO and BECPs, respectively, were positive for bacterial growth (Table 2). At 6 h post-challenge, no bacterial growth was recorded for the blood from fish injected with BWO, while 11/12 samples from the BECPs group had bacterial growth (Table 2). After this sampling time point, no bacterial growth was seen in the peritoneal exudates or blood (Table 2). As expected, the blood and peritoneal exudates from undisturbed controls and mock-treated fish did not show bacterial growth (Table 2). Bacterial cultures recovered from inoculated fish showed Tenacibaculum-like characteristics, with pale/translucent colonies with uneven edges, flat and adherent between them and PCR analysis confirmed that they corresponded to *T. maritimum* (amplification of a single product with the expected size) (Fig. S2, Appendix I).

Table 2: Bacterial growth in aseptically collected peritoneal exudates (PE) and blood from undisturbed fish (Control) or from fish i.p. challenged with 100 µL MB (Mock), 100 µL *T. maritimum*'s ECPs (ECPs), 100 µL MB containing 5.5 × 10^5 CFU *T. maritimum* without ECPs (BWO) or 100 µL MB containing 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs) (n = 12 per treatment).

	0 h		**3 h**		**6 h**		**24 h**		**48 h**	
	PE	Blood	PE	Blood	PE	Blood	PE	Blood	PE	Blood
Control	0/12	0/12	-	-	-	-	-	-	-	-
Mock	**-**	-	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
ECPs	**-**	-	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
BWO	**-**	-	12/12	2/12	12/12	0/12	0/12	0/12	0/12	0/12
BECPs	**-**	-	12/12	3/12	12/12	11/12	1/12	0/12	0/12	0/12

2.3.4. Peritoneal cell numbers and haematological parameters

No significant differences in the numbers of peritoneal cells (neutrophils, macrophages and lymphocytes) were observed after i.p. injection of ECPs when compared with the mock (Fig. 2). In what concerns the systemic response, a decrease in the total number of WBC was observed both in fish challenged with ECPs or MB (mock) when compared to the undisturbed control group, but no significant differences between ECPs or mock groups were recorded (Table S2, Appendix I). Despite the lack of differences in total WBC, the number of circulating neutrophils was significantly higher at 3 and 6 h post-challenge in the ECPs' group, when compared to the mock, decreasing afterwards in the 48 h sampling point (Table S2). In contrast, no differences were recorded for monocytes, lymphocytes and

thrombocytes (Table S3, Appendix I). Also, injection of ECPs did not affect the total number of RBC (Table S2) and failed to induce significant differences in hemoglobin concentration, haematocrit and the hematological ratios of mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (Table S2).

In the experiments involving i.p. inoculation of bacteria, an increase in the numbers of peritoneal neutrophils, macrophages and lymphocytes was observed in the BECPs group, when compared to the mock. In contrast, no major changes in peritoneal cell numbers were recorded after injection of BWO, relative to the mock-challenged group (Fig. 3).

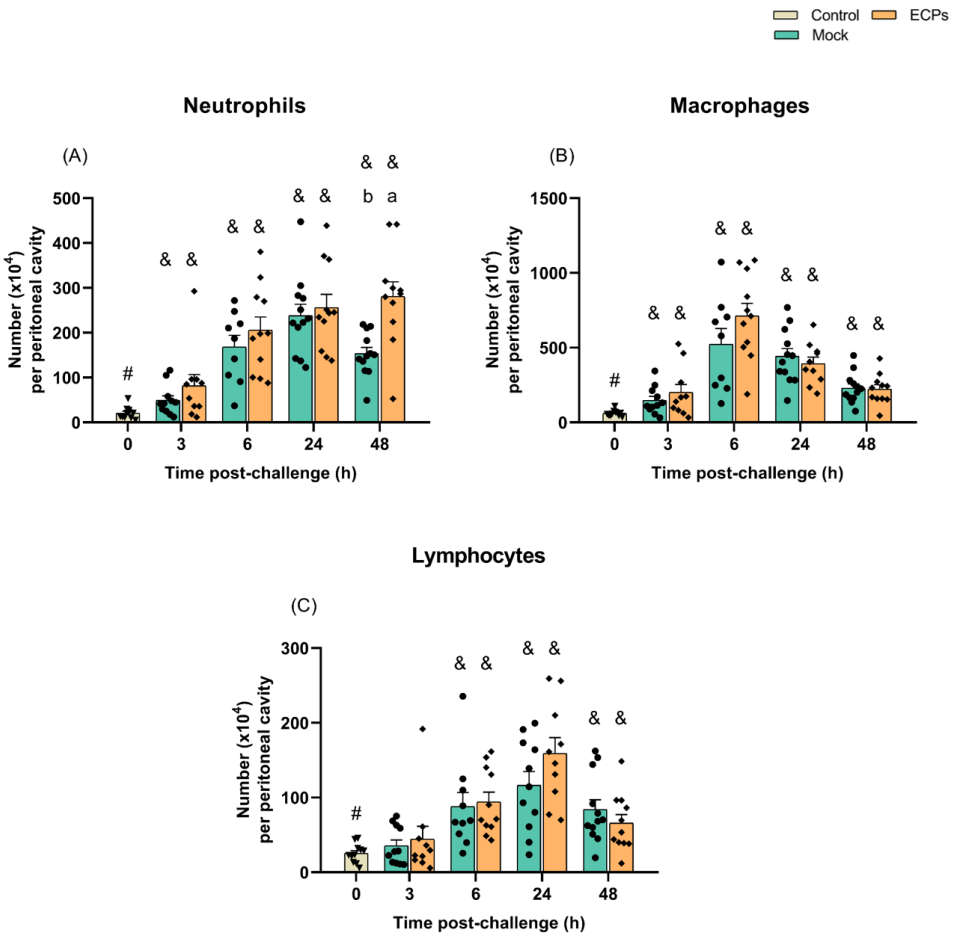

Figure 2: Numbers of neutrophils (A), macrophages (B) and lymphocytes (C) in the resting peritoneal cavity (Control – grey column) or in the peritoneal cavity of European sea bass i.p. challenged with 100 µL MB (Mock – blue columns) or 100 µL *T. maritimum*'s ECPs (ECPs – orange columns). Data are expressed as mean ± SEM (n = 12 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols (&) represent significant differences between the control group (undisturbed - #) and the remaining groups (Student's *t*-test; $p \leq 0.05$).

At the systemic level, an abrupt decrease in total WBC was observed in the BWO and BECPs treatments at 3 h post-challenge when compared with the mock-challenged and control groups (Table S4, Appendix I). Afterwards, the numbers of WBC in the BWO and BECPs groups remained low but similar to the numbers recorded in the mock-challenged fish (Table S4). The results of the differential cell counts revealed no significant differences between the mock and bacterial inoculated groups, for neutrophil and monocyte counts (Table S5, Appendix I). Changes in the numbers of circulating lymphocytes and thrombocytes showed an emphasised decrease at 3 h in the BECPs and BWO groups, relative to mock-treated and control animals (Table S5).

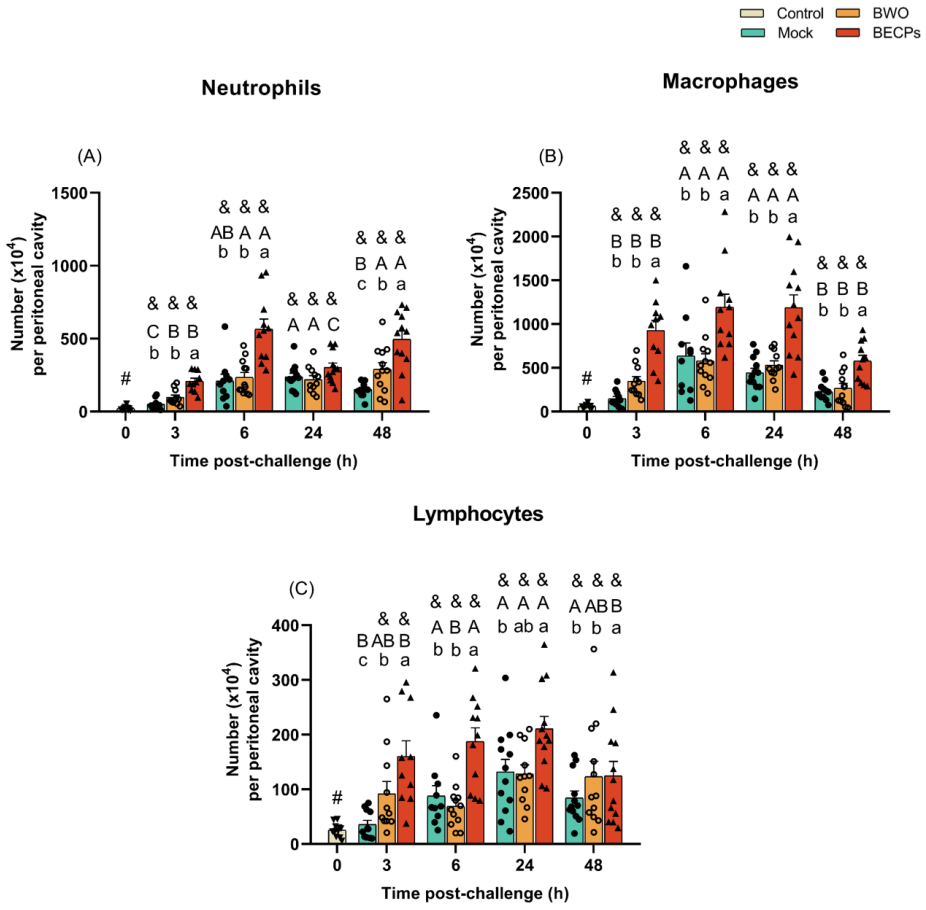

Figure 3: Numbers of neutrophils (A), macrophages (B) and lymphocytes (C) in the resting peritoneal cavity (Control – grey column) or in the peritoneal cavity of European sea bass i.p. challenged with 100 µL MB (Mock – blue columns), 100 µL MB containing 5.5 × 10^5 CFU *T. maritimum* without ECPs (BWO – orange columns) or 100 µL MB containing 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs – red columns). Data are expressed as mean ± SEM (n = 12 per treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, $p \leq 0.05$). Different

symbols (&) represent significant differences between the control group (undisturbed - #) and the different treatment groups (Student's *t*-test; $p \leq 0.05$).

Afterwards, the levels of lymphocytes and thrombocytes remained low in BECPs and BWO groups but were not significantly different to the ones in mock-treated fish (Table S5). A slight decrease in the RBCs counts was observed in the BECPs-treated group, when compared to the mock, with differences reaching significance at 3 and 6 h post-challenge, whereas no decrease was observed in the BWO group (Table S4). Injection of BECPs also led to a decrease in the haematocrit, when compared to the BWO or mock treatments at 48 h (Table S4). The remaining haematological ratios presented no major differences (Table S4).

2.3.5. Innate humoral parameters

Regarding the innate humoral parameters, no major differences were observed between the mock and ECPs treatments (Table S6, Appendix I). In what concerns the response to the injection of BWO or BECPs, an increase in the peroxidase activity was recorded at 48 h and a higher bactericidal activity was detected at 24 and 48 h, relative to the levels in mock-treated animals (Table S7, Appendix I). The other parameters analysed did not show major differences (Table S7).

2.3.6. Oxidative stress biomarkers

No changes in hepatic catalase activity were recorded in response to i.p. injection of ECPs when compared with mock treatment (Table S8, Appendix I). The same was observed for the BWO and BECPs treatments (Table S9, Appendix I). Superoxide dismutase activity in the liver significantly increased at 3 h post-challenge for the ECPs treatment, when compared to the mock and control groups, followed by a decrease at 6 h (Table S8). The same response pattern was also obtained for the BWO and BECPs treatments, which showed increased superoxide dismutase activity at 3 h post-challenge when compared to the mock challenge group (Table S9). No significant changes in lipid peroxidation were observed in fish i.p. injected with ECPs, when compared to mock-treated (Table S8). The BECPs treatment led to an increase of lipid peroxidation as quickly as 3 h post-challenge, with a prolonged effect, since at 24 and 48 h post-challenge the values continued significantly high when compared to the mock group (Table S9). Apart from an initial increase at 3 h post-challenge in the mock group in reduced: oxidized glutathione ratio when

compared with the remaining inoculated groups (ECPs and bacterial ones), no major differences were seen among the remaining analysed oxidative stress parameters.

2.3.7. Gene expression analysis

Although a peritoneal response was not seen for the fish i.p. injected with *T. maritimum*'s ECPs, the gene expression profile of this group pointed to a systemic inflammatory response, quite similar to the one observed for the fish i.p. injected with bacteria plus ECPs. A significant increase in interleukin 1 beta (*il1β*) expression was seen at 3 and 6 h post-challenge with ECPs compared to the control or mock groups (Fig. 4, A). At 3 h post-challenge, *il1β* expression in the ECPs-treated group was 80-fold higher than in mock challenge fish. After these sampling time points, the expression values of this inflammatory cytokine started to decrease, reaching values similar to the control group (Fig. 4, A). An identical response was observed for the fish i.p. injected with BECPs, recording an increase of *il1β* at 3 and 6 h, with a 305-fold increase at 3 h compared to the mock challenge group (Fig. 5, A). In this case, at 24 h post-challenge the immunogenic effect of BECPs treatment can still be seen, compared to the expression of BWO and mock treatments (Fig. 5, A). The interleukin 6 (*il6*), interleukin 8 (*il8*) and interleukin 10 (*il10*) responses in the course of this challenge were quite similar to the ones seen for *il1β*, for both ECPs and BECPs. For *il6* an exacerbated expression (an almost 38-fold increase regarding the mock group) was seen at 3 h post-challenge, followed by a decrease in the following time point (Fig. 4, B). The BWO and BECPs treatments also presented an increased expression at 3 and 6 h post-challenge for this cytokine when compared with the control and mock groups (Fig.5, B). As previously mentioned, *il8* showed an identical response to *il1β*, for the ECPs group, as well as for the BECPs (Fig. 4 and 5, C). The anti-inflammatory cytokine *il10* presented an increased expression at 3 and 6 h post-challenge, reaching identical expression values for both sampling points in the ECPs treatment, when compared to control and mock fish (Fig. 4, D). The ECPs group remained different from the mock group until 48 h (Fig. 4, D), indicating a slightly sustained immune response. A similar type of kinetics was seen for il10 for the bacteria injected groups, since at 3 h post-challenge no differences were recorded between BWO and BECPs treatments (Fig. 5, D), however, at 6 h post-challenge the BECPs group reached its maximum expression value and BWO group started to decrease (Fig. 5, D). Again until 48 h differences were recorded between the mock and BECPs groups (Fig. 5, D). Injection of ECPs induced the expression of tumour necrosis factor-alpha (*tnfα*) at 3 h post-challenge, with a 4-fold increase when compared to control and mock groups, followed by a decrease near basal levels afterwards (Fig. 4, E. The BECPs and BWO treatments also induced upregulation of tnfα expression at this time point, with an almost 3-

and 2.4-fold increase, respectively, regarding the mock and control groups (Fig. 5, E). Afterwards, the expression decreased in both groups, although slower in BWO, reaching basal levels at 24 and 48 h post-challenge (Fig.4 and 5, E).

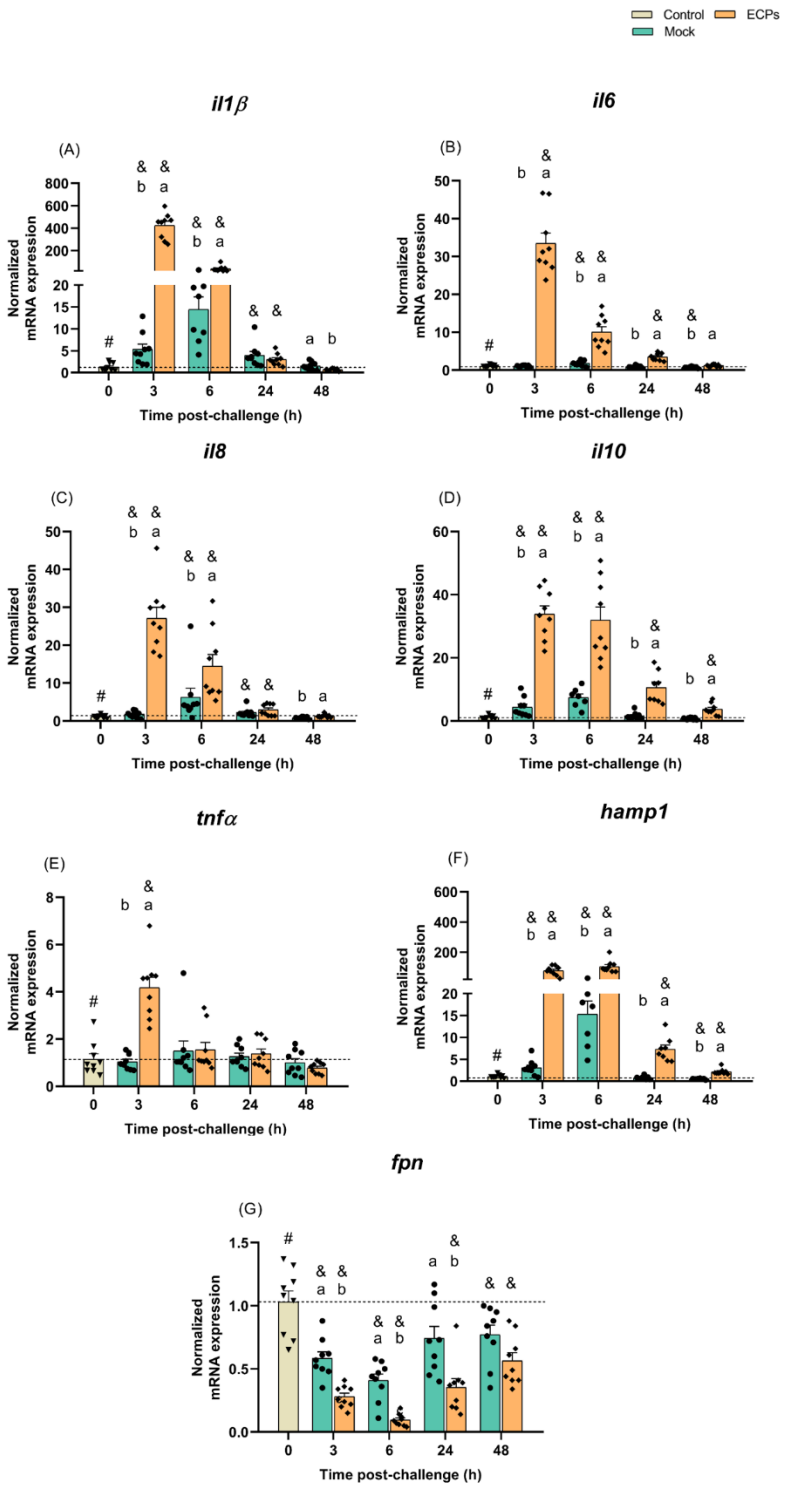

Figure 4: Quantitative expression of (A) *il1β*, (B) *il6*, (C) *il8*, (D) *il10*, (E) *tnfα*, (F) *hamp1* and (G) *fpn* (Control – grey column) in head-kidney of European sea bass i.p. challenged with MB (Mock – blue columns) or *T. maritimum*'s ECPs (ECPs – orange columns). Data are expressed as mean ± SEM

(n=9 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols (&) represent significant differences between the control group (undisturbed - #) and the remaining groups (Student's *t*-test; $p \leq 0.05$).

Usually, the expression of IL34 correlates with the expression of pro-inflammatory cytokines (e.g. IL1β and TNFα), but in this case, the values of expression of this cytokine in all i.p. injected groups remained low when compared to the control group. Initially, at 3 and 6 h post-challenge, mock and ECPs groups presented similar values. However, at 24 and 48 h, the expression of *il34* in the ECPs group was lower, when compared to the mock (Table S10, Appendix I). A similar response was observed after injection of BECPs, with decreased *il34* expression at 24 h and 48 h, when compared to the mock group (Table S11, Appendix I). The expression of the chemokine receptor *cxcr4* was also downregulated in the ECPs group at 3 and 6 h post-challenge when compared to control and mock (Table S10); the same kind of pattern was observed for BWO and BECPs groups at 3 and 6 h. However, *cxcr4* expression in the BWO group at 24 and 48 h was similar to the expression in mock and control groups, whereas the expression in the BECPs remained significantly low when compared to BWO, mock and control groups (Table S11).

When compared to control, the mock group showed increased *mmp9* expression at all times, peaking at 6 h post-challenge. In the ECPs group, *mmp9* expression was higher than in controls at 3, 6 and 24 h, but was lower than in mock group at all-time points, reaching a minimum at 48 h post-challenge (Table S10). Regarding the trial involving injection of bacteria, expression of *mmp9* was lower in the BECPs group, when compared to mock and BWO (Table S11).

Regarding the antimicrobial peptide hepcidin (*hamp1*), a significant increase in its expression was seen at all sampling time points for the ECPs group, when compared to mock or control groups (Fig. 4, F). This suggests that this iron withholding mechanism may have a preponderant role in the initial response against *T. maritimum*. The highest expression levels of *hamp1* were reached at 3 and 6 h post-challenge, with a 25 and 7-fold increase when compared to the mock group (Fig. 5, F). Moreover, significant upregulation was also seen at all sampling time points for the BECPs group when compared with the control and mock-challenged fish (Fig. 5, F). At 3 and 6 h post-challenge, the group i.p. injected with BECPs group showed approximately a 137- and 36-fold increase in *hamp1* expression, respectively, when compared to the mock group (Fig. 5, F). As expected, an opposite pattern was observed for the iron exporter ferroportin (*fpn*), since the ECPs group remained always with lower expression values than the control and mock-challenged groups (Fig. 4, G).

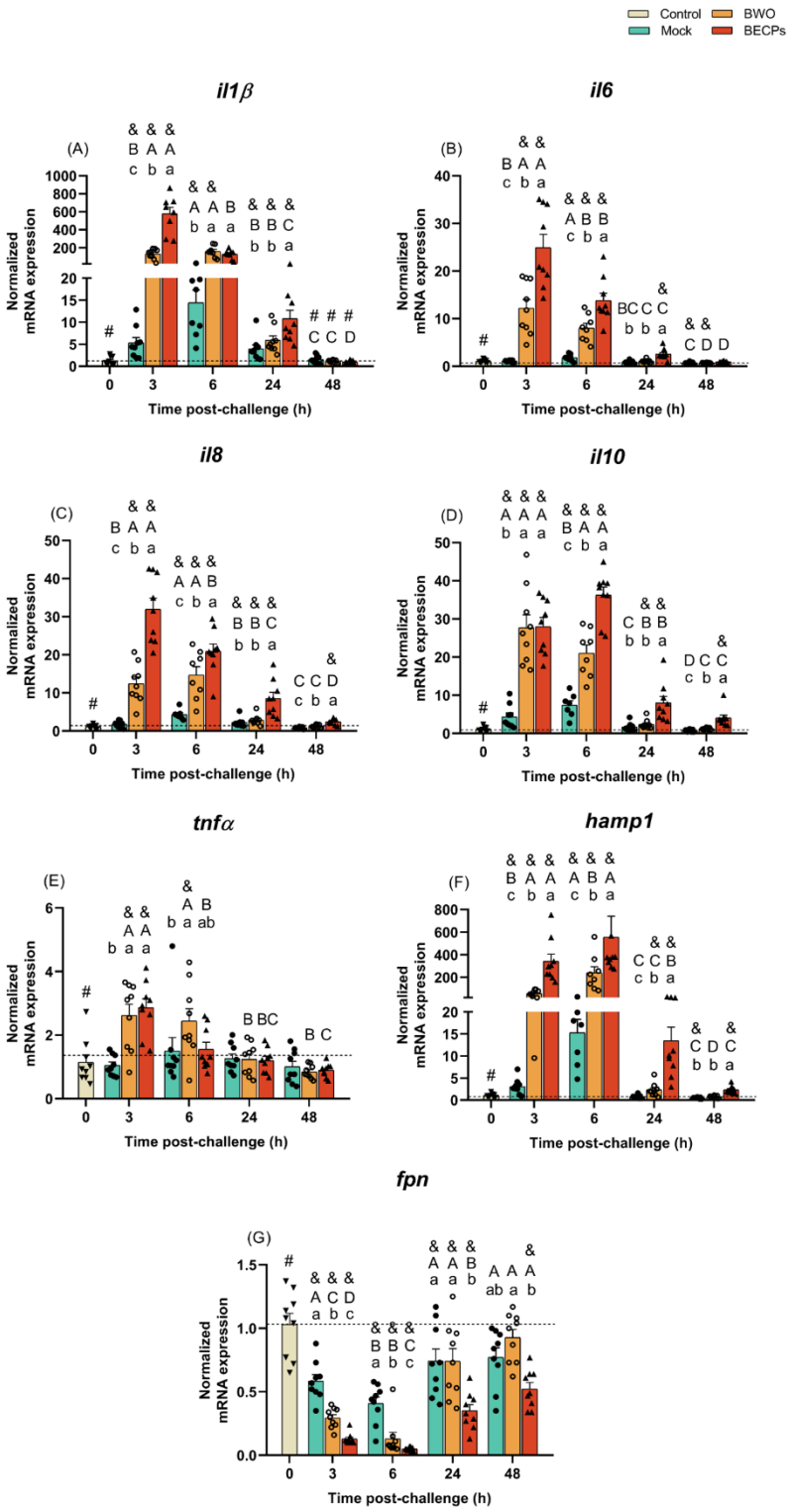

Figure 5: Quantitative expression of (A) *il1β*, (B) *il6*, (C) *il8*, (D) *il10*, (E) *tnfα*, (F) *hamp1* and (G) *fpn* (Control – grey column) for head-kidney of European sea bass i.p. challenged with MB (Mock – blue columns) or 5.5 × 10^5 CFU *T. maritimum* without ECPs (BWO – orange columns) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs – red columns). Data are expressed as mean ± SEM (n=9 per

treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, $p \leq 0.05$). Different symbols (&) represent significant differences between the control group (undisturbed - #) and the different treatment groups (Student's *t*-test; $p \leq 0.05$).

A similar trend was recorded for fish inoculated with bacteria. At 3 and 6 h, fpn expression in the BWO group was lower than in mock group, but afterwards (24 and 48 h) returned to levels similar to the mock group (Fig. 5, G). In contrast, expression of *fpn* in the BECPs group was downregulated, relative to mock, at all-time points analysed (Fig 5, G).
The expression of *mif*, *mcsfr* and *mhcII* did not show any major differences and no differences were recorded for *casp1* and *hsp70* expression (Table S10 and S11).

2.4. Discussion

One of the factors that can compromise new advancements in the characterization of the complex host-pathogen relationship operating in tenacibaculosis, which is essential for developing effective prevention measures against the disease is, undoubtedly, the establishment of a suitable infection model, able to mimic the natural infection. Despite the efforts made in the last decades to approach the specific traits and mechanisms of *T. maritimum* pathogenesis, no studies were performed to investigate the host's immune response against *T. maritimum* infection through different inoculation routes.
Many studies have explored several ways to develop challenge models for *T. maritimum* in different commercial fish species which included Atlantic salmon, rainbow trout and European sea bass, among others (Bernardet et al., 1994; Powell et al., 2004; Soltani et al., 1996). These studies allowed a better understanding of the clinical symptoms and/or mortality rates induced by the different inoculation routes for *T. maritimum*, but the host's immune response was not investigated. Moreover, despite the evidence pointing to an important virulence role of the *T. maritimum* ECPs (Avendaño-Herrera et al., 2006b; Escribano et al., 2023; Van Gelderen et al., 2009), there is a lack of studies addressing the immune response triggered by the ECPs in the host.
Since the '90s, several pathogenicity studies were developed with *T. maritimum*, some of which involved the i.p. route as an inoculation method. Studies involving different serotypes and doses reported that, regardless of the serotype or dose used, *T. maritimum* isolates were not able to induce disease when i.p. inoculated in turbot (Avendaño-Herrera et al., 2006a). In a more recent study, Faílde et al. (2013), demonstrated that the i.p. inoculation of 10^8 CFU $fish^{-1}$ led to septicaemia in turbot, but cutaneous lesions characteristic of natural

T. maritimum infections were not observed in the challenged fish. The toxicity of *T. maritimum*'s ECPs was also investigated by Van Gelderen et al. (2009) through i.p. administration of ECPs (1000, 500, 250, 125 and 62.5 µg protein $fish^{-1}$) in Atlantic salmon (average weight of 40 g), revealing a LD_{50} of 3.1 µg of protein g^{-1} of fish body weight. This study showed that i.p. injection of ECPs caused haemorrhages and ascites in the peritoneal cavity, and histological examination of organs collected from fish injected with 1 mg ECPs showed focal inflammation and necrosis in the liver (Van Gelderen et al., 2009).

In the present study, a 100% survival was recorded for all i.p. challenged fish, independently of the inocula used, although they displayed darkened skin during the first 24 h. These results are in agreement with the previously mentioned studies from Avendaño-Herrera et al. (2006a) and Faílde et al. (2013) (Avendaño-Herrera et al., 2006a; Faílde et al., 2013), where no mortality was recorded for turbot i.p. injected with *T. maritimum* or *T. maritimum* ECPs. The here reported findings suggest that bath infection is the best approach to induce tenacibaculosis in European sea bass since i.p. injection did not induce mortality or disease symptoms typically observed in fish suffering from natural *T. maritimum* infections.

Although i.p. inoculation of *T. maritimum* was not able to induce disease in European sea bass, viable bacteria were isolated at 3 h post-challenge from blood and peritoneal exudates of fish injected with BWO and BECPs, indicating that the bacteria were able to persist and reach the systemic circulation. At 24 h post-challenge, no bacterial growth was recorded in blood or peritoneal exudates, indicating that *T. maritimum* is cleared by the host between the 6 and 24 h post-challenge. This ability to clear *T. maritimum* can be related to the rapid and orchestrated response of the host's resident immune cells in the peritoneal cavity and of the immune cells that migrate to the peritoneal cavity after injection. In the peritoneal exudates, a more exacerbated response of neutrophils, macrophages, lymphocytes and thrombocytes was seen for the fish challenged with BECPs, especially at 6 h post-challenge, suggesting that bacteria and ECPs act synergistically and induce stronger chemotactic signals than bacteria alone. Moreover, the observed leukopenia at the beginning of the trial, associated with lymphopenia and thrombocytopenia, is consistent with acute inflammation, which is known to be triggered in fish by pathogens (including Gram-negative pathogenic bacteria) (Campbell, 2015; Clauss et al., 2008). Again, these effects appeared to be more pronounced in fish challenged with BECPs at 3 h post-challenge, suggesting the occurrence of a stronger immune stimulus and chemotactic effect triggered at the peritoneal cavity by that treatment. Also supporting the immune cells recruitment hypothesis is the enhanced expression of the pro-inflammatory biomarker, il8, known for its chemoattractant abilities of inflammatory cells and lymphocytes, which participate in the elimination of bacteria (Brennan & Zheng, 2007; Havixbeck & Barreda, 2015). Several studies in fish reported the chemotactic effect of recombinant IL-8 towards neutrophils,

macrophages, head-kidney leucocytes and peripheral blood lymphocytes (Wang et al., 2017; Wang et al., 2013; Wang et al., 2019; Zhao et al., 2022). The rapid clearance of i.p. injected *T. maritimum*, in contrast to the development of progressive disease after bath challenge, supports the possibility that the route of entry of this pathogen is crucial for its pathogenesis. *T. maritimum*'s adhesion and gliding motility capacities, iron uptake systems, type IX secretion system, as well as its ECPs production, have been suggested to be essential for the immune evasion of the host response, invasion, colonization and nutrient scavenging of these bacteria (Pérez-Pascual et al., 2017). However, *T. maritimum* was not able to proliferate and trigger the disease when inoculated by i.p. injection. It is likely that the fast-acting host response triggered after i.p. inoculation, with the recruitment of neutrophils, macrophages and other immune cells (Bruce et al., 2017; Shi et al., 2022), contributes to counteract the immune evasion ability of *T. maritimum*, and consequently to its rapid clearance. This can also explain the lack of significant responses in the evaluated immune and oxidative stress parameters. Nevertheless, the increase in the plasma bactericidal activity at the end of the trial in fish challenged with BWO and BECPs treatments denotes an attempt to prevent bacterial colonization, since the increase of bactericidal activity in fish was already associated with the detection of pathogens by the host's innate immune system (Biller-Takahashi et al., 2013; Mabrok et al., 2016). Furthermore, it may indicate that European sea bass plasma contains bactericidal compounds suitable to eliminate *T. maritimum*. The innate mechanisms against bacterial invasion include a plethora of broad-spectrum antibacterial compounds, which include acute phase proteins, cytokines, non-classical complement activation, phagocytosis and inflammation (Biller-Takahashi et al., 2013; Ellis, 2001). It is reasonable to speculate that in addition to the augmented bactericidal activity in plasma, the influx of phagocytic cells known to produce bactericidal compounds (do Vale et al., 2002; Machado et al., 2015) seen in the peritoneal cavity/infection site, may also have contributed to the elimination of *T. maritimum* after i.p. inoculation.

It is common for diseased fish to present a decrease in several haematological parameters, including RBCs, erythrocytes indices and haemoglobin when exposed to bacteria (Ahmed et al., 2020). In the present study, the only variations were recorded for the fish challenged with BECPs, with a tendency to decreased RBCs and haematocrit values from 24 h onwards. The lack of changes in the parameters related to humoral and cellular innate responses, as well as in the oxidative stress indicators, denotes a lack of systemic response to all treatments, which can indicate that regardless of the inoculum type, *T. maritimum* was quickly eliminated by the host's immune system. Despite the lack of studies approaching *T. maritimum*'s ECPs immunogenic capacity, Salati et al., (2005) used formalin-killed cells, crude lipopolysaccharides and ECPs preparations obtained from *T. maritimum* (strain

SPVId) as experimental vaccines against tenacibaculosis. After i.p. injection into European sea bass, all preparations, including the ECPs, triggered an immune response, inducing an increase in agglutinating antibody titter and *in vitro* phagocytosis by total blood leukocytes (Salati et al., 2005). In the present study, although displaying a damper chemotactic effect regarding the peritoneal cavity cells, the treatment with ECPs resulted in a pro-inflammatory response in the head-kidney as strong as the BECPs treatment. The profile and kinetics of the expression of pro-inflammatory cytokines revealed a marked up-regulation at short times (few hours) after i.p. inoculation of ECPs or *T. maritimum* with or without ECPs, which is congruent with the occurrence of an acute inflammatory process. Usually, acute inflammation is described to be enough to overcome an infectious challenge (Soliman & Barreda, 2023). In this process, the activated cells release pro-inflammatory cytokines, such as IL1β and tumour necrosis factor-alpha, and chemokines, like IL8 (Soliman & Barreda, 2023). As previously mentioned, this cocktail of cytokines ultimately culminates in the migration of neutrophils, macrophages and lymphocytes to the inflammation site, for infection clearance (Abdallah et al., 2017). In the present study, a fast increase in the expression of pro-inflammatory cytokine-related genes (*il1β*, *tnfα*, *il6*, *il8*) and of *hamp1* gene was detected after injection of BWO, BECPs and ECPs, with the strongest increase registered in the BECPs and ECPs treatments. This type of response is often triggered against bacterial pathogens (Reyes-Cerpa et al., 2012; Rodrigues et al., 2006; Shike et al., 2002). Moreover, interleukin 10 was also overexpressed in those same treatments with a slight delay regarding the expression of the other cytokines, which is consistent with its role in the control and resolution of inflammation (Forlenza et al., 2011). The downregulation of ferroportin (an iron exporter) seen in the concentrated ECPs and BECPs treatments throughout the trial is likely triggered by the increased hepcidin as a strategy to prevent iron from being accessible for bacterial growth and constrain bacterial invasion (Ward et al., 2011). Hepcidin can bind to ferroportin forming a complex that is internalized and degraded, allowing the iron to be retained in the erythrocytes (Nemeth et al., 2004; Ward et al., 2011). The comparison between BWO and BECPs treatments regarding the studied molecular markers suggests that fish challenged only with bacteria require more time to assemble an innate immune response, which is consistent with a slightly subtler immunogenic effect. *T. maritimum* is a proteolytic pathogen (Wakabayashi et al., 1986), relying on the secretion of ECPs, which include caseinases, gelatinases, amylases and hemolysins (Escribano et al., 2023; Van Gelderen et al., 2009), to successfully invade and colonize the host's tissues. The results of the present study suggest that this proteolytic cocktail of ECPs (also demonstrated by the ECPs identification and analysis in the present study), shapes the interaction of *T. maritimum* with its host, corroborating the role of ECPs as the main factors

in *T. maritimum*'s pathogenicity, even after inoculation by a route different from its natural route of entry.

Due to its widespread geographical distribution and ubiquitous host species, tenacibaculosis outbreaks have been rising in the last few years, with serious consequences for the aquaculture industry, namely the global salmonid aquaculture industry (Mabrok et al., 2023). Despite its current importance as a bacterial pathogen, there is still a lot to explore regarding the complex relationship between *T. maritimum* and its hosts. Although it is not a challenge model that mimics *T. maritimum*'s natural conditions to develop pathogenesis, the i.p. challenge provided a different insight regarding this pathogen's vulnerability when in contact with the fast and orchestrated host's innate immune response. The insipid host's systemic immune response supports the hypothesis of a triggered local acute inflammatory process, which rapidly controls *T. maritimum*'s invasion. The combination of bacteria and its ECPs triggered the most enhanced inflammatory response, although *T. maritimum*'s ECPs were also able to stimulate a similar response, as demonstrated by the pro-inflammatory molecular biomarkers. Undoubtedly, the route of entry of *T. maritimum* greatly influences the immune response triggered in the host and is a determinant factor for a successful host invasion and colonisation.

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CHAPTER 3

Early innate immune responses in European sea bass (*Dicentrarchus labrax* L.) following *Tenacibaculum maritimum* infection

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Early innate immune responses in European sea bass (*Dicentrarchus labrax* L.) following *Tenacibaculum maritimum* infection

Abstract

The marine aquaculture industry has been witnessing a worldwide emergence of tenacibaculosis, a poorly understood bacterial disease caused by *Tenacibaculum maritimum* that affects commercially important fish. So far, knowledge on the *T. maritimum* virulence mechanisms is scarce and the pathogen-host interaction operating in tenacibaculosis remain to be disclosed. This study aimed at contributing to a better understanding of this disease, by evaluating the early innate immune response triggered in European sea bass (*Dicentrarchus labrax*) by a bath-challenge with *T. maritimum*. Groups of sea bass were bath-challenged with *T. maritimum* (challenged fish) or mock-challenged. Undisturbed fish were used as controls (time 0). Samples of blood, liver and mucosal organs (skin, gills and posterior-intestine) were collected at 0 h (control) and at 6, 24, 48 and 72 h post-challenge (n=12). Mucosal organs were used for analysing the expression of immune-related genes by RT-qPCR, as well as blood samples for assessing haematological and innate humoral parameters and liver for oxidative stress assessment. An increased expression of *il1β*, *il*8, *mmp9* and *hamp1* was detected in all mucosal organs of infected fish when compared with control and mock-challenged fish, suggesting a pro-inflammatory response against *T. maritimum* transversal to all organs. The faster induction of these pro-inflammatory genes was observed in the gills. Regarding the systemic response, challenged fish presented neutrophilia, monocytosis, signs of anemia, and a decrease of bactericidal and lysozyme activities in plasma. Almost no variations were observed regarding hepatic oxidative stress.

The present study suggests that *T. maritimum* induces a local innate immune response upon bath infection not only in the skin of European sea bass, but also in the gills and posterior-intestine, likely triggered by the *T. maritimum*'s capacity to adhere, colonize and damage these organs that can function as entry ways to bacteria, leading ultimately to the seen host's systemic response.

Keywords: Tenacibaculosis; Aquaculture; Mucosal Immunity; Innate Immunity; Bacterial infection

Highlights:

- Bath-challenge with *T. maritimum* induces a pro-inflammatory response in fish mucosal organs;
- The response was faster in the gills than in the skin and posterior-intestine;
- Haemato-immunological parameters of challenged fish suggest a systemic response;

3.1 Introduction

Aquaculture is regarded as one of the fastest growing food production sectors, and, therefore, has the potential to fulfil the future demand for animal protein. This need is reflected in the tendency that aquaculture has to develop towards intensification (Ahmed & Thompson, 2019), which in turn could enhance the susceptibility of the farmed aquatic organisms to disease outbreaks. The introduction and translocation of fish stocks between aquaculture facilities can also lead to the spread of diseases (Peeler et al., 2011), which in association with the high stocking densities used in the aquaculture settings allow the thriving of several pathogens (Krkošek, 2010; Salama & Murray, 2011).

In the last decades, the marine aquaculture sector has been witnessing a worldwide emergence of tenacibaculosis (formerly known as marine flexibacteriosis), a relatively unknown pathology that affects several commercially important species (Avendaño-Herrera et al., 2020; Fernández-Álvarez & Santos, 2018; Flores-Kossack et al., 2020).

This disease has been responsible for countless losses, since it was first reported as a gliding bacterial infection affecting black seabream fry (*Acanthopagrus schlegeli*) reared in floating net cages in Japan (Masumura & Wakabayashi, 1977). Since then, this pathogen was able to spread between aquaculture sites, reaching Europe in the French Mediterranean Coast, where it affected European sea bass (*Dicentrarchus labrax*) rearing facilities (Pepin & Emery, 1993). Later on, cases of tenacibaculosis in cultured European sea bass were diagnosed in Italy, Greece and Turkey (Yardımcı & Timur, 2015; Kolygas et al., 2012; Salati et al., 2005), increasing the concern regarding this disease.

T. maritimum is the etiological agent of tenacibaculosis, and has been described as a Gram-negative filamentous bacterium able to induce small lesions, upraised spots, scale loss and some disintegration of the epidermis in the host's body surface, namely in the head, skin or fins (Haridy et al., 2014; Lopez et al., 2021; Van Gelderen et al., 2011). These lesions can establish a portal of entry for other opportunistic and frank pathogens, leading to mixed infections, which can ultimately lead to the host's death (Avendaño-Herrera et al., 2006c; Handlinger et al., 1997; Lopez et al., 2021). In order to cause such detrimental symptomatology, *T. maritimum* presents a plethora of virulence mechanisms that allows a successful adhesion and colonization of its hosts. These bacteria rely on the production of

exopolysaccharides, various adhesins and proteins with lectin or carbohydrate-binding motifs to strongly adhere to fish mucus, where they gather and accumulate the nutrients necessary for growth and proliferation (Avendaño-Herrera et al., 2006c; Magariños et al., 1995; Pérez-Pascual et al., 2017). *T. maritimum* has also been described as a pathogen able to agglutinate erythrocytes from a wide range of species (Pazos, 1997) and to directly compete with the host's iron-binding proteins. In a study developed by Avendaño-Herrera et al. (2005a), it was demonstrated that different *T. maritimum* strains have at least two different iron-uptake mechanisms, one related to the synthesis of siderophores and other involving the utilization of heme groups as iron sources (Avendaño-Herrera et al., 2005a). The proteolytic activity of several extracellular products (ECPs) has also been described and shown to include the ability to degrade gelatin, amylase, casein and nucleases (Pazos, 1997). Furthermore, the genome analysis of *T. maritimum* revealed several proteins homologous to proteins that in other bacteria are known to act as toxins and virulence factors, such as sphingomyelinase and ceramidase (Pérez-Pascual et al., 2017). Despite these studies, knowledge regarding *T. maritimum* pathogenesis is scarce, and very few studies have approached the interactions between this pathogen and the host.

Guardiola et al., (2019) focused on Senegalese sole (*Solea senegalensis*) mucosal and systemic immune responses following bath challenge with a sub-lethal dose of *T. maritimum* and further suggested the rudimentary systemic and the delayed host's mucosal responses (Guardiola et al., 2019). Plasma's antiprotease and bactericidal activities were mainly increased in challenged fish in the end of the trial, at 14-days post-challenge, and the same tendency was recorded for the haemolytic complement, lysozyme and peroxidase activities in skin mucus (Guardiola et al., 2019). This suggests that Senegalese sole immune response can be prolonged at least 14 days after being exposed to *T. maritimum*.

In a study developed by Faílde et al. (2014), the haematological profile of turbot (*Scophthalmus maximus*) challenged subcutaneously with *T. maritimum* showed some alterations, including granulocytosis, lymphopenia and thrombocytopenia as well as mild decrease of haematocrit values. Due to the seen distribution of immunoglobulin positive cells in spleen, kidney, thymus, skin and intestine, it is suggested that tenacibaculosis is able to induce a humoral immune response in turbot, through the synthesis of specific antibodies in the spleen that later on migrate to lesion areas in the skin (Faílde et al., 2014).

The present study aimed to bring more insights on the host responses against this fastidious bacterial pathogen by evaluating parameters of the short-term mucosal and systemic innate immune response in European sea bass after bath-challenge with *T. maritimum*. To the best our knowledge, this is the first study approaching the host's molecular immune response with focus on the three main mucosal organs (gills, skin and posterior-intestine).

3.2. Material and Methods

3.2.1. Bacterial culture and inoculum preparation

The *T. maritimum* strain (ACC13.1) used in this study was isolated from Senegalese sole and belongs to the serotype O3 (Avendaño-Herrera et al., 2005b). The strain was kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and stocks were kept frozen at −80 °C until use. Recovery from frozen stocks was achieved using marine agar (MA; Laboratories CONDA, Spain) at 25 °C for 48 h.

For inoculum preparation, bacteria were inoculated in 50 mL of marine broth (MB; Laboratories CONDA, Spain) in a 500 mL Erlenmeyer and grown at 25 °C, with continuous shaking (180 rpm) for 48 h. Turbidity was measured at 600 nm (Spectrophotometer, UV-1600PC, VWR) and exponentially growing bacteria (OD=0.886) were collected by centrifugation at 3,000 × *g* for 10 min and resuspended in MB at a concentration of 5×10^5 CFU mL^{-1}. The bacterial concentration was adjusted with the predetermined growth curve for this specific strain: $y = 2 \times 10^8 x + 4 \times 10^7$ (Mabrok, 2016).

3.2.2. Fish husbandry and experimental design

The current study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese Veterinary Authority following FELASA category C recommendations and in agreement with the guidelines for protection of animal used for scientific purposes according to European Union directive (2010/63/EU) (reviewed and approved by 0421/000/000/2020).

For this trial, European sea bass juveniles (45.45 ± 8.1 g) with no record of previous tenacibaculosis outbreaks were obtained from a commercial fish farm (Portugal) and were maintained in quarantine for 4 weeks at CIIMAR fish holding facilities in a recirculating aerated seawater system at 21.8 ± 0.4 °C, salinity of 34.2 ± 0.4‰, 8.2 ± 0.2 mg mL^{-1} dissolved oxygen and a 12 h light/12 h dark photoperiod. Water quality was maintained with mechanical and biological filtration, and fish were fed daily with a commercial diet (Aquasoja, Portugal) at 2% of body weight, distributed by two meals a day. Ammonia and nitrite levels were measured daily using commercial kits. For screening purposes and to assess the health status of the stock fish, ten randomly selected individuals were sampled for histopathological assessment. Before the bacterial challenge, fish were randomly distributed into two closed recirculating seawater systems (10 kg m^{-3} stocking density, n= 25 fish per tank, 0.11 m^3), one for the mock-challenged fish and another for the challenged

fish, each with four aquaria (4 replicates for each treatment) for sampling purposes and two aquaria (two replicates for each treatment) to follow cumulative mortality, and acclimated for one week.

At the challenge, water temperature was increased to 25 °C, to mimic temperature conditions at which tenacibaculosis outbreaks occur (Mabrok, 2016; Yamamoto et al., 2010). Fish, previously fasted for 24 h, were bath challenged for 2 h with *T. maritimum* (ACC13.1), prepared as described in the previous section (Mabrok, 2016), at a concentration of 5×10^5 CFU mL^{-1} (according to a pre-challenge to determine the LD_{30} for this strain). Challenge was performed in 50 L tanks with strong aeration at a stocking density of 25 kg m^{-3}. Mock-challenged fish were submitted to the same treatment, but MB was used instead of bacterial inoculum. After challenge, fish were returned to the recirculating system where they were acclimated. Bacteria was re-isolated from aseptically collected blood from randomly selected challenge fish at 24 h post-challenged and identified as *T. maritimum* as described elsewhere (Avendaño-Herrera et al., 2004).

3.2.3. Sampling

Fish were not fed during the trial period. Samples were collected post-mortem after euthanizing the fish with an overdose of anaesthetic, 0.7 mL L^{-1} (2-phenoxyethanol; Merck, ref. 807291, Germany). Sampling was performed before starting the bath challenge (n=12) (time 0, control) and at 6, 24, 48 and 72 h post-challenge. At each sampling time, three fish were removed from each tank (n=12 per treatment) and blood was collected from the caudal vein with heparinized 1 mL syringes and placed in heparinized 1.5 mL tubes. An aliquot was removed for haematological analysis, while the remaining blood was centrifuged for 10 min at 10,000 × *g* at 4 °C for plasma collection and storage at -80 °C. Skin (collected across the midline of the fish, beneath the dorsal fin, without any muscle), gills (portion of the second arch) and posterior intestine were also sampled and stored in RNA later (at a proportion of 1/10 w/v) at 4 ºC for the first 24 h, and then stored at -80 ºC for molecular biology analysis. Liver was collected and immediately frozen in liquid nitrogen, followed by storage at -80 ºC. Samples of skin (across the midline of the fish, beneath the dorsal fin, not previously sampled for mucus, including 1 cm of subjacent muscle), gills (portion of the second arch) and posterior intestine were also collected for histological analyses.

3.2.4. Haematological parameters

The haematological profile was conducted according to Machado et al. (2015). Total white (WBC) and red (RBC) blood cells were counted using a Neubauer chamber and haematocrit

(Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) were also assessed, as previously described (Machado et al., 2015). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated (Machado et al., 2015).

Blood smears were done with 3 µL of gently homogenized blood, air dried and fixed for 1 min in formol-ethanol (10% of 37% formaldehyde in absolute ethanol). For identifying neutrophils, the peroxidase detection method described by Afonso et al. (1998) was used (Afonso et al., 1998). Blood smears were then stained with Wright's stain (Haemacolor; Merck). Slides were examined under oil immersion (1,000 ×) and 200 leucocytes were counted and categorized, based on their morphology, as thrombocytes, lymphocytes, monocytes and neutrophils. The percentage of each cell population was calculated and multiplied by total number of WBC in order to determine the number of cells per mL.

3.2.5. Innate immune parameters

3.2.5.1. Antiprotease and protease activities

The antiprotease activity was determined as described by Ellis (1990) adapted for 96-well microplates. Shortly, 10 µL of plasma were incubated in microtubes with 10 µL of trypsin solution (5 mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3) (Sigma, USA) for 10 min at 22 °C. After incubation, 100 µL of phosphate buffer (115 mM NaH2PO4, pH 7.0) plus 125 µL of azocasein (20 mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3) were added and incubated again for 1 h at 22°C in the dark, with agitation. Then, 250 µL of 10% cold trichloroacetic acid (TCA) were added and incubated for 30 min at 22°C, followed by centrifugation at 10,000 × *g* for 5 min at room temperature (RT). Finally, 100 µL were transferred, in duplicate, to a 96-well plate containing 100 µL of 1N NaOH per well and the OD (optical density) read at 450 nm in a Synergy HT microplate reader. Phosphate buffered saline was used as positive control, instead of plasma, and the percentage of trypsin activity was calculated as follows: 100 − ((sample absorbance/reference absorbance) × 100).

To assess protease activity, the same protocol was followed, but the initial incubation of the plasma with trypsin was omitted and the incubation with azocasein and phosphate buffer was maintained for 24 h instead of 1h, in constant agitation. Plasma was replaced by trypsin (5 mg ml^{-1}, Sigma) as a positive control or by PBS as negative control. The percentage of trypsin activity compared to the positive control was calculated as follows: (sample absorbance/positive reference) × 100.

3.2.5.2. Peroxidase

Peroxidase activity was determined in plasma as described by Quade & Roth (1997). Briefly, in triplicates, 15 µL of plasma were placed into flat-bottomed 96-well plates and diluted in 135 µL of HBSS without Ca^{+2} and Mg^{+2} (Cytiva, USA). Then, 50 µL of 20 mM 3,3',5,5'- tetramethylbenzidine hydrochloride (TMB; Sigma, USA) were added to each well. After 2 min the reaction was stopped by adding 50 µL of 2 M sulphuric acid and the absorbance was measured at 450 nm (Synergy HT microplate reader). Peroxidase activity (units mL-1 plasma) was calculated by defining one unit of peroxidase as the amount needed to produce an absorbance change of 1 OD.

3.2.5.3. Lysozyme activity

Lysozyme activity was assessed as described by Costas et al., (2011). Firstly, *Micrococcus lysodeikticus* solution (0.5 mg mL^{-1} in 0.05 M sodium phosphate buffer, pH 6.2) was prepared. Then, 15 µL of plasma were added, in triplicates, to a microplate plus 250 µL of the *Micrococcus lysodeikticus* solution, for a final volume of 265 µL. After incubation at 25 °C, the absorbance (450 nm) was measured after 0.5 and 20 min in a Synergy HT microplate reader. Lyophilized hen egg white lysozyme (Sigma) was successively diluted in sodium phosphate buffer (0.05 M, pH 6.2) to obtain a standard curve. The amount of lysozyme in the sample was calculated using the standard curve.

3.2.5.4. Bactericidal activity

The bactericidal activity assay was performed using *T. maritimum* ACC13.1 strain. Bacteria were grown on MA at 25 °C for 24 h and resuspended in MB at a concentration of 1.6×10^8 CFUs mL^{-1}, by measuring the turbidity at 600 nm (Synergy HT microplate reader) and using the previously mentioned growth curve. Plasma bactericidal activity was then determined following the method described by Graham and Secombes (1988) with some modifications (Graham et al., 1988; Machado et al., 2015). In a U-shaped 96-well plate, 20 µL of plasma were added in duplicates, and as positive control, MB was added to the wells instead of plasma. In each well, 20 µL of bacteria were added to the plate followed by an incubation for 2.5 h at 25 °C. Afterwards, 25 µL of 3-(4, 5 dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg mL^{-1}; Sigma) were added to each well and the plate was incubated for 10 min at 25 °C. Plates were centrifuged at 2,000 × *g* for 10 min and formazan precipitate was dissolved with 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved precipitate was measured at 560 nm (Synergy HT microplate reader). In this method, the difference between the formazan present in samples and in the positive controls (100%) enables to calculate the viable bacteria in each sample and, consequently, the percentage of non-viable bacteria.

3.2.5.5. Nitrite concentration

To indirectly access the nitric oxide (NO) concentration in plasma, a Nitrite/Nitrate colorimetric kit (Roche, 11746081001, Germany) was used according to the manufacturer's instructions. Since nitrite and nitrate are endogenously produced as oxidative metabolites of the messenger molecule NO, these compounds are considered as indicative of NO production (Saeij et al., 2003). To measure nitrite/nitrate, the samples were previously diluted 1:10 in distilled H_2O in microtubes and the concentrations were expressed as µM.

3.2.6. Oxidative stress biomarkers

Liver tissue were homogenised 1/10 (w/v) in potassium phosphate buffer (0.2 M, pH 7.4). From the homogenised mixture, 200 µL were transferred to a microtube with 4 µL of 4% BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol for lipid peroxidation (LPO) assessment.

For determining superoxide dismutase, catalase and glutathione-S-transferase activities, for each volume of tissue homogenate, a volume of potassium phosphate buffer (0.2 M, pH 7.4) was added followed by a centrifugation at 10,000 × *g* for 20 minutes at 4 °C. The supernatants were collected and kept at −80 °C. Protein concentration was measured using Pierce™ BCA Protein Assay kit, with bovine serum albumin as standard, according to the manufacturer's instructions. For superoxide dismutase and catalase activity homogenates were diluted to achieve a final protein concentration of 0.3 and for total glutathione-S-transferase a concentration of 0.7 mg mL^{-1}. LPO was determined using the protocol described by Bird & Draper (1984) with some modifications (Peixoto et al., 2021). A volume of 100 µL of 100% TCA was added to the previously mentioned 204 µL of liver homogenate, and afterwards, 1 mL of 0.73% thiobarbituric acid solution (in Tris–HCl 60 mM, pH 7.4 with DTPA 0.1 mM). Samples were incubated for 1 h at 100 °C in a kiln and then microtubes were centrifuged for 5 minutes at 15,000 × *g*. A volume of 200 µL of supernatant was transferred to a 96-well plate in triplicates and the absorbance was measured at 535 nm. The LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per g of wet tissue. Catalase activity was quantified measuring the decrease in absorbance, through the consumption of H_2O_2, as described by Claiborne (1985) but adapting the protocol to microplates as described by Rodrigues et al. (2017). A sample of 10 µL was transferred to a UV light microplate in triplicates with 140 µL of potassium phosphate (0.05 M, pH 7.0) plus 150 µL of 30% H_2O_2. The absorbance was measured at 240 nm for 2 min. The catalase activity was quantified using H_2O_2 molar extinction coefficient at 240 nm of 40 M cm-1, expressed in U per mg of protein. Superoxide dismutase (SOD) activity was assessed following the protocol describe by Almeida et al. (2010), utilizing the cytochrome

C method with xanthine/xanthine oxidase (Almeida et al., 2010). A volume of 50 µL of each sample was transferred to a microplate in triplicates. Then, 200 µL of a reaction solution containing 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM Na-EDTA, 0.7 mM xanthine and 0.03 mM cytochrome C were added. Promptly, 50 µL of 0.03 U mL^{-1} xanthine oxidase with 0.1 mM Na-EDTA were also added to the microplate. Absorbance was measured at 550 nm (Synergy HT microplate reader) at 20 s intervals for 3 min. Activity is described as units of SOD per mg of protein. One unit of activity was defined as the quantity of enzyme necessary to produce a 50% inhibition of the cytochrome C reduction rate. Glutathione-S-transferase (GST) activity was accessed following the method of (Habig et al., 1974) adapted to microplate by Frasco & Guilhermino (2002). Briefly, a 250 µL of a reaction solution containing 0.2 M potassium phosphate buffer (pH 6.5), 10 mM reduced glutathione (GSH) and 60 mM 1-chloro-2,4-dinitrobenzene (CDNB) was added to 50 µL of liver homogenate in triplicates. Absorbance was recorded at 340 nm for 5 min with 20 s intervals in microplate. GST activity was expressed as mU per mg of protein, using the molar extinction coefficient at 340 nm of 9.6 × 106 M/cm. The reduced (GSH): oxidized (GSSG) glutathione ratio was determined using the microplate assay for GSH/GSSG commercial kit (Oxford Biomedical Research, UK) as previously described by Hamre et al. (2014). This method relies on the quantitative determination at 412 nm of the total amount of glutathione (GSH + GSSG) and GSSG (Tietze, 1969). Briefly, the determination of GSSG is obtained by adding a thiol scavenger (N-ethylmaleimide pyridine derivative solution, Oxford Biomedical Research, UK), which reacts with GSH to form a stable complex, therefore removing the GSH prior to the quantification of GSSG, without inhibiting GR activity. Through the addition of glutathione reductase, the available GSSG is reduced to GSH which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) allowing the measurement of pre-existent GSSG. The rate of the reaction is proportional to the GSH and GSSG concentration. The GSH/GSSG Ratio is calculated as follows: (GSHt – 2GSSG)/GSSG.

3.2.7. Histology and immunohistochemistry

At each sampling point, 12 fish per group (control, mock-challenged and challenged) were sampled. Tissue fragments from gills, skin and intestine were fixed with 4% buffered formaldehyde for 24-48 h, dehydrated and embedded in paraffin wax. Sections of 2-3 mm thickness were obtained and collected on silane coated slides, followed by drying overnight, dewaxing, hydration. Sections were then stained with haematoxylin and eosin (H&E) or used for immunohistochemistry (IHC). Regarding IHC, incubations were performed at RT in a humidified chamber and washing was performed by immersion for 5 min in phosphate-buffered saline (PBS; 8 mM Na_2HPO_4 3 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) containing

0.5% (v/v) Tween 20. Endogenous peroxidase activity was quenched by incubation with peroxidase blocking buffer (Vector Labs, Burlingame, CA) for 1 h. The sections were washed once and blocked for 20 min in 2.5% normal horse serum (Vector Labs, Burlingame, CA), followed by incubation with 1:1000 (concentrations of mg mL^{-1}) working dilution of rabbit anti-*T. maritimum* LL01.8.3.8 immunoadsorbed antibody (anti-Tm) for 1.5 h, according to Faílde et al. (2014).

After washing again, the sections were incubated with ImmPRESS®-VR Horse Anti-Rabbit IgG Polymer-HRP (Vector Labs, Burlingame, CA) for 30 min, rinsed, and colour development achieved with Vector® VIP Substrate Kit, Peroxidase (HRP) (Vector Labs, Burlingame, CA), as the chromogen. After a final wash, the slides were counterstained with haematoxylin, dehydrated and mounted.

3.2.8. Gene expression analysis

Target organs (gills, skin and posterior-intestine) were weighted (up to 300 mg of organ), placed in 500 µL of Trizol (NZYTech, Lisbon, Portugal) and homogenized in a Precellys Evolution homogenizer at 6000 × *g* (2 x 20 s, 4 ºC). After this step, 150 µL of chloroform were added at 4 ºC and the samples were vortexed, followed by a centrifugation at 12,000 × *g* for 15 min at 4 ºC. The aqueous phase was transferred to a clean tube with 300 µL of 70% ethanol, mixed, and placed in NZYSpin Binding columns. After this step, the total RNA isolation was conducted with NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) according to the manufacturer's specifications. RNA samples were quantified and purity was assessed by spectrophotometry using DeNovix DS-11 FX (Wilmington, DE, USA) with absorbance ratios at 260 nm/280 nm of 1.9–2.1. First-strand cDNA was synthesized and samples were standardized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) with further storage at -80°C. For reverse transcriptase, a Veriti DX 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used. Real-time Quantitative PCR (qPCR) was performed with CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA) using 4.4 µL of diluted cDNA mixed with 5 µL of iTaq Universal SYBR Green Supermix® (Biorad, Hercules, CA, USA) and 0.3 µL (10 µM) of each primer in a final volume of 10 µL. Primers were designed with NCBI Primer Blast Tool and IDT OligoAnalyzer ToolTM to amplify genes related with innate immune response in European sea bass. The known qPCR requirements (amplicon size, Tm difference between primers, GC content, and self-dimer or cross dimer formation) were respected. The template sequences used for the primer's design were obtained from both NCBI and the databases dicLab v1.0c sea bass genome (Kuhl et al., 2010). The efficiency of each primer pair was determined by calculating the slope of the regression line of the cycle thresholds (Ct) vs.

the relative concentration of cDNA, using serial 2-fold dilutions of cDNA. In order to ensure no amplification of primer dimers, melting curves were analysed. The standard cycling conditions were 95 ºC initial denaturation for 10 min, followed by 40 cycles of two steps (95 ºC denaturation for 15 s followed by primer annealing temperature for 1 min), 95 ºC for 1 min followed by 35 s at the annealing temperature, and finally, 95 ºC for 15 s.

The reactions were run in duplicates and target gene expression was normalized using the geometric mean of elongation factor 1β (*ef1β*) and ribosome 40s subunit (*40s*) and calculated according to the Pfaffl method (Pfaffl, 2001). Accession numbers, primer efficiencies and annealing temperatures for each organ, amplicon length and primer sequences are detailed in Table 1.

3.2.9. Statistical analysis

Mean and standard error of the mean (mean ± SEM) were calculated for all parameters. Data were analysed for normality and homogeneity of variance, when necessary outliers were removed and gene expression data was Log-transformed before being statistically analysed.

When all the assumptions were fulfilled, a T-student test or a One-Way ANOVA (Tukey *post hoc* test) was used under SPSS 27 program for WINDOWS. When the assumptions were not verified a Welch ANOVA (Games-Howell *post hoc* test) or a Kruskal-Wallis was performed. The level of significance used for all statistical tests was $p \leq 0.05$.

Table 1: Immune-related genes analysed by Real-time PCR.

Gene	Acron.[a]	Accession number	Eff[b]			AT[c] (°C)			Amplicon length	Primer sequence (5'-3')
			Gills	Skin	Pl[c]	Gills	Skin	Pl[d]		
Elongation factor 1-beta	*ef1b*	AJ866727.1	114.3	96.1	107.2	60	60	60	144	F: AACTTCAACGCCCAGGTCAT R: CTTCTTGCCAGAACGACGGT
40s Ribosomal protein	*40s*	HE978789.1	108.2	96.1	105.2	60	60	62	79	F: TGATTGTGACAGACCCTCGTG R: CACAGAGCAATGGTGGGGAT
Interleukin 1 beta	*il1β*	AJ269472.1	93.1	93.4	114.4	60	60	60	105	F: AGCGACATGGTGCGATTTCT R: CTCCTCTGCTGTGCTGATGT
Interleukin 8	*il8*	AM490063.1	93.1	90.6	100.1	60	60	60	140	F: CGCTGCATCCAAACAGAGAGCAAAC R: TCGGGGTCCAGGCAAACCTCTT
Interleukin 6	*il6*	AM490062.1	89.6	86.9	101.8	60	60	62	81	F: AGGCACAGAGAACACGTCAAA R: AAAAGGGTCAGGGCTGTCG
Tumour necrosis factor alpha	*tnfα*	DQ070246.1	104.9	91.3	110.7	60	55	55	112	F: AGCCACAGGATCTGGAGCTA R: GTCCGCTTCTGTAGCTGTCC
Interleukin 10	*il10*	AM268529.1	114.9	87.3	105.5	60	60	60	164	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC
Matrix metallopeptidase 9	*mmp9*	FN908863.1	104.5	90.5	107.8	57	62	60	166	F: TGTGCCACCACAGACAACTT R: TTCCATCTCCACGTCCCTCA
Hepcidin	*hamp1*	KJ890396.1	110.2	92.5	110.2	62	62	60	148	F: ACACTCGTGCTCGCCTTTAT R: TGTGATTTGGCATCATCCACG
Ferroportin	*fpn1*	KU599935.1	97.6	95.3	99.5	60	60	60	161	F: GCTAGAGTTGGCCTGTGGTC R: GGGTTCGGAGCCAGTATCAC
Nuclear factor kappa B	*nf-κB*	DLAgn_00239840[e]	113.3	106.8	106.8	60	60	55	136	F: GCTGCGAGAAGAGAGGAAGA R: GGTGAACTTTAACCGGACGA

Signal transducer and activator of transcription 3	*stat3*	DLAgn_00192560[e]	97.6	104.4	112.8	60	60	60	275	F: GACATCAGCGGAAAGACCCA R: GGGGTGACGCAGATGAACTT
Apoptosis regulator bcl-2-like	*bcl2-like*	DLAgn_00005980[e]	101.3	88.6	106.8	60	62	62	181	F: CTCCTCCTCCTCTTCCTCGT R: TCATCTGGTTGCTTCAGTCG
Nod-like receptor 1	*nod1*	DLAgn_00065300[e]	92.4	94.5	107.2	60	60	60	293	F: ACCCAAGCAATGACGTAGCA R: TTTTCCTACACCCGCATCCC
Nod-like receptor 2	*nod2*	DLAgn_00155640[e]	103.9	90.6	104.4	60	60	60	298	F: GAGGAAGCATCACAGGGACC R: TGCAATCCCCTCAAAGGCAA
Toll-like receptor 2	*tlr2*	KX399288.1	106.5	96.1	97.6	57	60	60	173	F: CAGTAGGCCAAGTCCGTCTC R: GGAGCTACGCTTGGCCTTTA
Toll-like receptor 9	*tlr9*	KX399289	104.5	102.7	108.2	60	55	55	100	F: TCTTGGTTTGCCGACTTCTTGCGT R: TACTGTTGCCCTGTTGGGACTCTGG

[a] Gene acronym

[b] Efficiency of PCR reactions, calculated from serial dilutions of organ RT reactions in the validation procedure.

[c] Annealing temperature for each organ (°C).

[d] Posterior intestine.

[e] Sequences obtained from databases dicLab v1.0c sea bass genome.

3.3. Results

3.3.1. Bacterial challenge

Bath-challenge with 5 x 10^5 CFU mL^{-1} *T. maritimum* ACC13.1 resulted in 32.1% cumulative mortality, whereas, as expected, no mortality was recorded in mock-challenged fish (Fig. 1; n=30 fish per treatment, X2<0.0008 for comparisons between treatments). Moreover, the mortalities in challenged fish occurred between days 3 and 4 after challenge (Fig. 1).

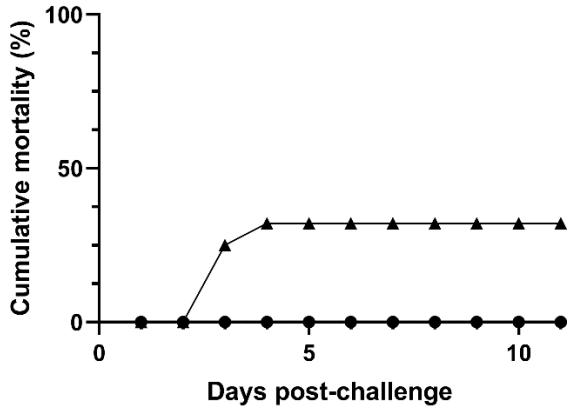

Figure 1: Mortality of European sea bass (*Dicentrarchus labrax*) after bath-challenge with 5 x 10^5 CFU mL^{-1} *T. maritimum* (▲) or with marine broth MB (●) (n=30 fish per treatment).

3.3.2. Haematological analysis

The concentration of red blood cells suffered a decrease in infected fish at 6, 24 and 48 h post-challenge, returning to a value similar to the control fish (0 h) at 72 h (Table S2, Appendix II). In contrast, in mock-challenged fish, a slight decrease in red blood cells was only observed at 48 h post-challenge. Furthermore, the concentrations of red blood cells at 6, 24 and 48 h in challenged fish were lower than in mock-challenged animals (Table S2). In agreement with this, haematocrit also decreased at 24 and 48 h post-challenged in infected fish, when compared to control value (0 h), and was lower in infected fish than in mock-challenged fish at all-time points analysed (Table S2). However, haemoglobin did not show any significant difference for the challenged fish (Table S2). The mean corpuscular volume was increased at 6 h in infected fish, when compared to controls (0h) and to mock-challenged fish (Table S2), whereas for mean corpuscular haemoglobin, an increase was observed in infected fish from 6 to 48 h post-challenge when compared to controls and mock-challenged animals (Table S2). Regarding mean corpuscular haemoglobin concentration, an increase at 24 and 48 h post-challenge was observed in infected fish, but

the values for the remaining time points were similar between mock and challenged fish (Table S2).

The white blood cells' counts in infected fish at 6, 24 and 48 h did not differ from the control value (0 h), but at 72 h, an increase in white blood cells number was observed (Table S2). Likewise, an increase in white blood cells at 72 h was registered in the mock-challenged group (Table S2). The differential counts showed that the number of circulating neutrophils increased at 6, 48 and 72 h post-challenge in infected fish, compared to controls (Table S3, Appendix II). Infected fish also presented monocytosis at 48 and 72 h, compared to controls and mock-challenged fish (Table S3). Lymphocytes' concentration did not significant differences between groups (despite a wave-like variation on its values), except at 72 h, where an increase was observed for both mock and challenged fish, compared to controls (Table S3). Regarding thrombocytes, a decrease was recorded at 6 h in infected fish compared to control and mock-challenged fish (Table S3).

3.3.3. Innate humoral parameters

An increase in plasma antiprotease was only observe in infected fish at 48 and 72 h post-challenge (Table S4, Appendix II). For plasma protease activity, a peak was reached at 24 h followed by a decrease at 48 h post-challenge for infected sea bass, returning to values similar to those from controls after 72 h (Table S4). Although not significant, a similar pattern was observed for mock-challenge fish at 24 h post-challenge (Table S4). Plasma peroxidase activity also increased over time reaching a peak at 48 h post-challenged for both mock-challenged and infected fish (Table S4), with both groups showing similar patterns of activity. A strong decrease in lysozyme activity was observed in infected fish from 24-72 h post-challenge, with a minimum at 48 h (Table S4). A similar tendency to decrease was seen for mock-challenged fish, although the values in infected fish at 24, 48 and 72 h were much lower than in mock-challenged animals. Plasma bactericidal activity decreased in infected sea bass from 6 to 48 h post-challenge, with values lower than the ones obtained for mock-challenged fish (Table S4). For plasma NO levels, no significant differences were found between mock and challenged groups, despite an increase was observed at 48 h post-challenge for both groups when compared with control values (Table S4).

3.3.4. Oxidative stress biomarkers

Hepatic catalase activity decreased over time until the end of the trial for both mock-challenged and challenged fish, with the two groups presenting similar values for each time

point (Table S5, Appendix II). Superoxide dismutase activity in liver was significantly higher for infected sea bass compared to control and mock-challenged fish at 6 h returning to basal values at the end of the trial (Table S5). For mock-challenged specimens, a peak of activity was reached at 48 h post-challenge (Table S5). No differences between time points were observed regarding lipid peroxidation, however, values were significantly lower for infected fish at 6 and 48 h post-challenge compared to mock-challenged group (Table S5). Hepatic glutathione-S-transferase levels decreased slightly in challenged fish until the end of the trial, reaching its minimum value at 72 h post-challenge (Table S5). Although a general tendency to decrease was also seen for mock-challenged fish, the values the values did not differ much from the basal ones (Table S5). Hepatic reduced glutathione decreased significantly between the control fish and both mock-challenged and infected fish after 72 h (Table S5). Oxidised glutathione decreased until 48 h post-challenged in the liver of mock-challenged fish, and returned to basal values at 72 h. No differences were recorded for challenged fish (Table S5). Regarding reduced: oxidised glutathione ratio in liver, no differences were recorded for challenged fish, but it was possible to distinguish a wave-like variation with a peak at 48 h post-challenge. The same was seen in the mock-challenge group, with a significantly higher value at that sampling time point, when compared to controls (0h) (Table S5).

3.3.5. Histology and immunohistochemistry analyses

The fish sampled before the trial for screening purposes did not display any histopathological changes and the same was recorded for the individuals from the control and mock-challenge groups.

No histopathological changes were observed in the analysed mucosal organs at 6 h post-challenge for bacteria-challenged fish. Instead, infected fish started to display typical tenacibaculosis symptoms at 24 h post-challenge, with ulcers in different areas of the skin and frayed fins. At 24 h the lesions in the skin of bath-challenged fish showed similar degrees of severity, presenting a considerable number of scattered inflammatory cells in the dermis and hypodermis, with severe necrosis of the dermis and detachment or loss of the epidermis (Fig. 2 A and B). In the samples of the remaining organs, no evidence of histopathological changes was observed for any of the specimens analysed through sampling time points. Immunoreactivity was detected only in the skin of infected fish at 24 h and 48 h post-challenge, being mainly distributed across the dermis, revealing an extensive and fast progression of the bacteria; along with necrosis and vacuolisation, it was possible to observe the recruitment of inflammatory cells to adjacent areas at 24 h (Fig.2 C

and D). No immunoreactivity was detected in the remaining organs for any specimens through sampling time points.

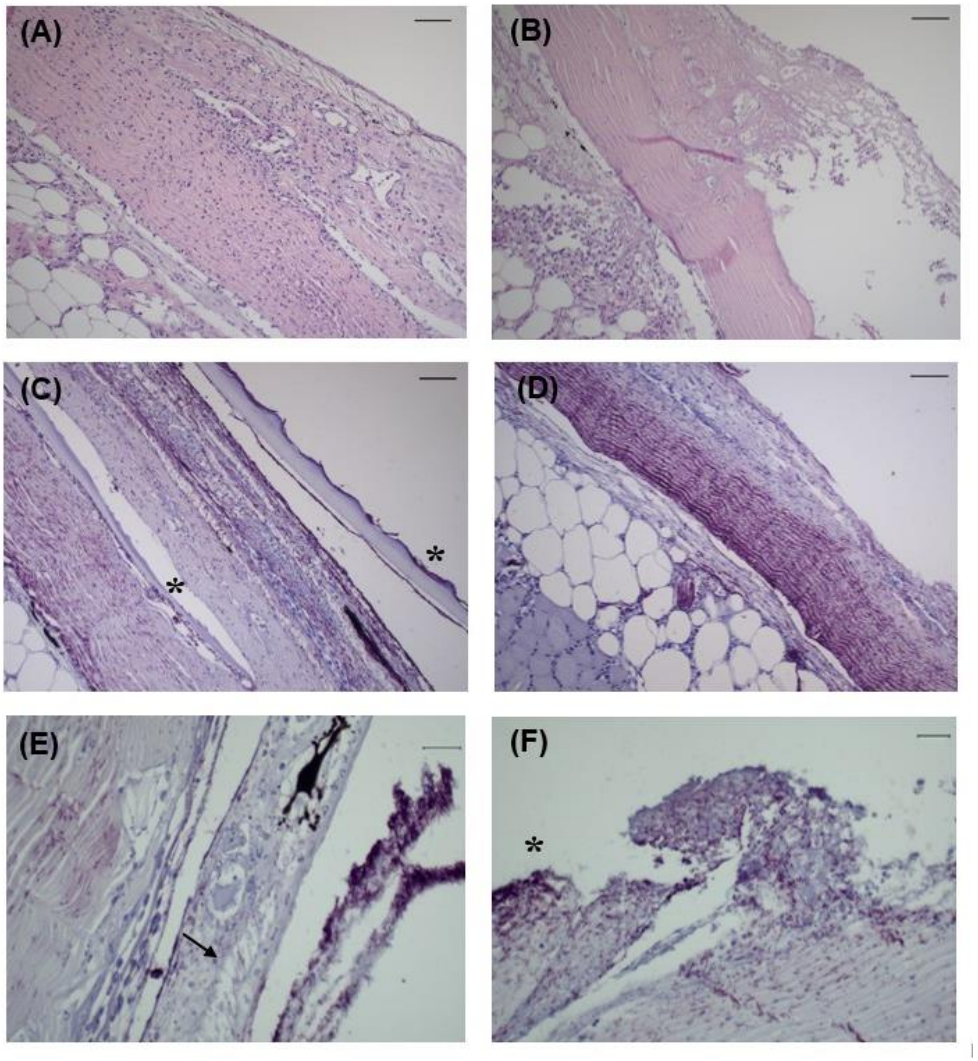

Figure 2: Representative images of skin tissue from European sea bass (*Dicentrarchus labrax*) bath-challenged with 5 x 10^5 CFU mL^{-1} *T. maritimum*. A) Heavy infiltration of inflammatory cells in the dermis of challenge fish at 24 h. H–E. Bar 50 µm. B) Extensive necrosis of the dermis associated with infiltration of inflammatory cells in the hypodermis of challenge fish at 24 h. H–E. Bar 50 µm. C) Immunohistochemistry against *T. maritimum* antigen, revealing extensive proliferation of *T. maritimum* in the dermis of challenged fish at 24 h, with agglomerates of bacteria in the epidermis and scale pockets (*) Bar 50 µm. D) Necrosis and agglomerates of bacteria in the dermis with infiltration of inflammatory cells in the hypodermis. Bar 50 µm. E) Vacuolization of epithelial cells from the epidermis of challenged fish at 24 h (arrow), with agglomerates of *T. maritimum* in the same area. Bar 50 µm. F) Proliferation of these bacteria in challenged fish at 48 h post-challenge. Bar 20 µm. Section subjected to immunocytochemistry against *T. maritimum* antigen.

3.3.6. Gene expression analyses

3.3.6.1. Gills

Infected sea bass displayed a greater than 23-fold increase in the expression of the pro-inflammatory cytokine *il1β* in the gills at 6 h post-challenge. At 24 h the expression was 9-fold higher than the expression in mock-challenged fish and returned to basal values after that sampling point (Fig. 3, A). A very similar pattern was also seen for *il8* and *mmp9* transcripts (Fig. 3, B and C). A high increase of *hamp1* expression was observed at 6, 24 and 48 h post-challenge in infected sea bass compared to control and mock-challenged fish, with a 30-fold peak at 24 h (Fig. 3, D). On the contrary, a slight, albeit significant decrease in *fpn* expression was noticed in infected specimens at all sampling points compared to control and mock-challenged fish (Fig. 3, E). The expression of the anti-inflammatory cytokine *il10* did not change in the mock-challenged fish, but was increased at 6 h post-challenge for the infected ones (Fig. 3, F).

The expression of the *tlr2*, *tlr9*, *nod1* and *nod2* receptors in the gills did not change significantly after bacterial exposure when compared to controls (0 h), although a tendency to decreased *tlr2* expression was seen at 6 and 24 h post-infection (Table S6, Appendix II). The mock-challenged fish showed higher *tlr2* expression than the control or challenged fish at all-time points (Table S6). Regarding tlr9 transcripts, the different treatment groups presented a similar pattern, with a downregulation at 24 h for infected fish (Table S6). For the intracellular receptor *nod1*, an upregulation was observed at 6 h for challenged fish, returning afterwards to basal expression values (Table S6). On the other hand, *nod2* suffered a significant downregulation in infected fish compared to control and mock-challenged groups, with the lower expression registered at 24 h post-challenge. Both transcription factors, *nf-κB* and *stat3*, presented higher expression values at 6 h post-challenge for infected fish followed by a decrease in the remaining time points (Table S6). In the mock-challenged fish, no changes in the *nf-κB* expression were observed (Table S6). The same pattern of expression was seen for *stat3* (Table S6). Regarding *bcl2-like*, the expression in mock-challenged fish did not differ from the expression in control fish, but infected sea bass showed decreased expression throughout all sampling points (Table S6). Expression of *il6* was downregulated in infected fish at 48 and 72 h post-challenge compared to control and mock-challenged animals (Table S6). The mock-challenged fish did not present any major differences for *il6* expression. For the pro-inflammatory cytokine tnfα, the expression values for mock-challenged sea bass increased until the end of the time-course trial reaching its maximum at 72 h post-challenge, whereas a tendency to decrease was observed in the infected fish from 24 h onwards (Table S6).

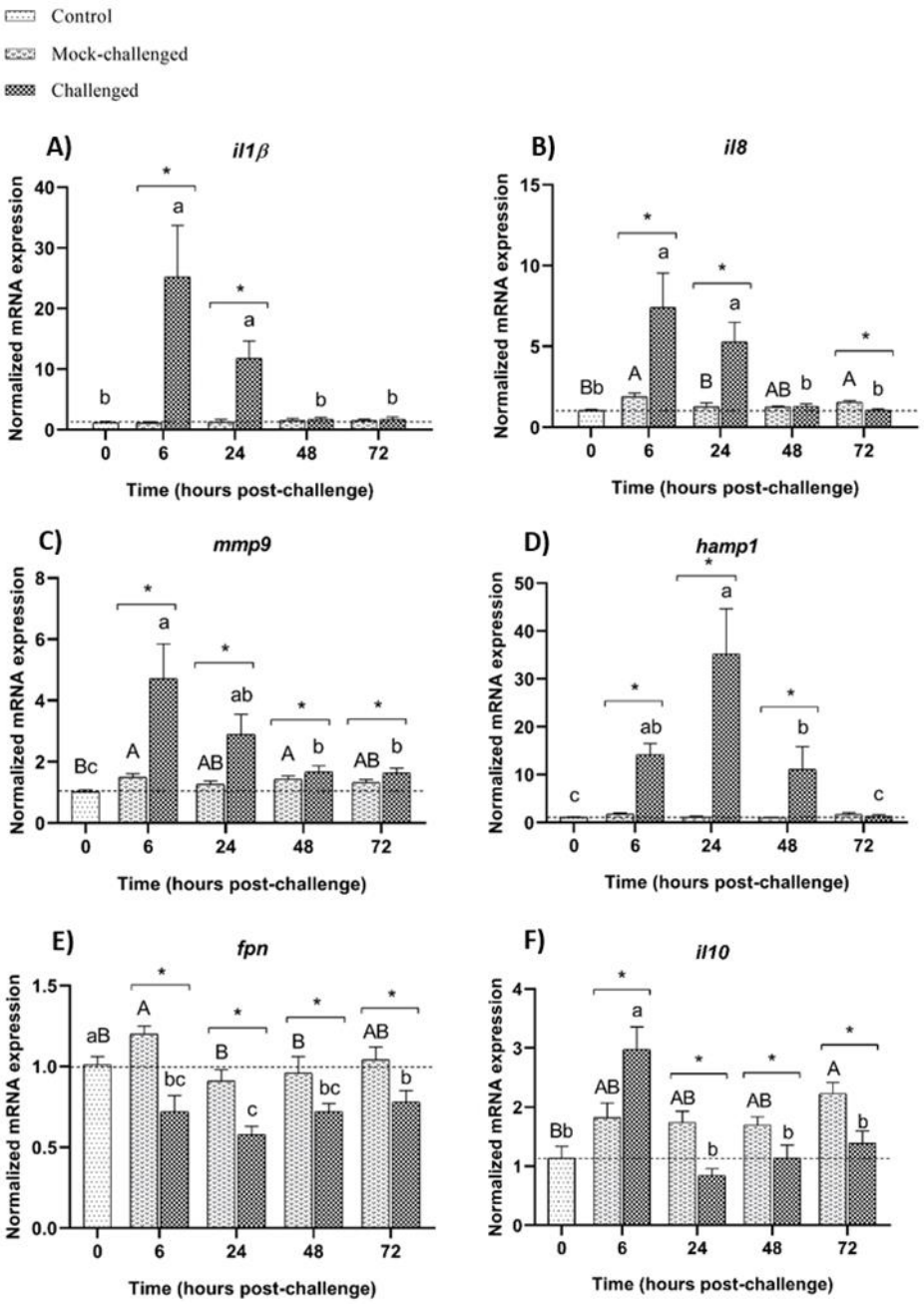

Figure 3: Expression of (A) *il1β*, (B) *il8*, (C) *mmp9*, (D) *hamp1*, (E) *fpn* and (F) *il10* in gills of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 x 10^5 CFU mL^{-1} *T. maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters indicate differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; p≤0.05).

3.3.6.2. Skin

In what concerns the expression of pro-inflammatory mediators, the skin responded similarly to the gills. An upregulation of *il1β* was observed at 6 h post-challenge in infected sea bass, with the higher expression (22-fold increase relative to control) recorded at 24 h (Fig. 4, A). A similar response was seen for *il8* and *mmp9* transcripts (Fig. 4, B and C). The antimicrobial peptide *hamp1* registered a significant increase in mRNA levels at 6, 24 and 48 h post-challenge for infected fish, reaching its maximum expression value at 24 h post-challenge with a 90-fold increase compared to mock-challenged fish (Fig. 4, D). Regarding *fpn* mRNA expression an opposite pattern was recorded, with a significant downregulation at 6, 24 and 48 h post-challenge for the infected group, returning to basal expression values after 72 h (Fig 4, E). The expression of the anti-inflammatory cytokine *il10* showed a significant, albeit moderate increase at 6 h post-challenge for both mock-challenged and infected groups, followed by a decrease at later time points to values similar to the basal level (Fig 4, F).

As in the gills, no major differences in the expression of the studied cell receptors were observed in the skin of sea bass bath exposed to *T. maritimum*. Infected fish showed a decrease in *tlr2* transcripts at 6 and 24 h compared to the control group, and a higher expression at 72 h (Table S7, Appendix II), whereas a tendency to increase was observed in the mock-challenged fish. No differences in the expression of the intracellular cell receptor *tlr9* were recorded (Table S7).

The *nod1* expression was slightly down-regulated in the mock-challenge group from 24 onwards. For the bath-challenged fish, *nod1* expression was decreased at 48 h and 72 h post-challenge (Table S7). Regarding *nod2* expression levels, a wave pattern was observed in infected fish, with a significant decrease at 24 h followed by an upregulation at 48 and 72 h when compare to basal levels. In the case of mock-challenged fish, an upregulation of *nod2* was seen from 24 h onwards (Table S7). The *nf-κB* mRNA levels were increased at 6 and 24 h infected fish, but decreased at 48 and 72 h to levels lower than the control ones. The mock-challenged fish had lower transcripts throughout the time-course study compared to the controls (Table S7). The same mRNA expression pattern was observed for *stat3* (Table S7). In the case of *bcl-2like*, a downregulation was seen throughout the time-course, especially at 24 h post-challenge for the infected fish compared to both control and mock-challenged groups. No differences were detected for mock-challenged ones (Table S7). Mock-challenged and infected groups presented a similar pattern of *il6* expression, with a significant upregulation at 6 h post-challenge, followed by a decrease in later time-points (Table S7). No significant differences in tnfα were recorded in mock-challenged and infected fish, despite a tendency to increase in the infected group (Table S7).

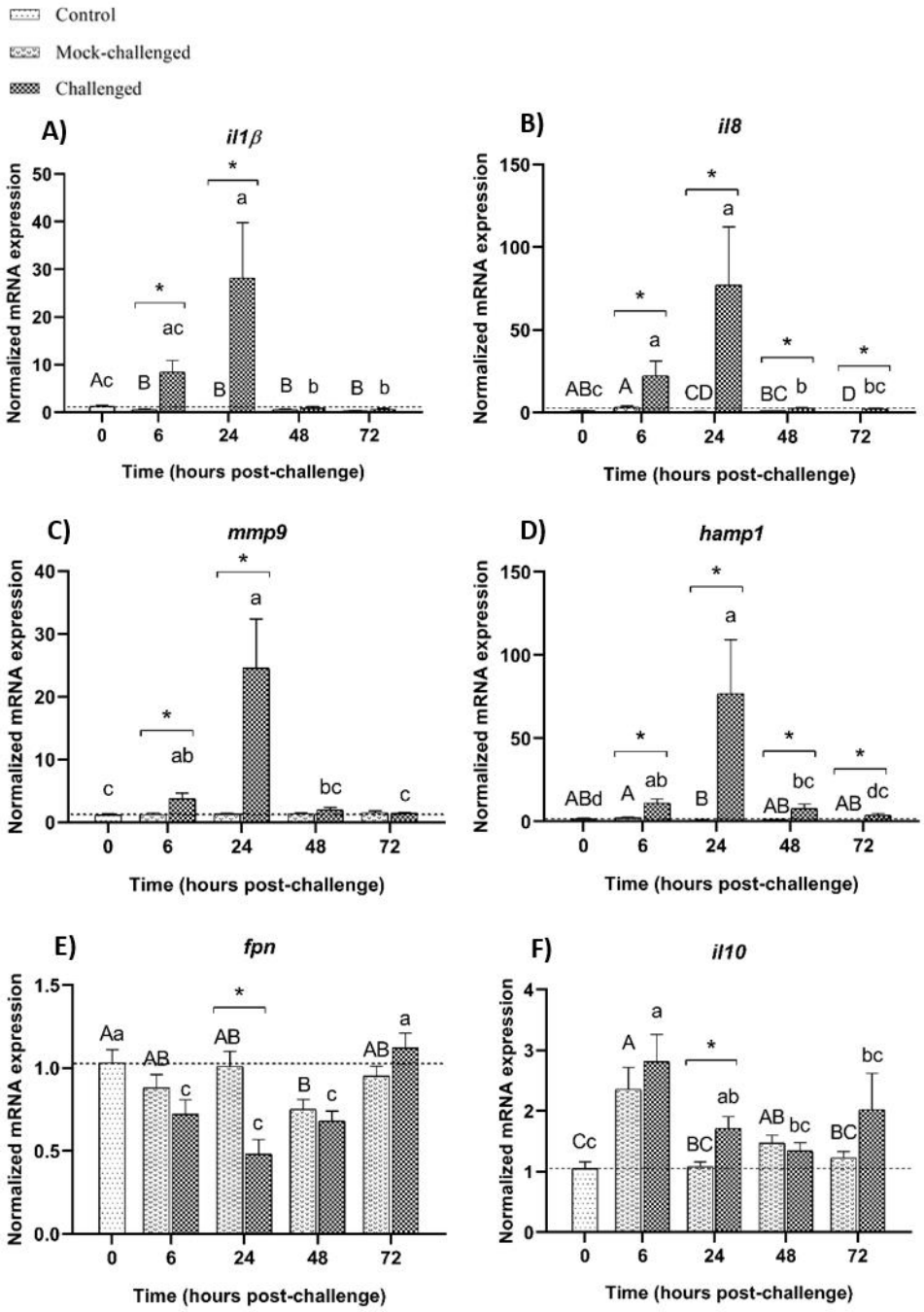

Figure 4: Expression of (A) *il1β*, (B) *il8*, (C) *mmp9*, (D) *hamp1*, (E) *fpn* and (F) *il10* in the skin of European sea bass (*Dicentrarchus labrax*) after bath-challenge with 5 x 10^5 CFU mL^{-1} *T. maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters indicate differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

3.3.6.3. Posterior-intestine

A clear increase in the levels of *il1β* transcripts was recorded at 6 and 24 h in infected sea bass (46-fold and 126-fold increase, respectively compared to mock-challenged fish), similar to what was observed in the gills and skin (Fig. 5, A). The same patter occurred for *il8* (Fig. 5, B), and *mmp9* (Fig. 5, C), with an upregulation in infected sea bass at all-time points compared to control and mock-challenged groups. The antimicrobial peptide *hamp1* showed marked increase in expression in challenged fish at 6 h (20-fold increase relative to mock fish), slowly decreasing after this time point, despite infected sea bass presented much higher values than the control or mock-challenged groups at all-time points (Fig. 5, D).

While *fpn* transcripts presented a tendency to be downregulated, especially for infected sea bass at 6 h post-challenge, no significant differences were found (Fig. 5, E). The cytokine *il10* expression demonstrates a slightly different expression pattern to what was observed for the gills and skin response, with a significant increase at 24 h post-challenge (20-fold increase relative to mock-challenge). At 72 h post-challenge, values from infected fish returned to basal levels, similar to control ones (Fig. 5, F). Similarly, to what was seen in the gills and skin, no major differences in the expression of the studied cell receptors were observed in the posterior-intestine. Expression of *tlr2* presents was slightly decreased in the challenged fish at 6 and 24 h, returning to basal values a 72 h. No differences were seen for mock-challenged group (Table S8, Appendix II).

Regarding *tlr9* expression, no changes were detected in infected specimens when compared to controls, and a minor increase was recorded for mock-challenged fish at all time-points (Table S8). For both *nod1* and *nod2*, no major differences in expression were noticed in infected animals. In mock-challenged fish, an increased *nod2* expression level was seen at 24 h post-challenge compared to control fish (Table S8). Expression of *nf-κB* decreased from 24 h onwards in infected fish, and was also decreased in mock-challenged fish at all-time points (Table S8). No differences in *stat3* expression were noticed in challenged and mock-challenged groups, when compared to controls (Table S8). The expression of *bcl2-like* in mock-challenged and infected groups was slightly lower than in control fish at all-time points (Table 8). For *il6* mRNA levels no major differences were record (Table S8). Regarding *tnfα*, no differences were recorded for mock-challenged fish, but a decreased expression was observed at 24 h post-challenge for infected fish, compared to control and mock-challenged groups (Table S8).

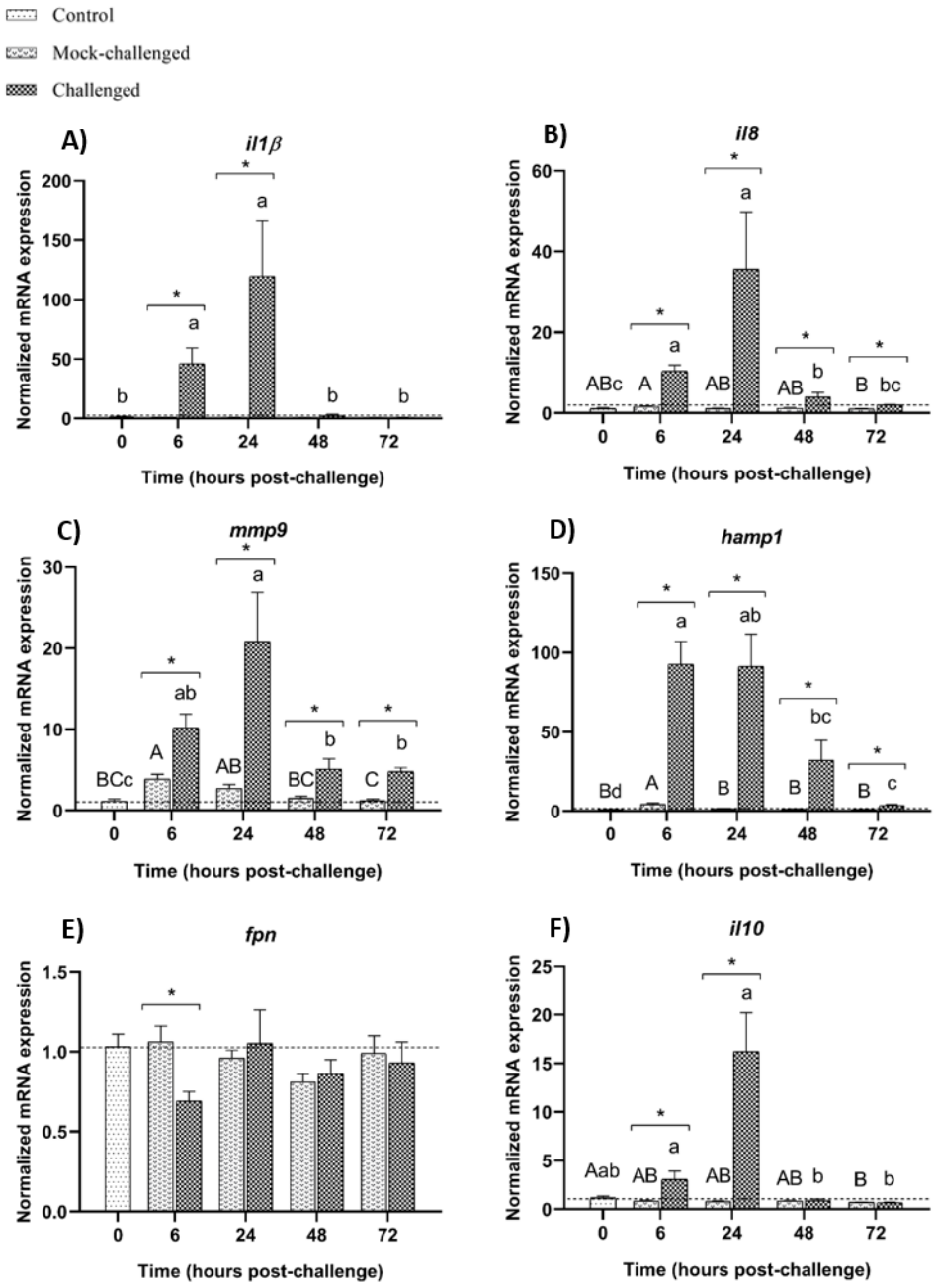

Figure 5: Expression of (A) *il1β*, (B) *il8*, (C) *mmp9*, (D) *hamp1*, (E) *fpn* and (F) *il10* in posterior-intestine of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 x 10^5 CFU mL^{-1} *T. maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters indicate differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

3.4. Discussion

With its ubiquitous distribution in marine environments, *T. maritimum* restrains the rearing of numerous fish species (Toranzo et al., 2005) and is considered one of the most threating bacterial infections (Cascarano et al., 2021) for aquaculture. However, available knowledge regarding *T. maritimum* pathogenesis is very limited. The present study evaluated cellular, humoral, oxidative and molecular short-term responses of European sea bass following *T. maritimum* bath challenge, providing an insight of the host's responses against this particular bacterium.

In 1990s a few pathogenicity studies were developed with commercial fish species, which included European sea bass (Bernardet et al., 1994), Atlantic salmon (Powell et al., 2004), rainbow trout, and greenback flounder (*Rhombosolea tapirina*) (Soltani et al., 1996), resulting in distinctive rates of cumulative mortality. Initially, prolonged immersion challenges of 18 h proved to be effective in reproducing tenacibaculosis in turbot (Avendaño-Herrera et al., 2006a). More recently, Mabrok et al. (2016) also used a prolonged bath of 24 h at 23°C to successfully challenge Senegalese sole with different strains of *T. maritimum* (ACC6.1, ACC13.1 and ACC20.1), resulting in cumulative mortalities ranging from 50% to 100%. In this same study, the ACC13.1 *T. maritimum* strain (the same used in the present study) (9.6×10^5 cells mL^{-1}) lead to cumulative mortalities of approximately 50% in Senegalese sole. In the last years, immersion challenge has been frequently used as a reliable method to experimentally reproduce tenacibaculosis in fish (Ferreira et al., 2023; Frisch et al., 2018; López et al., 2009; Nishioka et al., 2009; Resende et al., 2022; Valdes et al., 2021). In the present study, a mortality rate of 32.1% was obtained for the bath-challenged fish, while displaying tenacibaculosis clinical signs (e.g. ulcerative lesions in the skin and caudal fins, with haemorrhages, loose scales and abrasions). Since this challenge model was also able to successfully induce tenacibaculosis clinical signs and mortality in the bacteria exposed fish, it is suggested that immersion challenge (with a 2 h period of bacterial exposure) is an effective method to experimentally reproduce this disease in European sea bass. Similar mortality traits were also observed in previous studies following the same immersion challenge procedure (Ferreira et al., 2023; Resende et al., 2022).

Fish bath-challenged with *T. maritimum* presented a moderate decrease in total RBCs counts and a decreased haematocrit, suggesting the occurrence of anemia in response to infection. Further studies are required to elucidate if the observed anemia results from bacterial-induced destruction of RBCs or to an insufficient supply of healthy RBCs. The complete genome sequence of *T. maritimum* was able to offer some insights about virulence/associated genes which encode the biosynthesis of hemolysins (Pérez-Pascual

et al., 2017). The secretion of these hemolysins may be a possible explanation for the decrease of both RBCs and haematocrit in fish exposed to *T. maritimum*. However, these haematological parameters can also suggest anemia of inflammation, a host's off-target strategy, where erythrocyte lifespan is shortened by activating macrophages allowing the sequestration of iron from serum by these cells (Díaz et al., 2021; Ganz, 2018).

Analysis of the MCH showed that it increased after bacterial challenge, revealing a higher amount of hemoglobin inside the RBCs, also supported by a slight increase in MCHC. As previously mentioned, the presence of *T. maritimum* hemolysins may lead to RBCs lysis, which can lead to increased RBC production secondary to peripheral blood cell destruction, with the formed cells carrying more hemoglobin than normal-sized cells (Kaferle & Strzoda, 2009). Moreover, these incompletely processed RBCs, are slightly larger than the average RBC, increasing theses red cell indices (Borges & Sesti-Costa, 2022).

Neutrophils are responsible to assemble an early and potent antimicrobial response against invading pathogens, being the first leukocytes to be recruited to inflammatory sites (Havixbeck et al., 2016; Havixbeck & Barreda, 2015). Even though blood total leucocyte numbers did not change in response to bath-challenge with *T. maritimum*, there was an increase in neutrophils at 48 and 72 h, suggesting that they are involved in the response to *T. maritimum* infection. These data are in agreement with the observed increased expression of the pro-inflammatory cytokines at the mucosal organs already at 6 h following infection. IL8 has a potent chemotropic activity for neutrophils, monocytes, basophils and other immune cells (Remick, 2005), and several studies have demonstrated that increased expression of *il8* is related to acute inflammatory responses in teleosts upon infection with different bacterial species (Chen et al., 2018; Mohanty & Sahoo, 2010; Nguyen et al., 2017; Wang et al., 2017). Therefore, it is likely that the upregulation of *il8* expression in the mucosal organs of infected fish is related with the infiltration of inflammatory cells in the skin lesions, detected at 24 h post-challenge. The results obtained in the current study are in agreement with previous results obtained by Guardiola et al. (2019) in Senegalese sole, which revealed a significant increase of neutrophils at 48 and 72 h after bath-challenge with *T. maritimum* (Guardiola et al., 2019), as well as with results reported in other studies for several bacterial fish pathogens (Chen et al., 2020; Elbahnaswy & Elshopakey, 2020; Wang et al., 2019).

Moreover, besides being a key mediator of the immune system, neutrophils are also the main responsible for the production of myeloperoxidase in the plasma (Arnhold, 2020). Therefore, the increase of peroxidase in the plasma of infected specimens from the present study can be mostly explained by the neutrophilia observed in bath-challenged fish.

During inflammation, circulating monocytes migrate to infection sites, following conditioning by pro-inflammatory cytokines, microbial products and local growth factors, differentiating

afterwards into macrophages and dendritic cells (Shi & Pamer, 2011). The increased number of monocytes at 72 h post-challenge may indicate a host's attempt to increase the number of monocytes and their macrophage and dendritic-cell progeny, to fight against invading pathogens through phagocytosis, assist in the repair/regenerate of the damaged tissue, resolve the inflammation as well as to stablish the link with adaptive immunity by antigen presentation (Germic et al., 2019; Lichtnekert et al., 2013; Oishi & Manabe, 2018). Studies have demonstrated that thrombocytes are involved in innate immune and inflammatory responses in fish, participating in phagocytic activities and in the killing of internalized bacteria (Mirhaj et al., 2022; Nagasawa et al., 2014; Stosik et al., 2002). In the present study, the successive increase in circulating thrombocytes in bath-challenged fish up to 24 h following infection, allows to hypothesized that these cells are also migrating to infection sites. Since the teleost adaptive immune system implicates slow proliferation and maturation of lymphocytes (Whyte, 2007; Zhu & Su, 2022), their role in this time frame may not be as relevant as the other immune cells, as seen by the lack of variation during the first 72 h post-challenge. Plasma lysozyme activity decreased in infected fish, whereas bactericidal activity in the plasma only started to increase upon 48 h following bacterial bath challenge, which can be related with the late influx of phagocytes (e.g. neutrophils and monocytes) (Ellis, 2001) at the end of the time-course study. Many Gram-negative bacteria are able to produce lysozyme inhibitors that can significantly inhibited/decrease lysozyme activity in the host serum beginning from the early stages of host infection (Nishihara et al., 2022). Therefore, more studies would be needed to understand if *T. maritimum* could have similar evading mechanisms.

Pathogenic bacteria must be able to adapt to unpredictable environments and to cope with diverse stress-inducing factors, such as reactive oxygen species (ROS) produced by the host's macrophages (Pérez-Pascual et al., 2017). *T. maritimum*'s genome encodes three different superoxide dismutases (SodA, SodB, and SodC) and two catalases/peroxidase (KatA and KatG), which may imply that these bacteria use a complex mechanism to fight oxidative stress (Pérez-Pascual et al., 2017). Therefore, it is tempting to speculate that *T. maritimum* is able to use these enzymes to cope with and to modulate the host's immune response, resulting in the lack of changes regarding the analysed oxidative stress parameters.

In order to recognize bacteria, the host's immune system rely on pattern recognition receptors (PRRs), which are able to bind and recognize different pathogen-associated molecular patterns (PAMPs) and activate immune cells (Kawai & Akira, 2010; Li & Wu, 2021).

Although no major changes in the expression of the studied PRRs and transcription factors in response to *T. maritimum* infection were detected in the present study, an immune

response was indeed developed in bath-challenged fish, as a clear pro-inflammatory response was observed across all mucosal organs analysed.

Several studies revealed that, when challenged with pathogenic bacteria, teleosts upregulate the expression of *il1β* as initiation of the non-specific inflammatory response (Mohanty & Sahoo, 2010; Pressley et al., 2004; Rojo et al., 2007), with similar expression kinetics to the present study. The same upregulation is seen for mmp9, which increased expression was already linked to immune response against *Listeria monocytogenes* in infected zebrafish (Shan et al., 2016), *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) (Langevin et al., 2012), *Aeromonas hydrophila* in yellow catfish (*Pelteobagrus fulvidraco*) (Ke et al., 2015), and in peritoneal and peripheral blood leucocytes of stimulated common carp (*Cyprinus carpio*) (Chadzinska et al., 2008). The skin and posterior-intestine of challenged fish responded quite similarly, with *il1β*, *il8* and *mmp9* as the most highly induced genes, which provides evidence that an inflammatory response is activated upon infection with *T. maritimum*. The increase in *il1β* expression in these mucosal organs may result in increased mucus secretion (Lindenstrøm et al., 2004, 2006), which could be advantageous during a *T. maritimum*'s infection. The increased expression of *il8* and *mmp9* can also be intertwined with *il1β* expression, since this cytokine is able to promote the release of other cytokines and activate macrophages and other immune cells (Ellis, 2001; Secombes et al., 2001). Not only *T. maritimum* was able to trigger a pro-inflammatory response in the host, but also modulated the expression of genes related to iron metabolism regulation. The response of mucosal organs may suggest that one of the mechanisms employed by the host to withstand *T. maritimum* is associated with hepcidin, a small antimicrobial peptide that is involved in iron metabolism regulation in mammals (Anderson & Vulpe, 2009; Nicolas et al., 2002; Viatte & Vaulont, 2009). The iron control in the extracellular environment is a known innate immune strategy developed to deprive pathogens of iron, an essential nutrient for bacterial growth, replication, and metabolic processes (Page, 2019). This strategy, as a response to inflammatory stimuli, leads to high circulating levels of hepcidin, which in turn, negatively regulate the iron concentration in plasma (Nairz et al., 2010) through occlusion of the open-outward conformation (Aschemeyer et al., 2018) or by internalization and degradation of ferroportin, an iron exporter (Cassat & Skaar, 2013; Nemeth et al., 2004). Although there was no clear activation of the pro-inflammatory cytokine IL6, the main hepcidin inducer (Nemeth et al., 2004), other experiments already revealed that hepcidin can also be induced by IL1β (Katsarou & Pantopoulos, 2020; Lee et al., 2005). Therefore, it is possible that in this study, hepcidin regulation was due to the inflammatory signals conducted by IL1β, with the activation of NF-κB and JNK signalling pathways (Katsarou & Pantopoulos, 2020), leading to the seen transcriptional hamp induction and to the downregulation of fpn. The previously

described mild anaemic condition of challenged fish, that can be referred to as anemia of inflammation, could have some repercussions in the expression levels of hepcidin, since in an anaemic situation, hepcidin synthesis is suppressed (Nemeth & Ganz, 2006; Roy & Andrews, 2005; Viatte & Vaulont, 2009). However, this type of response was also recorded by Neves et al. 2011, where *Photobacterium damselae* spp. *piscicida* challenged fish demonstrated that hepcidin responds to infection by increasing its expression levels in sea bass liver, despite the anaemia demonstrated by the infected fish. This hepcidin dynamic was already described for other fish species (Bao et al., 2005; Douglas et al., 2003; Shen et al., 2019; Shike et al., 2002). These results are in accordance with other studies that also demonstrated the regulation of hepcidin and ferroportin by inflammatory signals induced by a pathogen (Agoro & Mura, 2016; Ma et al., 2020; Neves et al., 2011; Pulgar et al., 2015; Rodrigues et al., 2006).

Although it is known that IL1β is typically activated in situations where TNF-α is produced, no changes were detected in its expression after challenging sea bass with *T. maritimum* (Saperstein et al., 2009; Umare et al., 2014). In a study developed by Nascimento et al. (2007), a TNF-α up-regulation was briefly observed at 12 h post-infection with *P. damselae* subsp. *piscicida* in the head-kidney of European sea bass (Nascimento et al., 2007). Another study with *Streptococcus iniae*, presented the same results as the previous one for the head-kidney of European sea bass (El Aamri et al., 2015). Also, *tnfα* was significantly increased from 6 to 9 h post-infection in the head-kidney of European sea bass when intramuscularly infected with Betanodavirus (RGNNV), showing an early response of this gene (Vaz et al., 2022).

Since *tnf*-α and *il6* did not show any major differences in their expression, an earlier sampling time point could be valuable to add information about their possible upregulation in a response against *T. maritimum*. Due to its ubiquitous distribution and lack of host specificity, *T. maritimum* continuously inflicts significant losses among cultured marine species, as confirmed by the re-emerging nature of tenacibaculosis outbreaks in salmon farms globally (Mabrok et al., 2023). Although some progress has been made in the last decade regarding its pathogenic mechanisms, defining the host-pathogen relationship has proved to be very difficult to achieve. The present study offers a new insight regarding the mucosal innate immune response upon a pathogen inoculation pathway that mimics natural infection dynamics. In summary, the kinetics of the expression of molecular immune markers in gill, skin and posterior intestine of bath-challenged fish together with the findings observed for peripheral leucocytes demonstrate the occurrence of a pro-inflammatory response against *T. maritimum* in the studied mucosal organs, with a faster kinetic in the gills, which may suggest that this pathogen can use gill mucosa as a route of entry into the

fish. The analysis of the humoral parameters suggests that the local response at the mucosal organs is followed by a response at systemic level.

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CHAPTER 4

Mucosal transcriptome and proteome analyses in European sea bass (*Dicentrarchus labrax*) following *Tenacibaculum maritimum* bath challenge

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Mucosal transcriptome and proteome analyses in European sea bass (*Dicentrarchus labrax*) following *Tenacibaculum maritimum* bath challenge

Abstract

Tenacibaculum maritimum is a relevant fish pathogen that causes serious losses in the marine aquaculture industry. In recent years, this Gram-negative filamentous bacterium has been considered an emergent pathogen due to the increasing number of worldwide outbreaks in salmon industries. Additionally, it also affects the production of commercially important fish species, such as rainbow trout, chinook salmon, and European sea bass, underlying the need to uncover the cellular and molecular mechanisms behind its pathogenesis. *T. maritimum* has a preference for mucosal tissues, causing severe necrotic and ulcerative lesions on the body surface, leading to considerable economic losses. Therefore, the present study combined transcriptome and proteome analysis of skin tissue and mucus of European sea bass (*Dicentrarchus labrax*) to evaluate the mucosal immune response following *T. maritimum*'s bath challenge. The results indicate a complex local mucosal response in the skin and mucus upon *T. maritimum* bath challenge, involving modulation of re-epithelization and inflammatory processes. This suggests that the host responds to *T. maritimum* infection but also to the bacterial-induced wounds typically associated with tenacibaculosis.

Keywords: Tenacibaculosis; Aquaculture; Mucosal Immunity; Omics

Highlights

• Bath-challenged with *T. maritimum* up-regulates the expression of innate immunity genes (inflammatory mediators, acute phase response, iron withholding, and wound healing);

• Proteins related to wound healing processes and innate immunity were upregulated in challenged fish;

• Down-regulation of collagens and other ECM components, indicating re-epithelization processes;

4.1. Introduction

The rise of aquaculture as a solution for overexploited wild fish populations and as an alternative source of protein (Mavraganis et al., 2020) can be severely impacted by environmental and health aspects. Disease outbreaks contribute to significant economic losses to the aquaculture industry (Maldonado-Miranda et al., 2022). Many of these diseases are caused by pathogenic Gram-negative bacteria (Bondad-Reantaso et al., 2021; Maldonado-Miranda et al., 2022), such as *Tenacibaculum maritimum*. Since it was first described as a Flexibacter infection associated with mortalities recorded in reared red seabream (*Pagrus major*) and blackhead seabream (*Acanthopagrus schlegelii*) in Japan (Masumura & Wakabayashi, 1977), this disease has been causing high mortality rates in worldwide aquaculture (Avendaño-Herrera et al., 2006), affecting valuable species, such as Atlantic salmon (*Salmo salar*) (Apablaza et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Valdes et al., 2021), wedge sole (*Dicologlossa cuneate*) (López et al., 2009), gilthead sea bream (*Sparus aurata*) (Moustafa et al., 2015) and European sea bass (*Dicentrarchus labrax*) (Yardimci & Timur, 2015). European sea bass is a crucial species for the Mediterranean aquaculture industry (Stavrakidis-Zachou et al., 2019). In recent years, Mediterranean aquaculture production has been affected by bacterial diseases, which, according to Muniesa et al. (2020), account for 75% of the disease reports in fish production units. Among these diseases, tenacibaculosis was among the most frequently reported in European sea bass, demonstrating the potential to become a severe drawback for central, eastern, and western Mediterranean aquaculture (Muniesa et al., 2020).

Tenacibaculosis outbreaks are commonly associated with gross lesions on the body, which can include ulcerative and necrotic lesions, eroded/hemorrhagic mouth, necrotic gills, frayed fins, and tail rot, increasing the possibility of secondary infections by other opportunistic pathogens (Gourzioti et al., 2018; Mabrok et al., 2023). This pathology associated with *T. maritimum*'s infection indicates that the skin mucosa is one of the most affected tissues during tenacibaculosis. Several studies have shown the existence of skin lesions with loss of epithelial layers, with a detachment of skin dermis and epidermis, exposing collagen fibers, and deep necrosis reaching the musculature with inflammatory response around the affected areas (Ferreira et al., 2023; Vilar et al., 2012; Yardimci & Timur, 2015). The ulcerative areas can also exhibit whitish patches caused by *T. maritimum*, revealing, at a histopathological level, aggregations of filamentous bacteria and leucocyte infiltration (Lopez et al., 2022). Moreover, *T. maritimum* can survive in and deal with the bactericidal activity of the host's skin mucus, denoting that this matrix can act as a reservoir for these bacteria (Guardiola et al., 2019; Mabrok et al., 2016, 2023). It is reasonable to speculate that upon infection, the skin-associated lymphoid tissue's capacity to swiftly

respond against *T. maritimum* may determine the outcome of the infection, with decisive immune-related pathways and mediators being activated in this process. Nevertheless, the host response in the skin mucosa level against *T. maritimum* remains largely unexplored. Considering *T. maritimum*'s pathogenic potential, there is an urgent need to deepen the knowledge of the complex dynamic established between this pathogen and its hosts. Understanding the molecular mechanisms behind the immune response against *T. maritimum* in European sea bass may contribute to identifying relevant virulent factors and facilitate vaccine development, improving immunity and disease prevention.

In this study, transcriptome and proteome analyses of skin tissue and epidermal mucus from European sea bass were performed, to investigate the mucosal immune responses triggered by *T. maritimum*'s bath challenge. Considering that fish mucosa is one of *T. maritimum*'s targets for entering the host and establishing infection, the results of these analyses offer a unique perspective of the global gene regulation triggered by this pathogen during infection.

4.2. Material and Methods

4.2.1. Bacterial culture and inoculum preparation

T. maritimum strain ACC13.1, serotype O3 (Avendaño-Herrera et al., 2005), formerly isolated from Senegalese sole, was gently provided by Professor Alicia E. Toranzo of the Department of Microbiology and Parasitology at the Faculty of Biology (University of Santiago de Compostela, Spain). The stocks were stored at -80°C, and recovery from frozen stocks and inoculum preparation was performed according to Ferreira et al. (2023). Briefly, bacteria were grown in 50 mL of marine broth (MB, Laboratories CONDA, Spain) at 25°C with continuous agitation (180 rpm) for 48 h. Turbidity was measured and adjusted at 600 nm, and bacteria were collected, centrifuged (3,000 × *g*, 10 min, RT), and resuspended in MB adjusting concentration at 5×10^5 CFU mL^{-1} as described elsewhere (Mabrok, 2016).

4.2.2. Fish husbandry and experimental design

The experiments were approved by the CIIMAR Animal Welfare Committee (ORBEA-CIIMAR_26_2018) and DGAV (Portuguese Veterinary Authority) and were carried out under license number 0421/000/000/2020 in a registered facility (N16091.UDER). The current study was conducted under the supervision of researchers accredited in laboratory animal science by the Portuguese Veterinary Authority following FELASA category C

recommendations and in agreement with the guidelines on the protection of animals used for scientific purposes according to the European Union directive (2010/63/EU).

Juvenile European sea bass (45.5 ± 8.1 g) with no history of tenacibaculosis were acquired from a commercial fish farm in Portugal and kept in quarantine at CIIMAR fish-holding facilities for four weeks. A system of recirculating seawater was maintained at 21.8 ± 0.4 °C, 34.2 ± 0.4‰ salinity, 8.2 ± 0.2 mg mL^{-1} dissolved oxygen, and a photoperiod of 12 h light and 12 h dark. Water quality was maintained through mechanical and biological filtration, and fish were fed a commercial diet (Aquasoja, Portugal) consisting of 2% of their body weight divided into two meals per day. Every day, commercial kits were used to measure the amounts of ammonia and nitrite. Ten randomly chosen fish were sampled for histopathological evaluation. Previous to the bacterial challenge, fish were randomly placed into two closed, recirculating seawater systems (10 kg m^{-3} stocking density, n = 25 fish per 0.11 m^3 tank), one for the mock-challenged fish and another for the challenged fish, each with four tanks for sampling purposes (4 replicates for each treatment) and two tanks to follow cumulative mortality (two replicates for each treatment). The fish were allowed to acclimate for a week, and after fasting for 24 h were bath-challenged with 5×10^5 CFU mL^{-1} *T. maritimum* (ACC13.1) for 2 h (the bacterial doses were based on a pre-challenge to determine the LD_{30} for this strain) or mock-challenged with MB instead of bacterial inoculum, as control. The challenge was carried out in 50 L tanks with 25 kg m^{-3} of stocking density and vigorous aeration. To replicate the temperature circumstances at which outbreaks of tenacibaculosis occur, the water temperature was raised to 25°C throughout the challenge (Mabrok, 2016; Yamamoto et al., 2010).

4.2.3. Sampling

Samples were collected at 24 h post-challenge, after euthanizing the fish with an overdose of 0.7 mL L^{-1} (2-phenoxyethanol; Merck, ref. 807291, Germany). Three fish were removed from each tank (n=12 per treatment). A sample of the skin was collected across the midline of the fish, beneath the dorsal fin, without any muscle and was stored in RNA later (at a proportion of 1/10 w/v) at 4 ºC for the first 24 h, and then at -80 ºC. For skin mucus collection, excess water was removed with paper towels. Mucus samples were obtained by gently scraping the dorsolateral surface of fish on both sides using a cell scraper, with enough care to avoid scale removal and contamination with blood and urogenital and intestinal excretions. The samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

4.2.4. RNA isolation, library preparation, and sequencing

The skin (up to 300 mg) was weighed and homogenized in a Precellys Evolution homogenizer at 6000 × *g* (2 × 20 s, 4 ºC) in 500 µL of Trizol (NZYTech, Lisbon, Portugal). The samples were then mixed with 150 µL chloroform at 4 ºC, vortexed, and centrifuged at 12,000 × *g* for 15 min at 4 ºC. After transferring the resulting aqueous phase to a clean tube, 300 µL of 70% ethanol was added, mixed, and placed in NZYSpin Binding columns. The NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) was used to complete the remaining steps of total RNA isolation according to the manufacturer's instructions. Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) was used to determine sample RNA concentration, and the DeNovix DS-11 FX (Wilmington, DE, USA) to ensure that ratios of absorbance at 260 nm/280 nm were between 1.9–2.1. The RNA from three individual fish was pooled together (using the same RNA quantity for each fish) and quantified again as described above. Library preparation and sequencing were conducted by Novogene company (Cambridge, UK). The cDNA libraries were sequenced on an Illumina PE150 with an average of 20 M reads. All the analyzed samples passed the RNA quality control of Novogene (average RNA integrity number (RIN) of 9.94).

4.2.5. Analysis of differentially expressed genes

The quality of sequencing reads was controlled with FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Low-quality reads (Phred quality score < 15 and read length < 30 bp) were trimmed using the Fastp program (https://github.com/OpenGene/fastp). With RStudio and the biomaRt package, European sea bass transcriptome annotations and gene names were extracted from Ensembl (http://www.ensembl.org, accessed on 9th May 2023) using BioMart (http://www.biomart.org/, accessed on 9th May 2023). To quantify expression, the RNA-seq output files were pseudoaligned to the sea bass reference transcriptome with Kallisto (https://pachterlab.github.io/kallisto/, accessed on 9th May 2023).

We used the DESeq2 package to analyze differentially expressed genes (DEGs) (https://bioconductor.org/packages/release/bioc/html/DESeq2.html, accessed on 12th May 2023). Genes were evaluated as significantly differentially expressed if their adjusted *p*-value < 0.05. To better understand the functional relevance of the DEGs, all DEGs were further categorized by GO functional enrichment using ShinyGO (http://bioinformatics.sdstate.edu/go/, accessed on 2nd October 2023) platforms against the annotated genome for European sea bas.

The genes that remained unidentified were further characterized by the alignment of peptide sequences to the corresponding gene identification, generated through the Ensemble database (https://www.ensembl.org/index.html, accessed on 26th September 2023), against the NCBI (National Center for Biotechnology Information) database using the protein-protein BLAST (Basic Local Alignment Search Tool) tool (BLASTp, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 26th September 2023). Information regarding the best-matching protein sequences (plus accession number, E-value, query cover, percentage of identity, and query cover) was collected from the BLAST results. The number of unique DEGs (DEGs that only appear once) in each main GO functional Domain was determined. These were divided by the number of upregulated or downregulated total genes and named "unigenes".

4.2.6. Acquisition and analysis of proteomics data

Due to the limited amount of mucus collected from individual fish, mucus samples from three fish from the same tank were pooled to represent one biological replicate. Individual mucus samples were centrifuged (400 × *g*, 15 min, 4°C), the supernatants collected, and their total protein concentrations were estimated using a bicinchoninic acid Pierce assay kit (BCA; Thermo Fischer Scientific, USA). Mucus from three fish were pooled, to obtain samples containing 90 µg of protein (30 µg from each fish) before subsequent analysis.

Protein identification and quantitation were performed by nanoLC-MS/MS. This equipment is composed of an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Five hundred nanograms of peptides of each sample were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% ACN, 0.1% FA at 10 µL min^{-1}. After 3 min loading, the trap column was switched in-line to a 50 cm × 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 250 nL min-1. Separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99 % B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage

was 1.9 kV. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chrom. peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), AGC target 3 × 106, maximum injection time 120 ms; dd settings: minimum AGC target 8 × 103, intensity threshold 7.3 × 104, charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans 1, resolution 35 k (m/z 200), AGC target 2 × 105, maximum injection time 110 ms, isolation window 2.0 m/z, isolation offset 0.0 m/z, dynamic first mass, and spectrum data type profile. The protein identification analysis for European sea bass (*D. labrax*) taxonomic selection, the whole genome assembly, and the corresponding annotation file was retrieved from Ensemble (dlabrax2021), and isoforms were removed using AGAT software (Version v0.8.0). The raw data was searched against the retrieved non-redundant proteome. A common protein contaminant list from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry, Munich, Germany) was also included in the analysis. Two protein search algorithms were considered: (i) the mass spectrum library search software MSPepSearch, with the NIST human HCD Spectrum Library (1,127,970 spectra and (ii) the Sequest HT tandem mass spectrometry peptide database search program. Both search nodes considered an ion mass tolerance of 10 ppm for precursor ions and 0.02 Da for fragment ions. The maximum allowed missing cleavage sites was set as 2. Cysteine carbamidomethylation was defined as constant modification. Methionine oxidation, asparagine and glutamine deamidation, peptide N-terminus Gln->pyro-Glut, protein N-terminus acetylation, and loss of methionine and Met-loss+Acetyl were defined as variable modifications. Peptide confidence was set to high. The Inferys rescoring node was considered for this analysis. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target False Discovery Rate-FDR 1%; validation based on *q*-value. Protein-label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performed at the consensus step with the following parameters: unique plus razor peptides were considered, precursor abundance based on intensity, and normalization based on total peptide amount.

4.2.7. Differential enrichment analysis of proteomic data

Statistical differences between experimental groups were analyzed in R software (R Core Team, 2021). Only proteins identified in at least 3 of the 4 replicates were filtered for further analysis. Data were normalized with the *normalize_vsn* function, and missing values were imputed using the impute function from the DEP package (Differential Enrichment analysis of Proteomics data, accessed on 3rd February 2023). For this imputation, a probabilistic

minimum with the "MinProb" method, uses a random draw from a Gaussian distribution centered around a minimum fixed value. With these data of protein expression, differences between treatments were tested with multiple comparisons based on the Bayes sphericity test, implemented in the *test_diff* function from the DEP package (accessed on 3rd February 2023). Adjusted *p*-values for false discovery rate (*q*-values) were obtained and set to a $p \leq 0.05$. The presence of a significant *p*-value was used as a criterion to define differentially expressed proteins (DEPs).

The number of unique DEPs (DEPs that only appear once) in each main GO functional Domain was determined. These were divided by the number of upregulated or downregulated total proteins and named "uniproteins".

4.3. Results

4.3.1. Bacterial challenge

Fish bath-challenge with 5×10^5 CFU mL^{-1} *T. maritimum* (strain ACC13.1) showed 32.1% of cumulative mortality, while no mortality was recorded in the mock-challenged group (Fig. 1; n = 30 fish per treatment, X2 < 0.0008 for comparisons among treatments).

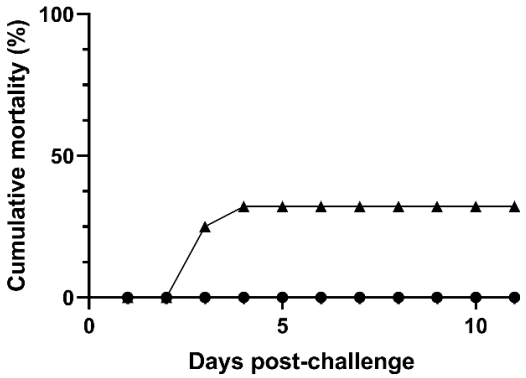

Figure 1: Mortality of European sea bass (*Dicentrarchus labrax*) (45.5 ± 8.1 g) after bath challenge with 5×10^5 CFU mL^{-1} *Tenacibaculum maritimum* (▲) or with marine broth MB (●) (n = 30 fish *per* treatment).

4.3.2. Identification and GO annotation of DEGs

An average of 46,263,845 ± 1.1559 clean reads with a Q30 value of 98.1825% ± 0.0457% were obtained from the European sea bass mock-challenged group, and 48,303,957 ± 4.1289 clean reads with a Q30 value of 94.4075% ± 0.1106% were obtained for the *T. maritimum* challenged group, respectively. The average GC% was 48.7975 ± 0.1352% and

48.4150 ± 0.3663% for the mock- and *T. maritimum* challenged groups, respectively (Table S1, Appendix III). These results indicate that the sequencing data is of high quality and could be used in subsequent analysis.

A total of 23,134 transcripts were detected in the skin tissue of European sea bass. From these, 3,843 were categorized as DEGs, 1,450 down-regulated, and 2,393 upregulated in the skin of infected fish (Fig. 2). A considerable portion of the down-regulated DEGs presented a relatively low Log_2 Fold change, with 864 genes (approximately 59.6%) displaying a Log_2 Fold change between 0.2 and 1. Only a small number of genes (61 genes, 4.2%) presented a Log_2 Fold change ≥ 2 (Fig. 2). The upregulated genes followed the same tendency, with only 270 genes (11.3%) of the total DEGs showing a Log_2 Fold change ≤ 2 (Fig. 2). Principal component analysis (PCA) of the gene expression data, separated and grouped animals based on mock or challenged with bacteria (Fig. S1, Appendix III), with one of the challenges replicates presenting a slightly deviated behavior. Nevertheless, this supports significant changes in the transcriptional regulation of DEGs between mock and challenged fish.

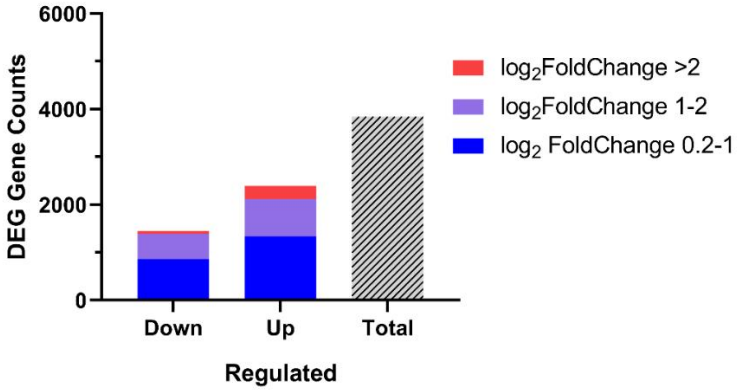

Figure 2: Number of differentially expressed genes in the skin tissue of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum*, according to displayed Log_2 Fold Change, for both down- and upregulated genes.

The distribution trends of the top 15 DEGs were mapped in a heat map plot (*q*-value < 0.05) (Fig. 3). Several of the upregulated DEGs are key mediator genes that participate in inflammation, eicosanoids metabolism, cell adhesion, and oxidative innate immune responses, like hepcidin-1 (*hamp1*), arachidonate 15-lipoxygenase (*alox15b*), CD209 antigen-like protein C (*cd209c*), interleukin 1β (*il1β*), microfibril associated protein 4 (*mfap4*), phospholipase A2 inhibitor and LY6/PLAUR domain-containing (*pinlyp*), interleukin-8 (*cxcl8*), and NADPH oxidase organizer 1b (*noxo1b*) (Fig. 3).

To acquire information about the biological functions of DEGs, the up-and down-regulated genes were categorized according to GO Terms (ShinyGO 0.77 software). The upregulated

set of genes was classified into three major functional GO domains: biological processes (31.13% unigenes), cellular component (13.37% unigenes), and molecular function (26.41% unigenes). Nevertheless, a considerable number of genes (29.1% unigenes) could not be categorized into these domains, possibly due to incomplete annotations and/or the lack of characterization of these genes. The biological processes domain had several dominant terms related to functions directed to RNA processing and protein production. These included ribosome biogenesis (GO:0042254), ribonucleoprotein complex biogenesis (GO:0022613), and NcRNA processing (GO:0034470) (Table 1). For the cellular component, the most enriched pathways were the peptidase complex (GO:1905368), proteasome complex (GO:0000502), and endopeptidase complex (GO:1905369) (Table 1). Regarding the molecular function category, unfolded protein binding (GO:0051082), catalytic activity, acting on a nucleic acid (GO:0140640), and RNA binding (GO:0003723) were also enriched (Table 1).

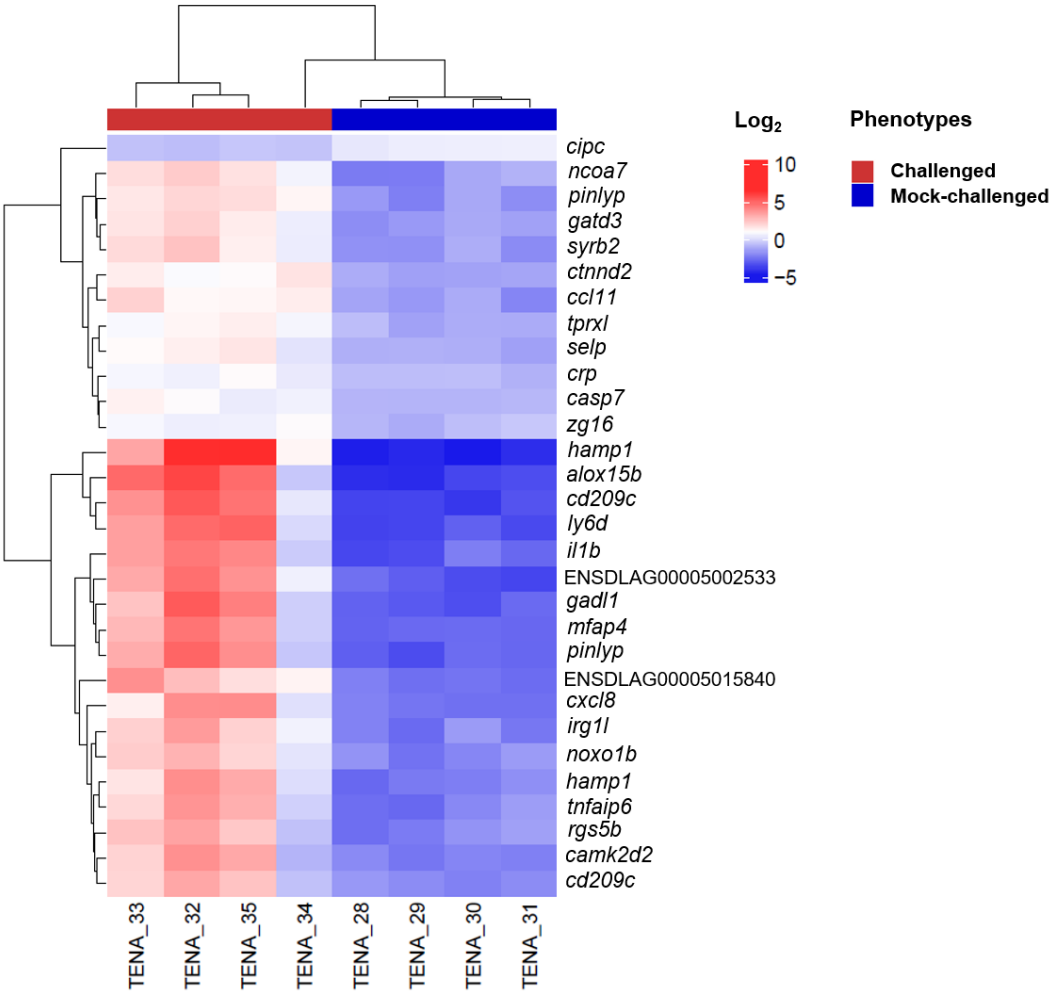

Figure 3: Heat map of the top 15 DEGs between mock-challenged (phenotype blue) and challenged (phenotype red) European sea bass (*Dicentrarchus labrax*) at 24 hours post-challenged (*q*-value < 0.05). Red: upregulated, and blue: downregulated genes.

To narrow down to the most highly modulated genes, the enriched pathways associated with the upregulated DEGs (Log_2 Fold Change ≥ 2) (Fig. 4) were selected and assigned to the three functional GO domains: biological processes (24.07% unigenes), cellular component (12.22% unigenes), and molecular function (14.44% unigenes). Briefly, the most dominant terms in biological processes corresponded to immune response such as defense response (GO:0006952), response to external stimulus (GO:0009605), biological process involved in interspecies interaction between organisms (GO:0009605), immune response (GO:0006955), and response to biotic stimulus (GO:0009607), all presenting an enrichment FDR value lower than 1.9×10^{-7} (Fig. 4). Apart from these immune-related pathways, other pathways, like regulation of plasminogen activation (GO:0010755), and collagen metabolic process (GO:0032963), were also enriched in biological processes. For the cellular component category, the most dominant subcategories were the extracellular region (GO:0005576) and extracellular space (GO:0005615) (Fig. 4).

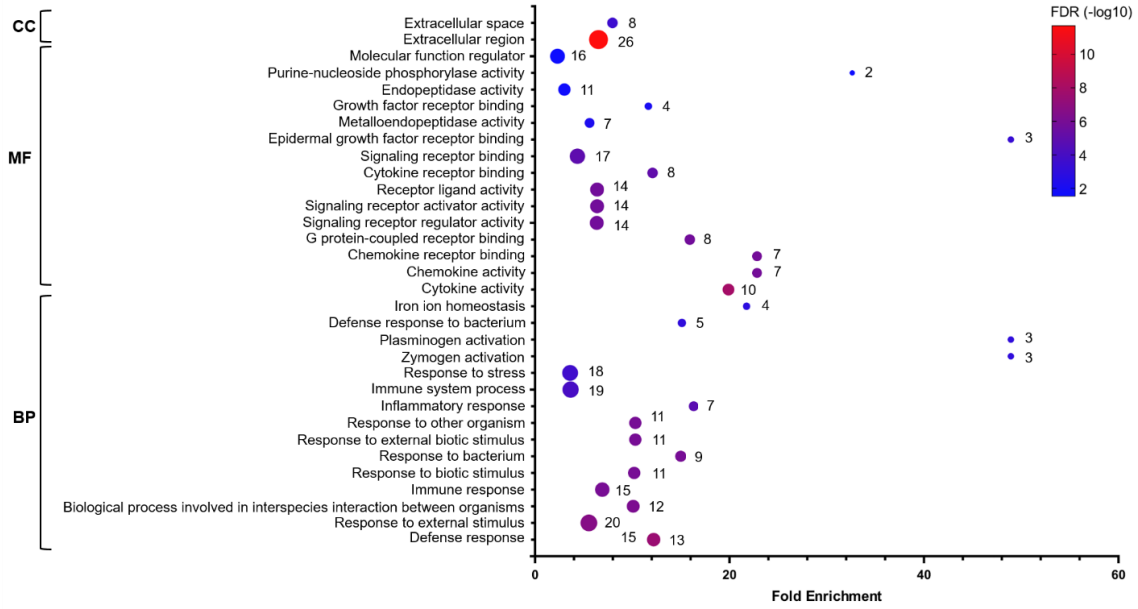

Figure 4: GO functional categories analysis of up-regulated DEGs (top 15) in the skin of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold change of expression ≥ 2 and *q*-value < 0.05). CC - Cellular Component; MF - Molecular Function; BP - Biological Processes; the numbers represent the gene count for each enriched pathway.

For the molecular function domain, the most enriched terms were majorly related to cytokine and signalling, such as cytokine activity (GO:0005125), chemokine activity (GO:0008009), chemokine receptor binding (GO:0042379), G protein-coupled receptor binding (GO:0001664), and signalling receptor regulator activity (GO:0030545), all presenting an enrichment FDR value lower than $1.2x10^{-6}$. The epidermal growth factor receptor binding (GO:0005154), and growth factor receptor binding (GO:0070851), and

metalloendopeptidase activity (GO:0004222) also revealed to be enriched. Furthermore, the down-regulated set of genes was only classified into two functional GO domains: biological processes (24.00%) and molecular function (17.66%). No pathways were enriched for the cellular component domain. Within the biological processes domain, the most enriched terms were related to the cell surface signalling pathway (GO:0007166) and regulation of several biological functions, including regulation of gene expression (GO:0010468) and RNA metabolic process (GO:0051252) (Table 1). Regarding the molecular function domain, extracellular matrix structural constituent (GO:0005201), calcium ion binding (8.87%, FDR of 1.5×10^{-2}; GO:0005509), and nuclear receptor activity (GO:0005509), were among the most enriched. ShinyGO could not perform the functional enrichment analysis for the down-regulated DEGs with Log_2 Fold Change ≤ 2.

Table 1: List of top 5 enriched pathways of each GO Domain (sorted by Enrichment FDR of each major functional GO Terms) of DEGs in the skin of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (BP - Biological Processes; CC - Cellular Component; MF - Molecular Function) (*q*-value ≤ 0.05). * The percentage of DEGs found, divided by the number of genes in the pathway.

Enrichment FDR	Fold Enrichment	Genes (%)*	Pathway	Associated GO Term	GO Domain	Regulated
4.4×10^{-25}	6.85	62.12	Ribosome biogenesis	GO:0042254	BP	Up
1.1×10^{-21}	5.85	48.28	Ribonucleoprotein complex biogenesis	GO:0022613	BP	Up
2.3×10^{-16}	4.71	36.61	NcRNA processing	GO:0034470	BP	Up
6.5×10^{-16}	3.02	23.76	RNA processing	GO:0006396	BP	Up
2.1×10^{-14}	3.74	31.19	NcRNA metabolic process	GO:0034660	BP	Up
4.8×10^{-14}	6.13	46.43	Peptidase complex	GO:1905368	CC	Up
1.3×10^{-13}	7.15	53.85	Proteasome complex	GO:0000502	CC	Up
1.3×10^{-12}	6.32	50.00	Endopeptidase complex	GO:1905369	CC	Up
7.7×10^{-11}	6.62	66.67	Nucleolus	GO:0005730	CC	Up
7.6×10^{-9}	1.65	12.33	Cytoplasm	GO:0005737	CC	Up
9.3×10^{-7}	5.04	41.46	Unfolded protein binding	GO:0051082	MF	Up
9.3×10^{-7}	2.20	19.23	Catalytic activity, acting on a nucleic acid	GO:0140640	MF	Up

1.5×10^{-6}	2.05	16.30	RNA binding	GO:0003723	MF	Up
1.5×10^{-6}	2.05	14.04	Hydrolase activity, acting on acid anhydrides	GO:0016817	MF	Up
2.2×10^{-6}	2.03	13.89	Hydrolase activity, acting on acid anhydrides and phosphorus-containing anhydrides	GO:0016818	MF	Up
5.4×10^{-6}	2.05	10.8	Cell surface receptor signalling pathway	GO:0007166	BP	Down
2.1×10^{-3}	1.55	7.5	Regulation of gene expression	GO:0010468	BP	Down
2.1×10^{-3}	1.58	7.64	Regulation of RNA metabolic process	GO:0051252	BP	Down
2.1×10^{-3}	1.60	7.68	Regulation of nucleic acid-templated transcription	GO:1903506	BP	Down
2.1×10^{-3}	1.60	7.68	Regulation of RNA biosynthetic process	GO:2001141	BP	Down
1.0×10^{-4}	5.98	34.29	Extracellular matrix structural constituent	GO:0005201	MF	Down
1.5×10^{-2}	1.66	8.88	Calcium ion binding	GO:0005509	MF	Down
4.0×10^{-2}	3.83	23.08	Nuclear receptor activity	GO:0004879	MF	Down
4.0×10^{-2}	2.27	14.38	Guanyl-nucleotide exchange factor activity	GO:0005085	MF	Down
4.0×10^{-2}	1.34	6.85	Metal ion binding	GO:0046872	MF	Down

4.3.3. Analysis of skin DEGs of European sea bass bath-challenged with *T. maritimum*

Among the DEGs in the skin, genes encoding several enzymes, collagens, and other components belonging to the extracellular matrix (ECM) were identified (Table 2). Essential regulators of homeostasis and coagulation (e.g., coagulation factor V, heparan - Table 2 - and platelet-activating factor receptor - Table 3) were increased in the skin of challenged fish.

Enzymes responsible for the degradation of ECM components and involved both in inflammation and tissue remodelling (e.g., matrix metallopeptidase 13a and 9) (Kudo et al., 2012; LeBert et al., 2015), as well as enzymes that have pivotal roles in wound healing (e.g., ADAMTS proteases 1 and 9) (Tayman & Koyuncu, 2023), were also upregulated after bath challenge with *T. maritimum* (Table 2). An upregulation was also observed for tissue inhibitors of metalloproteinase (TIMPs) and hyaluronidase. Different collagens, predicted to be present in different structures, were identified. Collagen types that compose the skin (e.g., type VII associated with the basement membrane or type XXVIII associated with the

peripheral nervous system) (Grimal et al., 2010; Theocharidis & Connelly, 2019) or act as nucleators for fibrillogenesis of collagens I (e.g., type XI) (Kadler et al., 2008) were down-regulated. Moreover, other associated structural proteins of ECM, such as tenascin N (1.73-fold) and claudin I (1.64-fold), displayed decreased expression. Growth factors that are regarded as impactful in the rate and quality of wound healing were upregulated, which included transforming growth factor alpha, (2.75-fold), together with epidermal growth factors (EGF)-family growth factors and transcription factors (Demidova-Rice et al., 2012). Fibroblast growth factor binding protein 1b, on the contrary, was decreased in expression (2.30-fold). Adhesion molecules (e.g., neuronal cell adhesion molecule and adhesion G protein receptors) were also down-regulated. These molecules not only participate in cell-cell interactions (i.e., adhesion and migration processes) (Lala & Hall, 2022) but also in the immune response (e.g., neuronal cell adhesion molecule is expressed in lymphoid cells and is induced by LPS) (Wu et al., 2015).

Table 2: List of extracellular matrix (ECM)-related DEGs in the skin of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Gene ID	Gene (Acron.)	Gene (name)	Log_2Fold Change	*q*-Value	Regulated
		Enzymes			
ENSDLAG00005008130	*mmp13a*	Matrix metallopeptidase 13a	6.55	2.3×10^{-8}	Up
ENSDLAG00005018682	*mmp9*	Matrix metallopeptidase 9	4.77	7.7×10^{-10}	Up
ENSDLAG00005001125	*adam8a*	ADAM metallopeptidase domain 8a	3.01	7.6×10^{-5}	Up
ENSDLAG00005022620	*adamts9*	ADAM metallopeptidase type 1 motif, 9	2.97	8.4×10^{-5}	Up
ENSDLAG00005004456	*mmp17*	Matrix metallopeptidase 25b	2.15	4.6×10^{-7}	Up
ENSDLAG00005008994	*adamts1*	ADAM metallopeptidase type 1 motif, 1	1.69	5.9×10^{-3}	Up
ENSDLAG00005006737	*ch25h*	Cholesterol 25-hydroxylase	1.69	4.3×10^{-5}	Up
ENSDLAG00005006990	*timp4*	TIMP metallopeptidase inhibitor 4	1.66	8.9×10^{-3}	Up
ENSDLAG00005022944	*adam28*	ADAM metallopeptidase domain 28	1.58	6.1×10^{-3}	Up
ENSDLAG00005007818	*adamts17*	ADAM metallopeptidase type 1 motif, 17	-1.73	9.7×10^{-4}	Down
ENSDLAG00005014230	*ADAMTS16*	ADAM metallopeptidase type 1 motif, 16	-1.79	4.1×10^{-2}	Down
		Collagens			

ENSDLAG00005018479	col28a1	Collagen alpha-1(XXVIII) chain isoform X1	-2.10	1.9×10^{-2}	Down
ENSDLAG00005023821	*col11a1a*	Collagen, type XI, alpha 1a	-1.75	3.0×10^{-2}	Down
ENSDLAG00005033274	*col7a1*	Collagen, type VII, alpha 1	-1.70	6.0×10^{-3}	Down
		Components associated with ECM			
ENSDLAG00005022480	*emilin2a*	Elastin microfibril interfacer 2a	1.88	1.35×10^{-3}	Up
ENSDLAG00005007510	*rspo1*	R-spondin 1	-1.63	1.4×10^{-2}	Down
ENSDLAG00005026355	*cldni*	Claudin I	-1.64	1.4×10^{-2}	Down
ENSDLAG00005005467	*tnn*	Tenascin N	-1.73	1.8×10^{-3}	Down
ENSDLAG00005003839	*matn4*	Matrilin 4	-2.01	2.5×10^{-3}	Down
ENSDLAG00005013722	*cilp2*	Cartilage intermediate layer protein 2	-2.30	3.6×10^{-7}	Down
		Adhesion related-molecules			
ENSDLAG00005021864	*nrcama*	Neuronal cell adhesion molecule a	-1.54	1.1×10^{-3}	Down
ENSDLAG00005018728	*adgrb2*	Adhesion G protein-coupled receptor B2	-1.55	4.5×10^{-2}	Down
ENSDLAG00005016946	*adgrl2a*	Adhesion G protein-coupled receptor L2a	-1.59	1.5×10^{-3}	Down
		ECM-associated growth factors			
ENSDLAG00005027901	*tgfa*	Transforming growth factor, alpha	2.75	2.4×10^{-2}	Up
ENSDLAG00005028532	*tcf21*	Transcription factor 21	2.72	6.3×10^{-4}	Up
ENSDLAG00005018941	*hbegfa*	Heparin-binding EGF-like growth factor a	2.08	5.9×10^{-4}	Up
ENSDLAG00005030781	*bmp10*	Bone morphogenetic protein 10	1.97	2.1×10^{-2}	Up
ENSDLAG00005020511	*efemp2a*	EGF containing fibulin extracellular matrix protein 2a	1.60	4.8×10^{-3}	Up
ENSDLAG00005033022	*serinc4*	Serine incorporator 4	1.58	1.6×10^{-2}	Up
ENSDLAG00005022186	*nog*	Noggin	-2.14	2.0×10^{-3}	Down
ENSDLAG00005027041	*fgfbp1b*	Fibroblast growth factor binding protein 1b	-2.30	1.8×10^{-2}	Down
		Coagulation factors			
ENSDLAG00005025738	*f5*	Coagulation factor V	3.17	3.5×10^{-4}	Up
ENSDLAG00005032596	*hs3st1*	Heparan sulfate-glucosamine 3-sulfotransferase 1	1.93	8.15×10^{-3}	Up

Besides these players of wound-healing, several genes that encode prostanoid biosynthesis enzymes (e.g., prostaglandin-endoperoxide synthase 2b, prostaglandin E synthase, prostaglandin I2 synthase) were also increased in the skin of challenged fish (Table 3).

A plethora of cytokines, chemokines, and regulators of lipid origin (eicosanoids) were differentially expressed, indicating an inflammatory response scenario (Table 3). Most cytokines and chemokines were increased in expression, especially the pro-inflammatory cytokine, interleukin-1 beta (7.38-fold). Others followed the same tendency, like interleukin 6 subfamily cytokine M17 and interleukin 12B (3.84 and 3.00-fold, respectively). Simultaneously, an important regulator of immune homeostasis and the signal transducer and activator of the transcription 3 (STAT3) pathway, suppressor of cytokine signalling 3a, was also upregulated (2.15-fold). TNF factor b and receptors and genes regulated by it (e.g., TNF receptor 11b and tumour necrosis factor, alpha-induced protein 6) were upregulated, indicating more evidence of a possible pro-inflammatory response in the skin. Tumor necrosis factor, alpha-induced protein 6 presented the most significant fold increase among this family, with a 5.19-fold change. The regulating transcription factor, Nf-kB polypeptide gene enhancer in B cells inhibitor, was also upregulated (1.73-fold), demonstrating a likely attempt to regulate cytokines production pathways. On the contrary, a member of the nuclear factors of activated T cells transcription complex, the nuclear factor of activated T cells 2a, known to induce gene transcription during the immune response (Feske & Vaeth, 2018), was down-regulated (1.72-fold). Several other genes encoding for pivotal molecules of the innate immune response were increased in expression. Other effectors, such as glucosaminyl (N-acetyl) transferase 3, mucin type, associated with O-linked glycosylation of mucins (Syed et al., 2022), were also upregulated. The peptidoglycan recognition protein 6 and formyl peptide receptor 1 are directly related to identifying bacterial pathogens, with a 2.26 and 2.16-fold increase. Selectin, lectins, peroxiredoxin, and other complement factors (e.g., complement 7b and 5) were identified as upregulated. Furthermore, several iron metabolism-related genes were increased in the skin, especially haptoglobin, with a 7.57-fold increase. Transferrin receptor 1b and heme oxygenase 1a were upregulated as well, with a fold increase between 1.56 and 2.35-fold. Antimicrobial peptides, such as hepcidin (isoform X2 and 1), displayed a major upregulation with a 10.37 and 6.49-fold increase. Studies performed in European sea bass have demonstrated the important role of both isoforms against pathogens: hepcidin type 1 is involved in iron homeostasis (i.e., regulation of ferroportin), and hepcidin type 2 is involved in antimicrobial activity (Barroso et al., 2021).

Table 3: List of immune-related DEGs in the skin of European sea bass (*Dicentrarchus labrax*) after bath challenge with *Tenacibaculum maritimum* (Log_2 Fold change of expression ≥ 1.5 and *q*-value ≤ 0.05).

Gene ID	Gene (Acron.)	Gene (name)	Log_2Fold Change	*q*-Value	Regulated
		Eicosanoid metabolism			
ENSDLAG00005007722	*alox15b*	Polyunsaturated fatty acid lipoxygenase ALOX15B-like isoform X1	9.03	5.9×10^{-17}	Up
ENSDLAG00005025759	*ptgs2b*	Prostaglandin-endoperoxide synthase 2b	3.52	1.5×10^{-5}	Up
ENSDLAG00005033508	*ptges*	Prostaglandin E synthase	3.13	1.4×10^{-5}	Up
ENSDLAG00005022212	*ptgis*	Prostaglandin I2 (prostacyclin) synthase	1.82	1.5×10^{-3}	Up
ENSDLAG00005014387	*ptger1a*	Prostaglandin E receptor 1a (subtype EP1)	1.75	5.4×10^{-4}	Up
ENSDLAG00005006757	*pparg*	Peroxisome proliferator activated receptor gamma	1.69	8.4×10^{-3}	Up
		Receptors, chemokines, and cytokines			
ENSDLAG00005021347	*Il 1b*	Interleukin 1 beta	7.38	7.5×10^{-12}	Up
ENSDLAG00005014206	*cxcl8*	Interleukin-8	6.65	6.4×10^{-17}	Up
ENSDLAG00005014333	*cxcr2*	C-X-C chemokine receptor type 2-like	5.95	1.4×10^{-6}	Up
ENSDLAG00005025383	*il11*	Interleukin 11 isoform X1	5.78	1.1×10^{-9}	Up
ENSDLAG00005019452	*il17C*	Interleukin 17C	5.65	5.0×10^{-8}	Up
ENSDLAG00005033919	*m17*	IL 6 subfamily cytokine M17	3.84	7.3×10^{-9}	Up
ENSDLAG00005001099	*il12b*	Interleukin 12B	3.00	4.6×10^{-7}	Up
ENSDLAG00005029539	*il11a*	Interleukin 11a	2.80	4.2×10^{-4}	Up
ENSDLAG00005018382	*socs3a*	Suppressor of cytokine signalling 3a	2.15	7.4×10^{-5}	Up
ENSDLAG00005020836	*il22ra2*	Interleukin-22 receptor, alpha 2	1.90	5.3×10^{-5}	Up
ENSDLAG00005000307	*angpt2a*	Angiopoietin 2a	1.90	5.8×10^{-3}	Up
ENSDLAG00005014882	*irak4*	Interleukin 1 receptor-associated kinase 4	1.55	7.3×10^{-4}	Up
		TNF-related			
ENSDLAG00005002929	*tnfaip6*	Tumour necrosis factor, alpha-induced protein 6	5.19	4.5×10^{-12}	Up
ENSDLAG00005033886	*tnfrsf11b*	TNF receptor superfamily member 11b	2.01	4.5×10^{-4}	Up

ENSDLAG00005009729	*tnfb*	Tumour necrosis factor b (TNF superfamily, member 2)	2.00	3.1×10^{-2}	Up
		Immune-related transcription factors			
ENSDLAG00005016141	*nfkbiaa*	*Nf-kB* enhancer in B-cells inhibitor, alpha a	1.73	5.1×10^{-4}	Up
ENSDLAG00005023269	*nfkbiab*	*Nf-kB* enhancer in B-cells inhibitor, alpha b	1.68	3.4×10^{-4}	Up
ENSDLAG00005016003	*nfil3-3*	Nuclear factor, interleukin 3 regulated, member 3	-1.53	5.6×10^{-3}	Down
ENSDLAG00005013321	*nfatc2a*	Nuclear factor of activated T cells 2a	-1.72	3.9×10^{-5}	Down
		Complement and other innate immune related-effectors			
ENSDLAG00005009616	*clec4f*	C-type lectin domain family 4 member M-like isoform X1	8.61	1.2×10^{-3}	Up
ENSDLAG00005005393	*cd209c*	CD209 antigen-like protein C isoform X2	8.06	2.4×10^{-18}	Up
ENSDLAG00005024758	*c7b*	Complement component 7b	6.49	7.7×10^{-10}	Up
ENSDLAG00005009077	*irg1l*	Immunoresponsive gene 1-like	5.48	4.1×10^{-16}	Up
ENSDLAG00005021570	*lepr*	Leptin isoform X1	5.83	2.7×10^{-5}	Up
ENSDLAG00005028437	loc107723506	Trypsin-3-like	4.96	2.1×10^{-2}	Up
ENSDLAG00005019032	*gcnt3*	Glucosaminyl (N-acetyl) transferase 3, mucin type	4.52	7.3×10^{-9}	Up
ENSDLAG00005026407	*ptafr*	Platelet-activating factor receptor	4.06	4.7×10^{-8}	Up
ENSDLAG00005026539	*c5*	Complement component 5	3.19	1.5×10^{-5}	Up
ENSDLAG00005002264	*selp*	Selectin P	2.67	5.6×10^{-12}	Up
ENSDLAG00005017983	*pglyrp6*	Peptidoglycan recognition protein 6	2.26	3.7×10^{-2}	Up
ENSDLAG00005013246	*malt3*	MALT paracaspase 3	2.23	2.5×10^{-5}	Up
ENSDLAG00005013305	*fpr1*	Formyl peptide receptor 1	2.16	1.1×10^{-3}	Up
ENSDLAG00005027234	*c1qtnf5*	C1q and TNF related 5	2.04	7.3×10^{-4}	Up
ENSDLAG00005007510	*rspo1*	R-spondin 1	-1.63	1.4×10^{-2}	Down
ENSDLAG00005015849	*znf385d*	Zinc finger protein 385D	-2.18	3.8×10^{-6}	Down
ENSDLAG00005014562	*nlrp3*	NLR family CARD domain-containing protein 3-like isoform X2	-5.17	6.7×10^{-4}	Down
		Iron-related			
ENSDLAG00005022256	*hp*	Haptoglobin	7.57	4.3×10^{-10}	Up

ENSDLAG00005021944	*tfr1b*	Transferrin receptor 1b	2.35	6.1×10^{-7}	Up
ENSDLAG00005025440	*hmox1a*	Heme oxygenase 1a	1.56	3.0×10^{-3}	Up
Antimicrobial peptides					
ENSDLAG00005028074	*hamp2*	Hepcidin isoform X2	10.37	1.6×10^{-7}	Up
ENSDLAG00005001556	*hamp1*	Hepcidin-1	6.49	2.6×10^{-15}	Up
ENSDLAG00005032399	*nkl.4*	NK-lysin tandem duplicate 4 isoform X2	-2.20	3.8×10^{-2}	Down
Oxidative stress					
ENSDLAG00005028429	*hspa1a*	Heat shock 70 kDa protein 1	5.88	6.0×10^{-3}	Up
ENSDLAG00005031640	*prdx1*	Peroxiredoxin 1	1.95	1.7×10^{-7}	Up
ENSDLAG00005032099	*cygb*	Cytoglobin-1	-2.45	7.9×10^{-5}	Down

4.3.4. Identification, analysis, and GO annotation of DEPs

A total of 3061 proteins were detected in the mucus of European sea bass, from which 823 were categorized as DEPs (371 down-regulated and 452 upregulated) when comparing mucus from challenged and mock-challenged fish (Fig. 5). A PCA was also conducted, showing a similar trend to the one observed for the DEGs (Fig. S2, Appendix III). Several down-regulated DEPs presented a low Log_2 Fold change, with 180 proteins (approximately 48.52%) displaying a Log_2 Fold change between 1 and 2. Only a small portion possessed a Log_2 Fold change ≥ 2 (52 proteins, 14.02%) (Fig. 5).

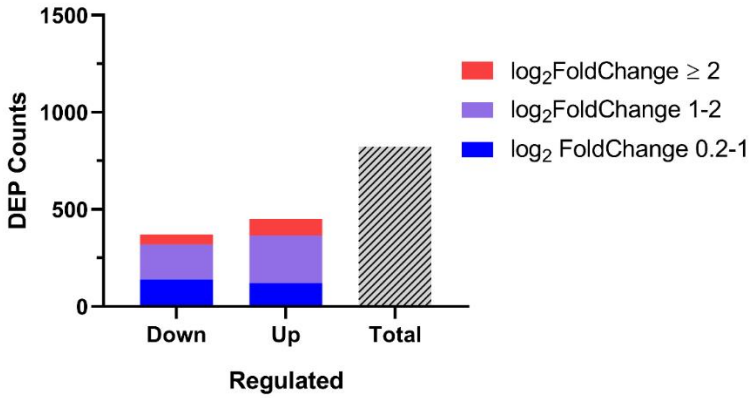

Figure 5: Number of differentially expressed proteins in the mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum*, according to displayed Log_2 Fold Change, for both down- and upregulated proteins.

The upregulated DEPs presented a slightly higher number than the one obtained for the down-regulated ones, with 246 (approximately 54.42%) proteins with a Log_2 Fold change between 1 and 2. For a Log_2 Fold change ≥ 2, 86 DEPs were detected (19.03%). The distribution trends of DEPs were represented in a heat map plot (*q*-value < 0.05) (Fig. 6). Similarly, to what was observed in DEGs, the most modulated proteins in the skin mucus were related to innate immune processes, like phospholipase A2 inhibitor and LY6/PLAUR domain-containing (PINLYP), pentraxin-related protein (PTX3), haptoglobin (HP), arachidonate 15-lipoxygenase (ALOX15B), serine protease 3 (PRSS3) and apolipoprotein B (APOB) (Fig. 6).

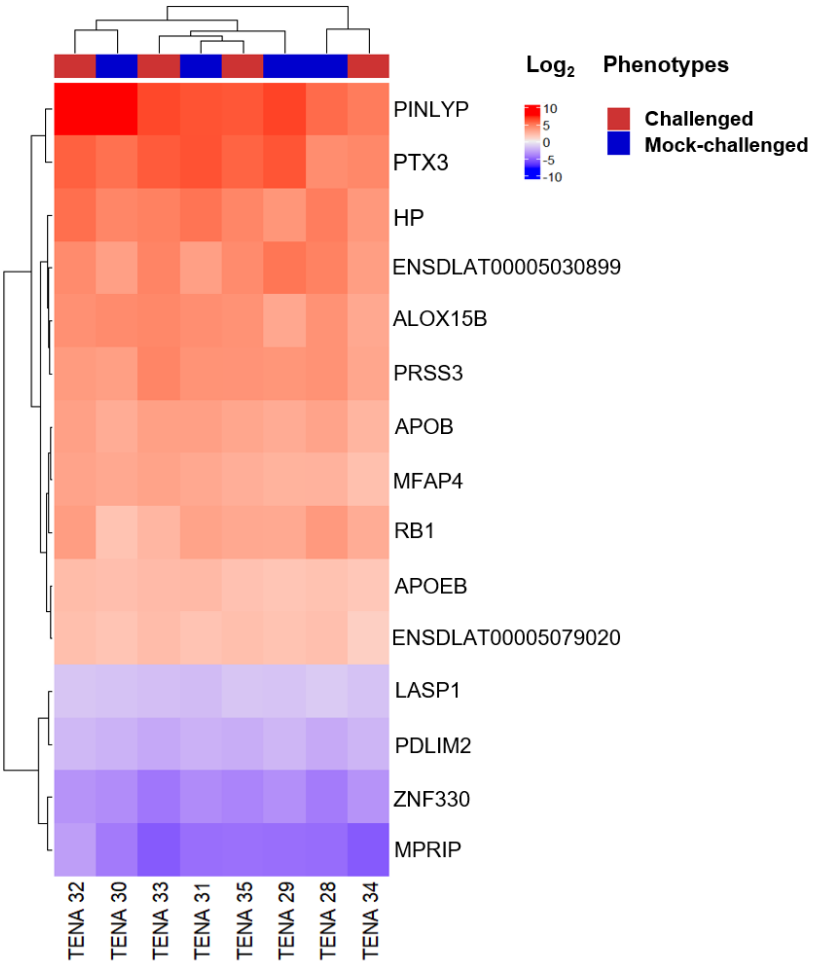

Figure 6: Heat map of the top 15 DEPs between mock-challenge fish (phenotype blue) and challenged (phonotype red) European sea bass (*Dicentrarchus labrax*) at 24 h post-challenged (*q*-value < 0.05). Red: upregulated, and blue: downregulated proteins.

To acquire more information about the biological functions of the DEPs, the up and down-regulated proteins were annotated with GO Terms (ShinyGO 0.77 software). The upregulated set of proteins was attributed to the three functional GO domains: biological

processes (18.81% uniproteins), cellular component (9.29% uniproteins), and molecular function (15.71% uniproteins). Similarly, to what happened to DEGs, a considerable number of these proteins (56.19% uniproteins) could not be categorized under functional GO domains possibly due to the incomplete annotations and/or categorization of such proteins. The biological processes of the modulated proteins were mainly related to immune system activation, such as the activation of immune response (GO:0002253), positive regulation of immune response (GO:0050778), and immune response (GO:0006955) (Table 4). Moreover, complement activation (GO:0006956), humoral immune response (GO:0006959), and protein activation cascade (GO:0072376) were also enriched.

Among the cellular component, the most enriched terms were related to the extracellular region (GO:0005576) and space (GO:0005615), and integral component of plasma membrane (GO:0005887 (Table 4). The upregulated DEPs were also enriched for molecular function processes namely peptidase regulator activity (GO:0061134), endopeptidase inhibitor activity (GO:0004866), and serine-type endopeptidase activity (GO:0004252). Other GO Terms were also enriched like metalloendopeptidase activity (GO:0004222) and glycosaminoglycan binding (GO:0005539). The enriched GO Terms for the upregulated DEPs presented similar functions to the ones enriched in the upregulated DEGs (e.g., immune-related terms, coagulation and wound healing processes). Again, to narrow down the most extremely modulated proteins (Log_2 Fold change ≥ 2), within the upregulated DEPs, several pathways were assigned to functional GO domains (Fig.7).

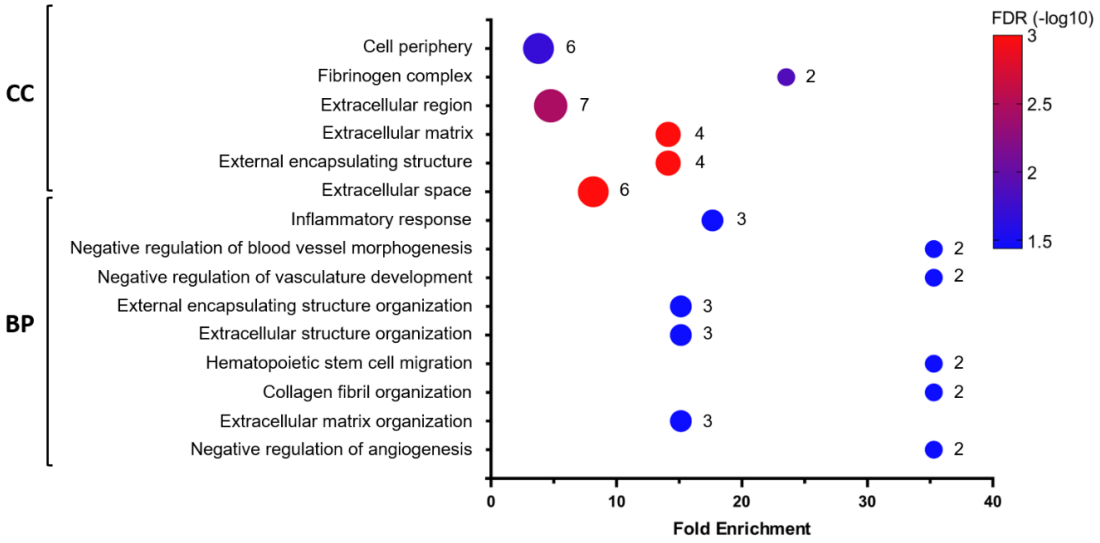

Figure 7: Go functional categories analysis: biological processes and cellular component for upregulated DEPs in the mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold change of expression ≥ 2 and *q*-value ≤ 0.05); CC – Cellular

Component; BP – Biological Processes; the numbers represent the protein count for each enriched pathway).

Enriched pathways were identified in biological processes: negative regulation of angiogenesis (GO:0016525), extracellular matrix organization (GO:0030198), collagen fibril organization (GO:0030199), hematopoietic stem cell migration (GO:0035701), and extracellular structure organization (GO:0043062) (Fig. 7). For the cellular component the extracellular space (GO:0005615), external encapsulating structure (GO:0030312), extracellular matrix (GO:0031012), extracellular region (GO:0005576), and fibrinogen complex (GO:0005577) (Fig. 7). The down-regulated DEPs were attributed to one major functional GO Term, molecular function (14.56% uniproteins). Within this function, several pathways were enriched, like nucleotide binding (GO:0000166), purine nucleotide binding (GO:0017076), and purine ribonucleotide binding (GO:0032555) (Table 4). ShinyGO was unable to perform the functional enrichment analysis for the DEPs with Log_2 Fold Change ≤ 2.

Table 4: List of top 5 enriched pathways of each GO Domain (sorted by Enrichment FDR) of DEPs (up and down-regulated) in the mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (BP – Biological Processes; CC – Cellular Component; MF – Molecular Function). * The percentage of DEGs found, divided by the number of genes in the pathway.

Enrichment FDR	Fold Enrichment	Genes (%)*	Pathway	Associated GO Term	GO Domain	Regulated
1.6×10^{-7}	6.62	37.93	Activation of immune response	GO:0002253	BP	Up
1.6×10^{-7}	6.62	35.48	Positive regulation of immune response	GO:0050778	BP	Up
1.6×10^{-7}	4.14	7.63	Immune response	GO:0006955	BP	Up
1.6×10^{-7}	6.62	55.00	Complement activation	GO:0006956	BP	Up
1.6×10^{-7}	6.62	42.31	Humoral immune response	GO:0006959	BP	Up
1.3×10^{-11}	3.95	6.38	Extracellular region	GO:0005576	CC	Up
3.9×10^{-8}	4.59	15.79	Extracellular space	GO:0005615	CC	Up
3.5×10^{-3}	4.42	2.64	Integral component of plasma membrane	GO:0005887	CC	Up
5.8×10^{-3}	3.73	2.83	Intrinsic component of plasma membrane	GO:0031226	CC	Up

9.6×10^{-3}	3.79	3.07	Plasma membrane protein complex	GO:0098797	CC	Up
3.4×10^{-5}	4.97	14.46	Peptidase regulator activity	GO:0061134	MF	Up
4.4×10^{-5}	4.86	15.94	Endopeptidase inhibitor activity	GO:0004866	MF	Up
4.4×10^{-5}	4.86	14.29	Peptidase inhibitor activity	GO:0030414	MF	Up
4.4×10^{-5}	4.86	15.94	Endopeptidase regulator activity	GO:0061135	MF	Up
9.8×10^{-4}	2.42	6.10	Endopeptidase activity	GO:0004175	MF	Up
1.7×10^{-3}	1.65	2.92	Nucleotide binding	GO:0000166	MF	Down
1.7×10^{-3}	1.68	2.82	Purine nucleotide binding	GO:0017076	MF	Down
1.7×10^{-3}	1.68	2.85	Purine ribonucleotide binding	GO:0032555	MF	Down
1.7×10^{-3}	1.71	2.88	Purine ribonucleoside triphosphate binding	GO:0035639	MF	Down
1.7×10^{-3}	1.65	2.92	Nucleoside phosphate binding	GO:1901265	MF	Down

4.3.5. Analysis of skin mucus DEPs of European sea bass bath-challenged with *T. maritimum*

Effectors related to hemostasis and thrombosis, like thrombospondins or clotting factors that participate in the coagulation cascade (e.g., coagulation factors VII and XIII) (Table 5), were upregulated in fish exposed to *T. maritimum*.

The collagen-degrading matrix metallopeptidase 13a and matrix metalloproteinase 9 were also upregulated (2.77- and 2.25-fold, respectively) (Table 6). Serine protease 1, which is an enzyme essential for processes like blood coagulation, apoptosis, inflammation, and tissue remodelling (Heutinck et al., 2010), was also upregulated (3.87-fold). Hardly any types of collagen were upregulated in the mucus of challenged fish. Only the collagen alpha VI chain and collagen alpha XII chain were increased. These dermal collagens participate in the regulation of dermal matrix assembly, fibroblast behaviour, and a wide range of cytoprotective effects (e.g., neutralizing apoptosis and oxidative damage) (Cescon et al., 2015), together with the assembly of larger macromolecules (Theocharidis & Connelly, 2019).

Table 5: List of wound healing-related DEPs in the skin mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Transcript ID	Gene (Acron.)	Protein (name)	Log_2Fold Change	*q*-Value	Regulated
ENSDLAT00005039059	*arhgap45b*	Rho GTPase activating protein 45b	2.16	1.7×10^{-2}	Up
ENSDLAT00005039059	*arhgap45b*	Rho GTPase activating protein 45b	2.16	1.7×10^{-2}	Up
ENSDLAT00005074331	*thbs4a*	Thrombospondin-4a	2.59	1.5×10^{-3}	Up
ENSDLAT00005075628	*thbs1b*	Thrombospondin-1	2.36	1.1×10^{-2}	Up
ENSDLAT00005027426	*f13a1b*	Coagulation factor XIII, A1 polypeptide b	1.99	2.3×10^{-3}	Up
ENSDLAT00005078458	*f7*	Coagulation factor VII	1.55	3.0×10^{-4}	Up

Moreover, other crucial components of the ECM, such as aggrecan protein (3.36-fold), vitronectin (2.89-fold), nidogen (2.29-fold), and hyaluronan and proteoglycan link protein-1 (2.00-fold) were found with increased abundance (Table 6). Besides these proteins, other important components of the ECM were equally upregulated, like laminins that not only participate in homeostasis but are critical for re-epithelialization (Santoso et al., 2024). Other upregulated proteins include the EGF-containing fibulin extracellular matrix protein (3.28-fold), emilin (1.96-fold), and periostin (3.10-fold), relevant for vascular smooth muscle cells proliferation, homeostasis, cell adhesion or collagen fibrillogenesis and interaction established between ECM components (Djokic et al., 2013; Huang et al., 2020; Kudo, 2011; Schiavinato et al., 2016). On the contrary, it was observed downregulation of receptors such as EPS8 Signalling Adaptor L1 (1.94-fold), involved in actin remodelling, adhesion, and angiogenesis (Cappellini et al., 2015; Giampietro et al., 2015), and periplakin (1.52-fold), responsible for connecting cellular junctions (Boczonadi & Määttä, 2016). Claudin 1 was also downregulated (1.83-fold) in the mucus of challenged fish. This may indicate some degree of epidermal barrier function impairment since claudins are tight junctions responsible for regulating epithelial permeability (namely against pathogen invasion) (Kolosov et al., 2013). The set of DEPs in the mucus of challenged fish reveals a modulation in several proteins that possess an essential role during inflammation and wound healing in the skin. This suggests that exposure to *T. maritimum* triggers a rapid response in the host aimed at re-establishing epidermal homeostasis.

Table 6: List of extracellular matrix (ECM)-related DEPs in the skin mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Transcript ID	Gene (Acron.)	Protein (name)	Log_2Fold Change	*q*-Value	Regulated
		Enzymes			
ENSDLAT00005031442	*prss1*	Serine protease 1	3.87	1.2×10^{-6}	Up
ENSDLAT00005080201	*timp2*	Metalloproteinase inhibitor 2b	3.46	1.2×10^{-3}	Up
ENSDLAT00005018605	*mmp13a*	Matrix metallopeptidase 13a	2.77	2.1×10^{-4}	Up
ENSDLAT00005044851	*mmp9*	Matrix metalloproteinase-9	2.25	2.7×10^{-4}	Up
ENSDLAT00005004364	*mmp2*	Matrix metallopeptidase 2	2.07	5.1×10^{-3}	Up
		Collagens			
ENSDLAT00005068141	*col6a6*	Collagen alpha VI chain	2.29	1.4×10^{-3}	Up
ENSDLAT00005038665	*col6a3*	Collagen alpha VI chain-like	2.28	1.2×10^{-2}	Up
ENSDLAT00005059551	*col12a1b*	Collagen alpha XII chain	1.92	1.0×10^{-3}	Up
		Other components of ECM			
ENSDLAT00005003539	*acanb*	Aggrecan core protein	3.36	7.8×10^{-3}	Up
ENSDLAT00005056966	*postnb*	Periostin, osteoblast specific factor b	3.10	1.7×10^{-3}	Up
ENSDLAT00005068629	*vtna*	Vitronectin a	2.89	1.1×10^{-2}	Up
ENSDLAT00005062095	*fgg*	Fibrinogen gamma chain	2.77	7.7×10^{-4}	Up
ENSDLAT00005079415	*fbn2*	Fibrillin-2b isoform X1	2.43	2.6×10^{-2}	Up
ENSDLAT00005062212	*nid1a*	Nidogen 1a	2.29	3.9×10^{-2}	Up
ENSDLAT00005075363	*hapln1b*	Hyaluronan and proteoglycan link protein 1-like	2.00	1.5×10^{-2}	Up
ENSDLAT00005055272	*emilin2a*	Emilin2a	1.96	1.5×10^{-3}	Up
ENSDLAT00005085606	*paplna*	Papilin	1.95	7.8×10^{-3}	Up
ENSDLAT00005000356	*vtnb*	Vitronectin b	1.75	4.8×10^{-3}	Up
ENSDLAT00005000920	*bcam*	Basal cell adhesion molecule	1.65	4.2×10^{-2}	Up
ENSDLAT00005037273	*lamc2*	Laminin subunit gamma-2	1.66	2.8×10^{-3}	Up
ENSDLAT00005081156	*cldni*	Claudin 1	-1.83	1.1×10^{-2}	Down
		Growth and differentiation factors and other regulators			

ENSDLAT00005049648	*efemp2a*	EGF containing fibulin extracellular matrix protein 2a	3.28	2.8×10^{-4}	Up
ENSDLAT00005013318	*csrp2*	Cysteine and glycine-rich protein 2	1.56	2.1×10^{-2}	Up
ENSDLAT00005033844	*ppl*	Periplakin	-1.52	7.8×10^{-4}	Down
ENSDLAT00005003314	*bdh2*	3-Hydroxybutyrate dehydrogenase, type 2	-1.64	2.6×10^{-2}	Down
ENSDLAT00005002916	*eps8 like 1b*	EPS8 Signalling Adaptor L1	-1.94	9.2×10^{-4}	Down

Many proteins that participate in the innate immune response, mainly related to inflammation and its regulation, were also found to be upregulated (Table 7). The well-known interleukin 1 beta was upregulated (2.43-fold), together with the coiled-coil domain containing 88B (2.47-fold), likewise required for inflammation and T cell maturation, activation, and proliferation (Fyfe-Desmarais et al., 2023). Pathogen recognition receptors like toll-like receptor 5 (TLR5) and CD209 antigen-like protein C (CD209C), which participate in bacterial recognition and influence the phagocytosis process during pathogen infections, were also increased (3.19 and 2.75, respectively) (Jiang & Sun, 2017; Zhang et al., 2023). On the contrary, the anti-inflammatory properties of apolipoprotein D (3.46-fold), providing oxidation and stress resistance (El-Darzi et al., 2021), may have an important role against the deleterious effects exerted by *T. maritimum* in the skin mucus. A downregulation scenario is seen for the zinc finger protein 330 (3.89-fold), a mediator of the mitochondrial apoptotic pathway, as well as for PDGFA-associated protein 1 (2.83-fold), a regulator of cellular homeostasis in mature B cells, and ceramide-1-phosphate transfer protein (2.68-fold) which regulates cellular sphingolipid homeostasis (e.g., through programmed cell death and inflammation) (Delgado-Benito et al., 2020; Gao et al., 2021; Peng et al., 2023). Complement factors were increased in relative abundance, including complement component 7b (3.69-fold) and 7a (1.50-fold), and complement component 6 (1.58-fold), among others like pentraxin (5.56-fold) and trypsin (3.87-fold), known for their role against pathogens and regulation of inflammation (Santoso et al., 2020). Another cluster of proteins with high relative abundance was related to iron metabolism, including haptoglobin (4.63-fold), heme-binding protein soul5 (2.29-fold), hemopexin a (1.83-fold), and transferrin receptor 1b (1.73-fold). These proteins are involved in the binding of hemoglobin (for prevention of iron loss), antioxidant and antibacterial activities, chemotaxis, downregulation of the pro-inflammatory cytokines released from macrophages, and mediation of transferrin and ferritin uptake (Kawabata, 2019; Liang et al., 2009; Lin et al., 2012; Wassell, 2000).

4.3.6. Combined analysis of transcriptomic and proteomic

A total of 3843 DEGs were identified in transcriptomic and 823 DEPs in proteomic approaches. From these, 248 DEGs and DEPs matched, suggesting that, in many cases, differences in gene expression in the skin are accompanied by differences in the levels of the corresponding protein in the mucus. Among the 248 DEGs and DEPs that matched, there were some upregulated that are associated with critical biological functions related to innate immunity, including pro-inflammatory response (i.e. interleukin 1 beta), complement pathways (i.e., complement component 7b) iron-related metabolism (haptoglobin and transferrin receptor 1b) and other immune-related effectors (i.e., phospholipase, lipoxygenase, and pentraxin-related protein PTX3) (Table S2, Appendix III). A matched upregulation of skin DEGs and mucus DEPs was also observed for genes/proteins related to ECM-remodelling (elastin, fibulin, and filamin) (Table S3, Appendix III). The cell-cell junction component, claudin, was downregulated both at gene and protein levels in challenged individuals (Table S3, Appendix III).

Table 7: List of immune-related DEPs in the skin mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Transcript ID	Gene (Acron.)	Protein (name)	Log_2Fold Change	*q*-Value	Regulated
		Interleukins			
ENSDLAT00005051888	*il-1b*	Interleukin-1 beta	2.43	1.1×10^{-4}	Up
		Recognition process			
ENSDLAT00005002419	*tlr5*	Toll-like receptor 5	3.19	1.8×10^{-3}	Up
ENSDLAT00005011298	*cd209c*	CD209 antigen-like protein C isoform X2	2.75	1.0×10^{-4}	Up
		Immune-regulatory function			
ENSDLAT00005059819	*apoda.1*	Apolipoprotein Da, duplicate 1	3.46	2.4×10^{-2}	Up
ENSDLAT00005065164	*cd59*	CD59 glycoprotein	3.12	4.4×10^{-3}	Up
ENSDLAT00005016224	*ccdc88b*	Coiled-coil domain containing 88B	2.47	1.0×10^{-2}	Up
ENSDLAT00005037865	*znf330*	Zinc finger protein 330	-3.89	5.1×10^{-5}	Down
ENSDLAT00005072551	*pdap1a*	Pdgfa-associated protein 1a	-2.83	1.9×10^{-2}	Down
ENSDLAT00005069544	*cptp*	Ceramide-1-phosphate transfer protein	-2.68	6.3×10^{-3}	Down

ENSDLAT00005076740	*lsp1a*	Lymphocyte-specific protein 1 a	-2.06	4.3×10^{-5}	Down
ENSDLAT00005071480	*tbk1*	TANK-binding kinase 1	-1.64	2.8×10^{-2}	Down
		Eicosanoid metabolism			
ENSDLAT00005030899	*pinlyp*	Phospholipase A2 inhibitor and Ly6	6.67	3.4×10^{-5}	Up
ENSDLAT00005017239	*alox15*	Polyunsaturated fatty acid lipoxygenase ALOX15B-like isoform X1	3.94	4.1×10^{-6}	Up
ENSDLAT00005077021	*ptgr2*	Prostaglandin reductase 2	2.01	1.6×10^{-2}	Up
		Complement and other innate immune related-effectors			
ENSDLAT00005078468	*ptx3*	Pentraxin-related protein PTX3	5.56	6.0×10^{-6}	Up
ENSDLAT00005031442	*prss3*	Trypsin-3	3.87	1.2×10^{-6}	Up
ENSDLAT00005062134	*c7b*	Complement component 7b	3.69	8.2×10^{-4}	Up
ENSDLAT00005067967	*sele*	Selectin E	2.17	1.5×10^{-2}	Up
ENSDLAT00005062219	*c6*	Complement component 6	1.58	3.5×10^{-3}	Up
ENSDLAT00005031016	*c3b.2*	Complement component c3b, tandem duplicate 2	1.52	1.1×10^{-3}	Up
ENSDLAT00005033865	*c7a*	Complement component 7a	1.50	2.7×10^{-3}	Up
		Cell death and apoptosis			
ENSDLAT00005006562	*niban2b*	Niban apoptosis regulator 2b	2.00	7.4×10^{-5}	Up
ENSDLAT00005039085	*baxa*	BCL2 associated X a	-2.07	7.4×10^{-3}	Down
		Iron-related			
ENSDLAT00005054501	*hp*	Haptoglobin	4.63	2.2×10^{-6}	Up
ENSDLAT00005039980	*soul5*	Heme-binding protein soul5	2.29	2.2×10^{-4}	Up
ENSDLAT00005028346	*wap65*	Hemopexin a	1.83	6.4×10^{-3}	Up
ENSDLAT00005053599	*tfr1b*	Transferrin receptor 1b	1.73	2.7×10^{-2}	Up
ENSDLAT00005019519	*tfa*	Transferrin-a	1.47	8.9×10^{-3}	Up

4.4. Discussion

In this work, it is shown that bath infection with *T. maritimum* triggers the activation of immune-related and inflammation response at a transcriptional level in the skin and leads

to altered levels of mucus proteins involved in the immune response, including complement and humoral immune response, and wound healing-related processes. This suggests that these pathways play an important role in the interaction between *T. maritimum* and its host during the initial stages of infection.

A cascade of pathways is activated upon skin teleost damage, which includes the processes related to wound repair: re-epithelialization and homeostasis, inflammation, cell proliferation with granulation tissue formation, and tissue remodelling (Richardson et al., 2013). Since in teleost, the absence of a blood clot is considered a typical characteristic in the skin healing process, the upregulation of genes and proteins involved in blood coagulation (e.g., coagulation factors V, VII, and XIII) may present other functions in fish (Sveen et al., 2019). Despite the unknown functions of coagulation effectors in wound healing, it can be speculated that its intervention may help in the formation of a fibrin-like mesh, which could initially help to protect the wound surface and provide a smooth matrix that could help in epithelial migration (Sveen et al., 2019).

In teleosts, besides contributing to hemostasis and coagulation, thrombocytes have important immunological functions, being able to express proinflammatory and antigen presentation-related genes and phagocyte microorganisms (Ortiz & Esteban, 2024). These cells can secrete proinflammatory (e.g., IL1β and tumour necrosis factor - TNFα) and anti-inflammatory mediators (e.g., interleukin 10 - IL10), chemokines, interferon-γ (INF-γ), and toll-like receptors (TLR) (e.g., TLR5 and TLR8), and eicosanoids (Ortiz & Esteban, 2024). Platelet-activating factors (PAFs) are the stored growth factors essential for wound repair, enhancing cell proliferation, extracellular matrix deposition, and remodelling (Nurden et al., 2008; Rozman & Bolta, 2007). Besides its importance in mammals, PAF receptors (*ptafr*) have already been demonstrated to mediate tissue repair in zebrafish caudal fin injury (Oremeke, 2021). Therefore, the upregulation of *ptafr* in the skin of European sea bass exposed to *T. maritimum* suggests an early attempt to activate the host wound repair mechanisms.

Prostaglandins and other eicosanoids are bioactive lipid mediators released by injured cells that are fundamental in supporting the regenerative and inflammatory processes (Campos-Sánchez & Esteban, 2021; Yasukawa et al., 2020). In the present study, *T. maritimum* bath-challenge fish displayed an up-regulation of several genes that participate in the eicosanoid metabolism. Among the upregulated mediators of the eicosanoid metabolism, it was possible to identify arachidonate 15-lipoxygenase type B. The hydroperoxy fatty acids produced by lipoxygenase (LOX) isoforms are subsequently converted to bioactive lipid mediators that include leukotrienes (Savari et al., 2014), lipoxins (Romano et al., 2015), protectins (Serhan & Petasis, 2011), among others. Prostaglandin E synthase (PGE synthase), responsible for generating prostaglandin E (PGE), which is another potent

mediator of inflammation (increasing vascular permeability and smooth muscle contraction or dilation), is also involved in tissue regeneration and repair following injury in diverse organ systems (Cheng et al., 2021). This modulation, together with the upregulation of prostaglandin I2 (prostacyclin, PGI2), known as a potent vasodilator and inhibitor of platelet aggregation (Kelton & Blajchman, 1980), may be related to the haemorrhage commonly associated with tenacibaculosis infections. Studies have already presented remarkable similarities in the role of eicosanoids initiating and controlling hemostasis-related processes of both fish thrombocytes and mammalian platelets (Hill et al., 1999; Jagadeeswaran et al., 1999; Khandekar et al., 2012; Rowley et al., 1995). Lipid-signalling eicosanoids have been described as having an important role in vasoconstriction in the gills of rainbow trout (Sundin & Nilsson, 1998); Sveen et al. (2019) also reported an upregulation of several genes involved in eicosanoid metabolism immediately after wounding (by biopsy punch). The action of eicosanoids is also associated with the production of cytokines (and vice-versa). A plethora of different cytokines and chemokines with pro-inflammatory roles were increased in sea bass bath-challenged with *T. maritimum*, with *il1β* having the highest change. This key mediator of inflammation, crucial for host-defense responses to infection and injury, is frequently associated with acute inflammation (Kaneko et al., 2019). Many studies investigated the functions of this interleukin in teleosts (Cui et al., 2020; Guo et al., 2022; Jiang et al., 2022; Wang et al., 2021; Y. Wu et al., 2019), indicating its crucial role in antibacterial innate immunity. Together with *il1β*, many other increased DEGs comprised cytokines (e.g., *m17*, *il12*b, *il11a*, *irak4*) that have been associated with an innate type response against a stimulus in the skin (Gao et al., 2023; Li et al., 2021; Soliman & Barreda, 2023; Wang & Secombes, 2009; Zhang et al., 2022). The synergism established between IL1 and TNF family cytokines is commonly reported during inflammatory responses (Dinarello, 2000). Both cytokines, secreted at infection sites, are then able to induce endothelial cells to express selectin and stimulate cells to produce chemokines (e.g., C-X-C motif chemokine ligand 8 and C-C motif ligand 2) (Campos-Sánchez & Esteban, 2021). This expression of selectins facilitates neutrophil and monocyte diapedesis and adhesion (Scopelliti et al., 2022), which may contribute to the host response against the bacteria. The importance of selectins in cell recruitment has been previously demonstrated by showing that when genetically blocked, immune cell infiltration and wound healing were significantly compromised (Denis et al., 2001; Yukami et al., 2007). C-X-C motif chemokine ligand 8, or IL8, promotes inflammation and is associated with inflammatory and infectious diseases (Baggiolini & Clark-Lewis, 1992; Bernhard et al., 2021). IL8 regulates the activity of macrophages and monocytes via attaching to glycosaminoglycans (GAGs) on the cell surface through activation of two G protein-coupled receptors (GPCRs), namely CXC chemokine receptor 2 (CXCR2) (Kuschert et al., 1998), also part of the DEGs from the

present study. Such chemokines can promote wound healing by activating the angiogenic response and triggering endothelial cell proliferation, survival, and recruitment (Li et al., 2003). Interleukin (IL)17 is a pro-inflammatory cytokine that is produced by epithelial cells, and it is involved in barrier maintenance of the mucosal tissues and antimicrobial protective responses, mainly against bacteria and fungus (Okamura et al., 2023). IL17 can induce the production of IL1β and IL8, chemokines (e.g., CXCL1, CXCL2, CXCL3), and antimicrobial peptides in human keratocytes stimulated with *E. coli* or PAMPs (Ramirez-Carrozzi et al., 2011). Moreover, it enhances the expression of tight junction molecules, including claudin 1, to improve barrier function against pathogens (Reynolds et al., 2012). Several fast-acting components of the innate immune, such as complement factors and pentraxin, were found to be upregulated in European sea bass after bath infection with *T. maritimum*. This pattern of the innate immune response against bacterial pathogens has already been described in many fish species (Bavia et al., 2022; Cammarata et al., 2014; Parisi et al., 2015; Tang et al., 2021; Zhang et al., 2016). A peptidoglycan recognition protein (PGRP), which belongs to a group of pattern recognition receptors (PRRs) that recognize peptidoglycan from the bacterial cell wall (Esteban, 2024), was also found to be increased in the infected fish from the present study. Other identified antibacterial components, such as formyl peptide receptors (FPRs), are released upon cellular damage and promote neutrophil chemotaxis to the injury site, releasing LTB4 and further neutrophil migration (Afonso et al., 2012; McDonald et al., 2010; Sokol & Luster, 2015). The complement signalling pathway plays a role in the fast elimination of pathogens and modulation of inflammatory responses (Mokhtar et al., 2023). This signalling pathway was significantly enriched in the skin mucus of sea bass after *T. maritimum* challenge. Evidence has already demonstrated the increased expression of several complement effectors in teleost fish upon bacterial infection (Wang et al., 2014; Wu et al., 2022; X. M. Wu et al., 2019). C7, a pore-forming glycoprotein involved in host immunity and bacterial pathogenesis, rapidly responded to *A. hydrophila* bacterial infection in grass carp (Shen et al., 2012). Besides forming the first portion of the complement membrane attack complex (MAC), C5 is a potent chemoattractant (Denk et al., 2017). In addition, Syahputra et al. (2019) reported that C5 had a necessary role in the mucosal immunity of rainbow trout against *I. multifiliis* infection (Syahputra et al., 2019). In the present study, complement 7 (C7) and 5 (C5) showed the most increase in expression and abundance following the *T. maritimum* challenge, suggesting their participation in the nonspecific immune response against the first stages of invasion by this pathogen. During infection or inflammation, the host employs strategies to withhold iron in order to prevent bacterial growth and limit iron from participating in redox reactions that can lead to unwarranted damage (Golonka et al., 2019; Gozzelino & Arosio, 2016). Therefore, and since iron is an essential nutrient for development of *T. maritimum* infection (Avendaño-

Herrera et al., 2005), it can be expected that contact with *T. maritimum* activates the host iron withholding mechanisms to avoid bacterial proliferation. Indeed, an increased expression and abundance of the iron metabolism-related glycoprotein haptoglobin was observed in bath-challenged European sea bass, which may indicate that this protein has a dominant role against *T. maritimum*. Many studies have already demonstrated how haptoglobin can be pivotal for fish survival during infection, hindering bacteria from accessing iron (Charlie-Silva et al., 2019; Kurpe et al., 2022; Peatman et al., 2007). Transferrin receptors also play an essential role in iron-withholding host strategies against bacteria (Ding et al., 2015; Neves et al., 2009; Poochai et al., 2014; Yin et al., 2020). These receptors are gatekeepers for regulating iron uptake and storage from transferrin, which transports iron in circulation to the intracellular environment (Ponka & Lok, 1999). The increase seen for both haptoglobin and transferrin receptors may be a host strategy to decrease available circulating iron, to try to undermine or delay bacterial invasion. Both hepcidin isoforms, *hamp1*, and *hamp2*, which have previously been shown to be highly upregulated during in vitro bacterial infections and exhibit antimicrobial activity against different bacteria (Neves et al., 2015), were also found to be upregulated after bath infection with *T. maritimum*.

The inflammatory phase in injured tissue is followed by proliferation and matrix remodelling, involving a coordinated network of several enzymes and growth factors (Richardson et al., 2013). In this study, it is shown that 24 h after exposure of European sea bass to *T. maritimum*, there was an enhancement of collagen-degrading metalloproteinases, MMP9 and MMP13, possibly potentiated by the increased expression of *il8* (Ågren & Auf Dem Keller, 2020; Chakrabarti & Patel, 2005). At 24 h post-challenge, wounds and skin abrasions were observed in infected fish (data not shown). Besides directly degrading ECM proteins, MMP9 is one of the significant chemokine regulators during wound healing (Chadzinska et al., 2008). MMP13 participates in the maturation of granulation tissue, with modulation of myofibroblast function, inflammation, angiogenesis, and matrix degradation (Caley et al., 2015). Similarly, to what was previously seen for mammals and some fish species, the increase of these enzymes, together with the increase of ADAM metalloproteinases and trypsin, could suggest a possible protective role of these enzymes against tenacibaculosis (Chi et al., 2023; Krampert et al., 2005; Murakami et al., 2006; Shan et al., 2016). Growth factors like transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), and EGF-containing fibulin-like extracellular matrix protein 2 (also known as fibulin-3), are closely related to wound healing processes, stimulating proliferation, migration of keratocyte, fibroblast and endothelial cells assisting in dermal regeneration in elastic and assembly of fibre (Djokic et al., 2013; Shanmugam et al., 2022). Together with other growth factors, these can increase the wound healing rate by augmenting collagen

deposition and neovascularization (Djokic et al., 2013; Shanmugam et al., 2022). The overall decrease of some types of collagens and components associated with ECM may be explained by the tissue re-epithelization process itself. A study focusing on the skin and scale regeneration process after mechanical damage demonstrated that several collagen types (i.e., collagen type I, type V, and type X) were significantly decreased in regenerating skin from 6 h until a maximum of 3 days after the mechanical damage (Costa & Power, 2018). Moreover, a study developed by Sveen et al. (2019) demonstrated that, in Atlantic salmon, several genes involved in collagen synthesis, fibril maturation, and growth factors were only activated from 36-57 days post-mechanical wounding. Claudin I, relevant to maintaining tight junctions to hamper pathogens from passing through, was also decreased in the bath-challenged sea bass. Many pathogens secrete toxins that can disrupt the tight epithelial cells' junctions, blocking epithelial cell growth (McGuckin et al., 2011). Further studies will be necessary to explore such potential virulence mechanisms in *T. maritimum*.
In summary, this study provided important information regarding the inflammatory and wound-healing process behind tenacibaculosis, highlighting potential players involved in tissue repair and its most important innate immune-related effectors. It showed that shortly after exposure to *T. maritimum*, European sea bass increases the transcription of innate immunity genes related to several inflammatory mediators, inflammation, and the re-epithelialization process. Moreover, typical pathways associated with acute innate response, complement factors, and iron withholding mechanisms were also found to be enriched at both transcription and protein levels in the skin and mucus of European sea bass bath-challenged with *T. maritimum*. Altogether, this suggests that in addition to the presence of bacteria, the skin wounds developed as a consequence of *T. maritimum* infection have a prominent role in triggering the fast innate immune response observed in the skin.

4.5. Acknowledgements

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CHAPTER 5

Identification of *Tenacibaculum maritimum* extracellular products: *in vitro* and *in vivo* proteomic approaches

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Identification of *Tenacibaculum maritimum* extracellular products: *in vitro* and *in vivo* proteomic approaches

Abstract

Among the numerous bacteria that can affect fish species, *Tenacibaculum maritimum* has consistently been a cause for concern due to re-emergent outbreaks in aquaculture sites. Recent evidence suggests that the extracellular products (ECPs) secreted by *T. maritimum* are among these bacteria's main virulence mechanisms. However, despite their importance to pathogenesis development, the protein content of *T. maritimum*'s ECPs produced *in vitro* is limited to very few strains, and there is no information about the ECPs components produced *in vivo* by this pathogen. In this work, proteins abundant in in *vitro* produced ECPs and secreted *in vivo* by the *T. maritimum* virulent strain ACC13.1 were identified. For identification of proteins secreted *in vitro*, bacteria were cultured in marine broth, supernatants collected at 6, 12, 24, 36, and 48 h and subjected to SDS-PAGE. Selected protein bands were excised from the gels and analysed by NanoLC-MS/MS. The identification of the proteins secreted *in vivo* during infection was performed using the raw data obtained in the proteomics analysis of skin mucus samples of European sea bass (*Dicentrarchus labrax*) bath-challenged with *T. maritimum* (strain ACC13.1) presented in Chapter 4. In ECPs produced *in vitro*, several proteins were identified, including sialidase, metalloproteases, outer membrane proteins (e.g., TonB-dependent receptors), lipoproteins, and type IX secretion system (T9SS)-related proteins. The presence of a C-terminal domain secretion signal (CTD) in these proteins suggests that they are being actively secreted by the bacterium. The analysis of the *in vivo*-produced proteins in the skin mucus of bath-challenged fish (cumulative mortality 32.1%) led to the identification of metalloproteases with CTD, sialidases, lipoproteins, and other proteins related to oxidative stress resistance (e.g., superoxide dismutase, catalase, thioredoxin). Further studies are required to clarify the role of these proteins during tenacibaculosis infections. Uncovering the complex host-pathogen cross-talks between *T. maritimum* and its hosts at the mucosal level may reveal promising target antigens for developing vaccination strategies against tenacibaculosis.

Keywords: *T. maritimum*; ECPs; Proteomics; *In vitro*; *In vivo*

5.1 Introduction

Several advances in functional genomics and proteomics led to the identification and characterization of a wide range of bacterial virulence-associated secreted proteins, which include enzymes, toxins, or cell surface proteins such as outer membrane proteins (OMPs), and lipoproteins (Zubair et al., 2022). These can perform several functions essential for pathogenesis development, such as nutrient provision and scavenging, cell-to-cell communication, attachment and disruption of targeted cells, and invasion and modulation of host defences (Dwivedi et al., 2016; Green & Mecsas, 2016; Johnson, 2018). Among the numerous bacteria able to infect fish species, *Tenacibaculum maritimum* has consistently been a cause for concern in marine environments due to its ubiquitous distribution and re-emergent outbreaks (Mabrok et al., 2023). This Gram-negative gliding bacterium is the primary etiological agent of tenacibaculosis, an infection that leads to considerable economic losses in aquacultures worldwide (Småge et al., 2016) (Avendaño-Herrera et al., 2006). Despite the negative impacts of *T. maritimum* infections, knowledge of the virulence factors/mechanisms displayed by this pathogen is still scarce.

A study developed by Pérez-Pascual et al. (2017) allowed the sequence of the complete genome of *T. maritimum* type strain NCIMB 2154T, offering a new understanding of specific virulence-associated genes. *T. maritimum* also appears to be a cohesive bacterial species since the genomic characterization of 25 strains revealed a high genomic identity, similar genome size, and a moderate level of nucleotide divergence (maximum of 1.52% in pairwise core-genome sequence comparisons) (Bridel et al., 2020). *T. maritimum*'s genome encodes several toxins that are predicted to be membrane-damaging enzymes, able to disrupt the host cells, inducing lysis (Pérez-Pascual et al., 2017; Sarkar et al., 2021). Among the numerous predicted toxins encoded in its genome, it is possible to find proteins like sphingomyelinase, ceramidase, hemolysin, chondroitin sulfate lyase, and C10 family peptidase (Pérez-Pascual et al., 2017). These putative *T. maritimum* proteins bear homology to proteins from other bacterial pathogens that are involved in bacterial colonization, invasion, nutrient acquisition during infection, and destruction of host tissues (Flores-Díaz et al., 2016; Li et al., 2015; Rawlings & Bateman, 2019; Zhang et al., 2023). Moreover, genes encoding a type IX secretion system (T9SS), which is commonly present in members of the phylum Bacteroidetes, were also identified in *T. maritimum*'s genome (Pérez-Pascual et al., 2017). The T9SS is known to participate in the delivery of proteins, including virulence factors, to the cell surface and extracellular space (McBride, 2019; McBride & Zhu, 2013) (Song et al., 2022), assisting in gliding motility, adhesion, and biofilm formation (Eckroat et al., 2021; Gorasia et al., 2020).

It has also been proposed that *T. maritimum* virulence is associated with the proteolytic activity of its extracellular products (ECPs). Escribano et al. (2023) analysed the proteolytic and lipolytic activities of the ECPs from 64 *T. maritimum* strains, belonging to serotypes O1-O4, highlighting the intra-specific heterogeneity in the ECPs' enzymatic activity, especially among serotype O4. The analysis of the ECPs also revealed the presence of a large amount of outer membrane vesicles (OMVs) (Escribano et al., 2023). While some outer membrane proteins appeared to be related to the OMVs fraction, others seemed to be mainly present in the soluble fraction of the ECPs (Escribano et al., 2023). Both OMVs and soluble ECPs fractions induced alterations in fish cells, but a combination of both fractions was needed to achieve maximum cytotoxicity. Some preliminary *in vivo* studies also demonstrated the proteolytic potential of *T. maritimum*'s ECPs (Baxa et al., 1988; Van Gelderen et al., 2009). Nevertheless, these studies could not correlate the *in vivo* toxicity of the ECPs with a definitive toxic factor present in the inoculated mixture. Despite the available studies suggesting that *T. maritimum*'s ECPs may be crucial for tenacibaculosis pathogenesis, there is still a significant lack of knowledge regarding the detailed composition and function of its components, especially *in vivo*.

The present study aimed to disclose the identity of some of the proteins secreted by *T. maritimum* strain ACC13.1 *in vitro* and *in vivo*. This, together with the global proteomic analysis of the ECPs produced *in vitro* by the same strain, used in the trial presented in Chapter 2, allowed to identify secreted proteins putatively important for *in vivo* virulence.

5.2. Material and Methods

5.2.1. Bacterial strain

The *T. maritimum* strain ACC13.1 used in this study was isolated from Senegalese sole (Solea senegalensis) during a farm outbreak and belongs to the serotype O3 (Avendaño-Herrera et al., 2005). The strain was kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and stocks were kept frozen at −80 °C (Frilabo, Portugal) until use. Recovery from frozen stocks was achieved using marine agar (MA; Laboratories CONDA, Spain) at 25 °C for 48 h.

5.2.2. Bacterial growth and collection of ECPs

T. maritimum was inoculated in 50 mL of marine broth (Laboratories CONDA, Spain) and grown at 25 °C, with continuous shaking (180 rpm) for 48 h in a 500 mL Erlenmeyer. Two

independent experiments were performed. In experiment 1, 1.5 mL aliquots of the culture were collected at 6, 12, 24, and 48 h post-inoculation and in experiment 2 at 6, 12, 24, 36 and 48 h post-inoculation. At each sampling point, turbidity was measured at 600 nm (Spectrophotometer, UV-1600PC, VWR) to estimate the bacterial concentration using a predetermined growth curve for this specific strain: $y = 2 \times 10^8 x + 4 \times 10^7$ (Mabrok, 2016) where x is measured turbidity (OD 600 nm) and y is the bacterial concentration (CFU mL^{-1}). The aliquots were centrifuged at room temperature (RT) for 30 min at 19,000 × *g* to obtain bacterial pellets and culture supernatants that were kept at −80 °C until use.

5.2.3. TCA protein precipitation and SDS-PAGE

Proteins from 1 mL cell-free culture supernatants were precipitated with 10% (w/v) trichloroacetic acid (TCA) for 30 min on ice, recovered by centrifugation (4 °C for 15 min at 19,000 × *g*), washed with 10% (w/v) TCA followed by a washing step with acetone. The precipitated protein pellets were air-dried at RT and stored at −80 °C. Precipitated proteins from cell-free supernatants and whole-cell pellets were solubilized in SDS-sample buffer (50 mM Tris-HCl (pH 8.8), 2% SDS, 0.017% bromophenol blue, 10% glycerol, 2 mM EDTA (pH 8.8), and 100 mM DTT), heated for 5 min at 95 ºC and subjected to SDS-PAGE in 8% or 14% polyacrylamide gels using the Laemmli discontinuous buffer system (Laemmli, 1970). A sample corresponding to 1 mL of culture was loaded per lane, whereas in the case of whole-cell pellets, the amount loaded per lane corresponded to 0.1 mL culture. Gels were stained with Coomassie Brilliant Blue (0.2% Coomassie R-250, 50% methanol, 10% acetic acid).

5.2.4. NanoLC-MS/MS analysis of ECPs' protein produced *in vitro*

After SDS-PAGE of culture supernatants' TCA precipitates, selected protein bands were excised from the gels and subjected to NanoLC-MS/MS at the i3S Proteomics platform, using an equipment composed of an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The bands excised from the gels were reduced, alkylated, enzymatically in-gel digested with trypsin, and purified by C18 tips (ZipTips, Millipore). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm x 300 µm i.d., 160454, Thermo Scientific) in a mobile phase of 2% ACN, 0.1% FA at 10 µL min^{-1}. After 3 min loading, the trap column was switched in-line to a 50 cm by 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 250 nL min^{-1}. Separation was generated by mixing A: 0.1% FA, and B: 80%

ACN, with the following gradient: 2 min (2.5% B to 10% B), 50 min (10% B to 35% B), 8 min (35% B to 99% B), 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from the full scan. The raw data was processed using Proteome Discoverer 2.4.0.305 software (Thermo Scientific) and searched against the UniProt database (2019_11) for the Tenacibaculum taxonomic selection. The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragment ions. Maximum allowed missing cleavage sites was set 2. Cysteine carbamidomethylation was defined as a constant modification. Methionine oxidation, protein N-terminus acetylation, methionine loss, and methionine loss plus acetylation were defined as variable modifications. Peptide confidence was set to high. The processing node target decoy PSM validator was enabled with a maximum delta Cn 0.05 and decoy database search target FDR 1%. Protein label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performed at the processing step, including unique and razor peptides. The precursor abundance was based on intensity. Raw data hits from each excised band with unique peptides above 2 were selected, and the obtained hits were automatically assigned the corresponding GO Terms using the UniProt tool ID Mapping (https://www.uniprot.org/id-mapping, accessed 7th Feb 2024).

5.2.5. Identification of *T. maritimum* proteins in mucus collected from infected fish

The identification of *T. maritimum* proteins expressed *in vivo* was performed using the NanoLC-MS/MS raw data obtained in Chapter 3 for the mucus samples collected from bath-challenged sea bass. The same methodology and parameters used in Chapter 3 were applied, except that the Proteome Discoverer 2.4.0.305 software (Thermo Scientific, Scientific, Bremen, Germany) was used to search against the UniProt database (2022_11) for the *T. maritimum* taxonomic selection, NCIMB2154 Proteome (2022_03).

Raw data hits with at least 2 unique peptides and present in at least 3 of the 4 replicates were filtered and were automatically assigned the corresponding GO Terms using the UniProt tool ID Mapping (https://www.uniprot.org/id-mapping, accessed 15th Jul 2024). Protein-protein interaction (PPI) analysis was obtained from the Search Tool for the Retrieval of Interacting Genes (STRING 12.0) database (http://string-db.org) based on an uploaded list of proteins present in the mucus, using Markov Clustering (MCL) algorithm

(inflation parameter=3) to retrieve the top five enriched clusters against UniProt database (NCIMB 2154) for the taxonomic selection of *T. maritimum* (accessed 15th Jul 2024).

5.3. Results

5.3.1. SDS-PAGE and selection of protein bands for NanoLC-MS/MS

Analysis of the cell-free supernatants (ECPs) from experiments 1 and 2 (Table 1) showed a complex protein profile in samples collected from 12 to 48 h cultures, with band sizes ranging from over 120 kDa to less than 12 kDa and no visible bands in supernatants collected at 6 h (Fig. 1). The highest molecular weight bands were only detected in samples from 24, 36, and 48 h cultures (Fig. 1, A) and B)). Some bands visible at 12 and 24 h were still detected and sometimes enriched at 36 and 48 h (Fig. 1, C) and D)). Also, at 48 h, distinctive bands not visible in the other sampling points could be observed (Fig. 1, A) and B)).

Table 1: Turbidity (OD 600 nm) and concentration (CFU mL^{-1}) of *Tenacibaculum maritimum* cultures at the sampling times in experiments 1 and 2.

Experiment 1		
Time (h)	**OD 600 nm**	**Bacterial concentration (CFU mL^{-1})**
6	0.271	9.4×10^{7}
12	-	-
24	0.296	9.9×10^{7}
48	1.651	3.7×10^{8}
Experiment 2		
Time (h)	**OD 600 nm**	**Bacterial concentration (CFU mL^{-1})**
6	0.076	5.5×10^{7}
12	0.116	6.3×10^{7}
24	0.679	1.8×10^{8}
36	0.972	2.3×10^{8}
48	0.849	2.1×10^{8}

Since some distinctive bands detected from 24 h onwards in lanes loaded with ECPs were not visible in lanes loaded with whole cells, it was predicted that those bands could correspond to proteins secreted by *T. maritimum*. Therefore, 5 bands (identified as B1-B5) of the 24 h-samples (Fig. 1) were excised and processed for proteomic analysis.

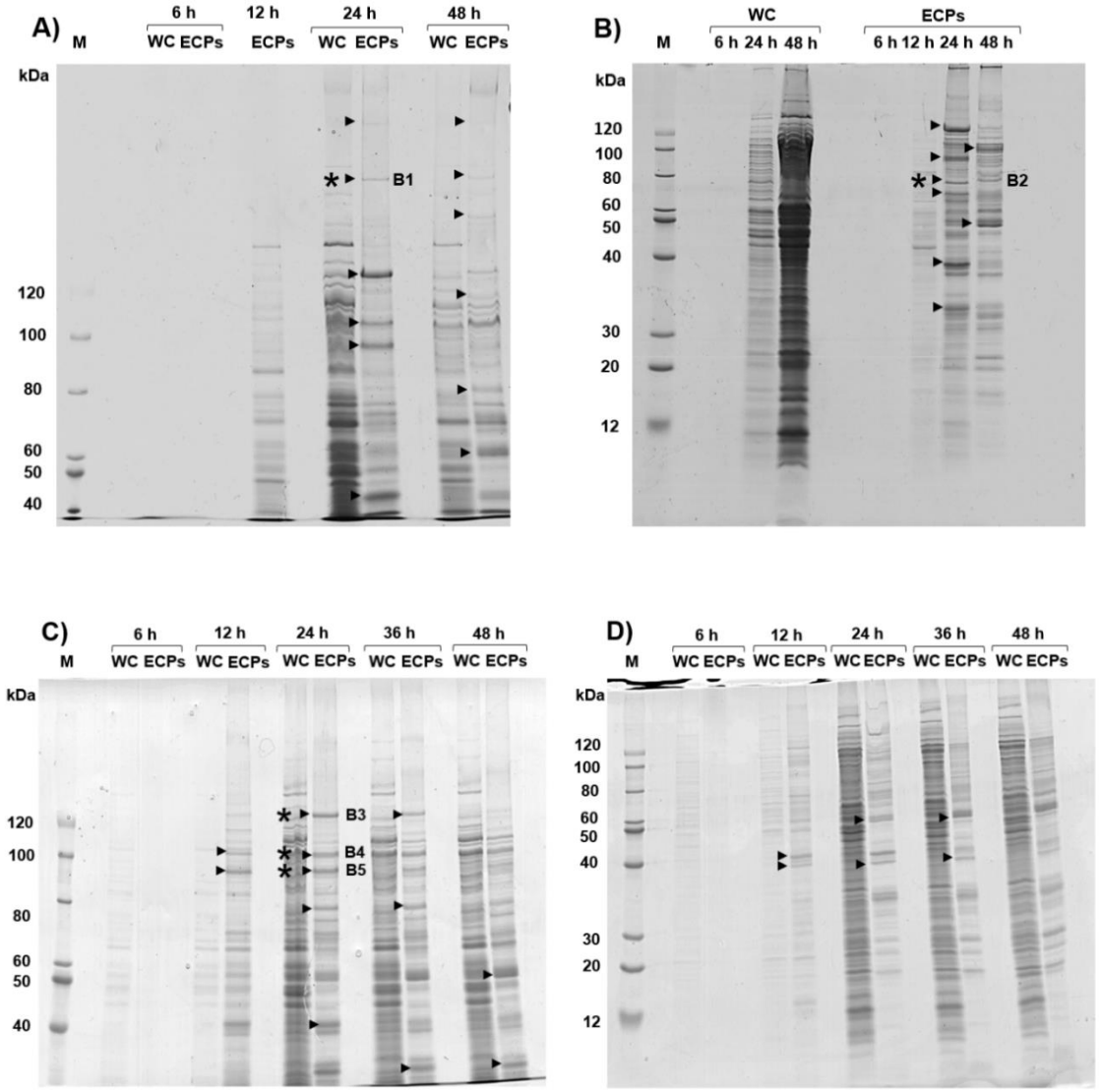

Figure 1: SDS-PAGE analysis of whole cells (WC) and cell-free culture supernatants (ECPs) of *Tenacibaculum maritimum* strain ACC13.1 (A and B- experiment 1; C and D- experiment 2). Analysis was performed in 8% (A and C) or 14% (B and D) polyacrylamide gels. For ECPs and WC, samples equivalent to 1 mL and 0.1 mL of bacterial culture, respectively, were loaded per lane. The gels were stained with Coomassie Brilliant Blue. M-molecular weight marker (GRS Unstained Protein Marker, GRiSP); numbers on the left indicate the molecular weight of the markers in kDa. Arrowheads indicate putative secreted proteins (highly enriched or exclusively present in ECPs). The excised bands analyzed by NanoLC-MS/MS are marked with an asterisk.

5.3.2. Identification of proteins in ECPs produced *in vitro*

The identity of the most abundant proteins (proteins with relative abundance > 5%) identified in each band excised from the gels is presented in Table 2 and the full list of identified proteins is available elsewhere (https://figshare.com/s/b6e7eb32f67bd93e0cd2).

It is worth noting that, as expected, most of the proteins present in the samples possess a predicted C-terminal secretion signal or correspond to putative outer membrane proteins (https://figshare.com/s/b6e7eb32f67bd93e0cd2). Bands B1, B3 and B5 corresponded to exo-alpha-sialidase (MARIT_2686; relative abundance 89.5%), M14 family carboxypeptidase (MARIT_2507; relative abundance 95.78%) and M43 family metalloprotease (MARIT_3130; relative abundance 89.75%), respectively, indicating that these proteins are amongst the most abundant proteins secreted *in vitro* by *T. maritimum* strain ACC13.1. In band B2, the most abundant protein was the gliding motility lipoprotein GldJ (MARIT_0896; 46.75% relative abundance), although it also contained significant amounts of a M12B family metalloprotease (MARIT_2638; 5.89% relative abundance). Band B4 contained mainly the M14 family carboxypeptidase present in Band 3 (MARIT_2507; relative abundance 55.20%) and a M4 family metalloprotease (MARIT_3129; relative abundance 31.94%). Since the M14 family carboxypeptidase has a predicted molecular weight of ~148 kDa (as determined by the Expasy ProtParam tool) (Gasteiger et al., 2005), its detection in band B4 (apparent molecular weight ~100 kDa) may be due to proteolytic processing of the protein, and consequent formation of a lower molecular weight form that co-migrates with the M4 family metalloprotease (predicted molecular weight of the mature form ~115 kDa). The presence of a conserved C-terminal Por secretion signal in M43 metallopeptidase, M14 family carboxypeptidase, M12B family metalloprotease, and M4 family metallopeptidase supports that these proteins are actively secreted by *T. maritimum*.

5.3.3. Identification of *T. maritimum* proteins expressed *in vivo*

A proteomics approach was used to identify potential virulence factors of *T. maritimum* present in the mucus of European sea bass shortly after bath infection with the pathogen. According to the criteria described above, 102 proteins were selected and identified (https://figshare.com/s/b6e7eb32f67bd93e0cd2). For a better interpretation of the results, the protein hits were classified according to their associated GO Term using the UniProt ID mapping platform.

Table 2: Most abundant proteins identified by NanoLC-MS/MS in bands B1, B2, B3, B4, and B5 (hits with relative abundance >5%).

Accession	Protein names (mapping)	Gene ID	Predicted MW (kDa)	Abundance	Abundance (%)	Gene Ontology
			B1			
A0A2H1ECB7	Exo-alpha-sialidase containing a C-terminal secretion	*siaA* MARIT_2686	245.5	5.6×10^{8}	89.56%	Carbohydrate metabolic process exo-alpha-(2->3)-sialidase activity; exo-alpha-(2->6)-sialidase activity; exo-alpha-(2->8)-sialidase activity; sialate 4-O-acetylesterase activity; sialate 9-O-
			B2			
A0A2H1E7X7	Gliding motility lipoprotein GldJ	*gldJ* MARIT_0896	65.3	4.5×10^{8}	46.75%	-
A0A2H1EDA6	Succinate dehydrogenase, flavoprotein subunit	*sdhA* MARIT_3013	74.8	6.6×10^{7}	6.83%	Fumarate reductase (menaquinone); succinate dehydrogenase (ubiquinone) activity
A0A2H1EC88	Probable M12B family metalloprotease containing a C-terminal secretion signal	MARIT_2638	107.3	5.7×10^{7}	5.89%	Proteolysis; metallopeptidase activity

			B3			
A0A2H1ECV9	Probable M14 family carboxypeptidase containing a C-terminal secretion signal	MARIT_2507	147.6	2.4×10^{9}	95.78%	Proteolysis; metallocarboxypeptidase activity; zinc ion binding
			B4			
A0A2H1ECV9	Probable M14 family carboxypeptidase containing a C-terminal secretion signal	MARIT_2507	147.6	8.9×10^{8}	55.20%	Proteolysis; metallocarboxypeptidase activity; zinc ion binding
A0A2H1EDL2	M4 family metallopeptidase containing a C-terminal secretion signal	MARIT_3129	125.0	5.2×10^{8}	31.94%	Proteolysis; metalloendopeptidase activity
			B5			
A0A2H1EER9	Probable M43 family metalloprotease containing a C-terminal secretion signal	MARIT_3130	95.6	3.6×10^{8}	89.75%	Proteolysis; metallopeptidase activity

Several putative virulence factors containing a C-terminal secretion signal and predicted to be secreted by the T9SS were identified in this analysis, including the M43 and M12B family metalloproteases (MARIT_3130 and MARIT_2638, respectively) and the exo-alpha-sialidase (MARIT_2686) (Table 3), which were also amongst the most abundant proteins *in vitro* produced ECPs (Table 2). Proteins related to the membrane were also found, such as the outer membrane protein (Omp) OmpH-like (MARIT_2671), OmpA family protein (MARIT_2995), and probable OmpA family (MARIT_0298) (Table 3). Type VI secretion system needle protein Hcp (MARIT_0833) and Type VI secretion system contractile sheath protein TssC (MARIT_0798), belonging to the machinery of the Type VI secretion system (T6SS), were also found. The T6SS is a nanomachine used by Gram-negative bacteria to translocate effector proteins directly into target cells, playing a key role in inter-bacterial competition (Coulthurst, 2019). Many proteins related to oxidative stress were identified in the mucus, including probable cold shock protein (MARIT_2838), superoxide dismutase (*sodA*, MARIT_3105), peroxidase (MARIT_1631), thioredoxin-dependent peroxiredoxin (*ygaF*, MARIT_2105), heat shock protein 70 (*dnaK*, MARIT_1078), catalase-peroxidase (*katG*, MARIT_0946), and thioredoxin (*trxA*, MARIT_2619) (Table 3). Nevertheless, the majority of the proteins identified in the mucus were intracellular proteins related to translation processes (e.g., *tuf* MARIT_0973, *tsf* MARIT_0398, and *fusA* MARIT_2372), tricarboxylic acid cycle (TCA cycle) (e.g., *mdh* MARIT_0889, and *sucC* MARIT_2292, and *fumC* MARIT_1906), and other carbon-related metabolism (e.g., *pck* pckA MARIT_0656, and *fbaA* MARIT_2642) (Table 3).

Table 3: Proteins putatively involved in the survival/virulence of *Tenacibaculum maritimum* identified in the mucus of bath-challenged European sea bass (*Dicentrarchus labrax*).

Accession	Protein ID	Gene ID (Acron.)	Predicted MW (kDa)	Gene Ontology (GO)
			Translation	
A0A2H1E892	Elongation factor Tu (EF-Tu)	*tuf MARIT_0973*	43.1	Cytosol; GTP binding; GTPase activity; translation elongation factor activity
A0A2H1E6B2	Elongation factor Ts (EF-Ts)	*tsf MARIT_0398*	35.0	Cytoplasm; translation elongation factor activity
A0A2H1ECR5	Elongation factor G (EF-G)	*fusA MARIT_2372*	77.4	Cytosol; GTP binding; GTPase activity; translation elongation factor activity; ribosome disassembly
A0A2H1E9Y2	30S ribosomal protein S1	*rpsA MARIT_1611*	66.0	Cytosolic small ribosomal subunit; mRNA binding; structural constituent of ribosome; translation

A0A2H1E908	Large ribosomal subunit protein bL12	*rplL MARIT_0979*	12.7	Cytosolic large ribosomal subunit; mRNA binding; structural constituent of ribosome; translation
A0A2H1EDB0	Translation initiation factor IF-2	*infB MARIT_2884*	104.9	Cytoplasm; GTP binding; GTPase activity; translation initiation factor activity
		Tricarboxylic cycle and carbon metabolism		
A0A2H1E656	Glutamate dehydrogenase	*gdhA MARIT_0249*	49.1	Cytosol; glutamate dehydrogenase (NADP+) activity; nucleotide binding; glutamate biosynthetic process;
A0A2H1E821	Phosphoenolpyruvate carboxykinase (ATP) (PCK) (PEP carboxykinase) (PEPCK)	*pck pckA MARIT_0656*	59.5	Cytosol; ATP binding; kinase activity; metal ion binding; phosphoenolpyruvate carboxykinase (ATP) activity; gluconeogenesis; phosphorylation
A0A2H1E8E8	Malate dehydrogenase	*mdh MARIT_0889*	32.4	L-lactate dehydrogenase activity; L-malate dehydrogenase activity; lactate metabolic process; pyruvate metabolic process; tricarboxylic acid cycle

A0A2H1ECH2	Succinate-CoA ligase subunit beta	*sucC* *MARIT_2292*	43.1	Cytosol; succinate-CoA ligase complex; ATP binding; magnesium ion binding; succinate-CoA ligase (ADP-forming) activity; succinate-CoA ligase (GDP-forming) activity; succinyl-CoA metabolic process; tricarboxylic acid cycle
A0A2H1EAM1	Fumarate hydratase class II (Fumarase C)	*fumC* *MARIT_1906*	49.9	Tricarboxylic acid cycle enzyme complex; fumarate hydratase activity; fumarate metabolic process; malate metabolic process; tricarboxylic acid cycle
A0A2H1EB88	Isocitrate dehydrogenase	*icd* *MARIT_2139*	81.7	Isocitrate dehydrogenase (NADP+) activity; metal ion binding; glyoxylate cycle; tricarboxylic acid cycle
A0A2H1EC77	Fructose-bisphosphate aldolase (FBP aldolase)	*fbaA* *MARIT_2642*	39.4	Cytosol; fructose-bisphosphate aldolase activity; zinc ion binding; gluconeogenesis; glycolytic process
		Cell membrane-related proteins		
A0A2H1ECP6	Outer membrane protein (OmpH-like)	MARIT_2671	18.9	Outer membrane-bounded periplasmic space; unfolded protein binding; chaperone-mediated protein folding; protein insertion into membrane from inner side; protein maturation by protein folding; protein stabilization

A0A2H1ED77	OmpA family protein	MARIT_2995	48.7	Cell outer membrane; pore complex; calcium ion binding; porin activity; cell adhesion; monoatomic ion transport
A0A2H1E6E7	Probable outer membrane protein, OmpA family	MARIT_0298	72.8	Membrane
A0A2H1EAQ0	Protein translocase subunit SecA	*secA MARIT_1895*	127.1	Cell envelope Sec protein transport complex; cytosol; plasma membrane; ATP binding; intracellular protein transmembrane transport; protein import; protein targeting; protein transport by the Sec complex
A0A2H1EBV9	Tetratricopeptide repeat protein	*MARIT_2190*	46.9	Cell outer membrane; receptor-mediated virion attachment to host cell
A0A2H1EBZ4	SusC/RagA family TonB-dependent receptor	*MARIT_2376*	113.5	Cell outer membrane

Oxidative stress-related proteins

A0A2H1E8F5	Chaperone protein DnaK (HSP70)	*dnaK* *MARIT_1078*	68.3	ATP binding; ATP-dependent protein folding chaperone; unfolded protein binding
A0A2H1E7S2	Catalase-peroxidase (CP)	*katG* *MARIT_0946*	80.3	Cytosol; catalase activity; heme binding; metal ion binding; cellular response to hydrogen peroxide; hydrogen peroxide catabolic process
A0A2H1EB86	Thioredoxin-dependent peroxiredoxin	*ygaF* *MARIT_2105*	16.8	Cytoplasm; thioredoxin peroxidase activity; cell redox homeostasis; cellular response to oxidative stress
A0A2H1E827	Alkyl hydroperoxide reductase, subunit C	MARIT_0947	23.6	Cytosol; thioredoxin peroxidase activity; cell redox homeostasis; cellular response to stress; hydrogen peroxide catabolic process; response to oxidative stress
A0A2H1E906	Thiol peroxidase (Tpx) (Thioredoxin-dependent peroxiredoxin)	*tpx* *MARIT_1280*	17.6	Thioredoxin peroxidase activity

A0A2H1EDI6	Superoxide dismutase	*sodA* *MARIT_3105*	22.4	Metal ion binding; superoxide dismutase activity
A0A2H1EAL8	Peroxidase	MARIT_1631	23.6	Cytosol; peroxiredoxin activity; cell redox homeostasis; cellular response to oxidative stress
A0A2H1EC90	Thioredoxin	*trxA* *MARIT_2619*	11.5	Cytosol; protein-disulfide reductase activity; cell redox homeostasis
A0A2H1ED92	Probable cold shock protein	MARIT_2838	7.0	Cytoplasm; nucleic acid binding
A0A2H1E8U9	DNA protection during starvation protein	*dps* MARIT_1360	17.9	Ferric iron binding; oxidoreductase activity, acting on metal ions

A0A2H1ED62	AhpC/TSA family protein	MARIT_2806	55.6		-
			Lipoproteins		
A0A2H1EAQ3	Lipoprotein	MARIT_1673	14.9		-
A0A2H1E8U3	Lipoprotein	MARIT_1345	17.0		-
A0A2H1E9Z7	Lipoprotein	MARIT_1666	43.9		-
A0A2H1E772	Lipoprotein	MARIT_0725	17.6		-

Predicted virulence factors				
A0A2H1EER9	Probable M43 family metalloprotease containing a C-terminal secretion signal	MARIT_3130	95.6	Metal ion binding; metallopeptidase activity; proteolysis
A0A2H1ECB7	Exo-alpha-sialidase containing a C-terminal secretion signal	*siaA* *MARIT_2686*	245.5	Cytoplasm; intracellular membrane-bounded organelle; membrane; exo-alpha-(2->3)-sialidase activity; exo-alpha-(2->6)-sialidase activity; exo-alpha-(2->8)-sialidase activity; sialate O-acetylesterase activity; ganglioside catabolic process; oligosaccharide catabolic process
A0A2H1EC91	Heme binding lipoprotein HmuY-family	MARIT_2478	28.8	-
A0A2H1E8R3	Adhesin SprB	*sprB* *MARIT_1321*	625.5	-
A0A2H1EC88	Probable M12B family metalloprotease containing a C-terminal secretion signal	MARIT_2638	107.3	Metallopeptidase activity; proteolysis

Secretion systems-related proteins				
A0A2H1E8L3	Type VI secretion system needle protein Hcp	MARIT_0833	15.0	Type VI protein secretion system complex; toxin transport
A0A2H1E7E0	Type VI secretion system contractile sheath protein TssC	MARIT_0798	52.0	Type VI protein secretion system complex; protein secretion by the type VI secretion system.

5.3.4. Protein-protein interaction analysis of the *T. maritimum* proteins expressed *in vivo*

A putative protein-protein interaction (PPI) network was constructed, and a visual representation was created using STRING 12.0 (Fig. 2). This analysis revealed a significant enrichment of the PPIs (7.0×10^{-11}) among the several *T. maritimum* proteins identified in the skin mucus of bath-challenged European sea bass. This network of PPIs included GO Terms related to the tricarboxylic acid cycle (FDR of 7.9×10^{-3}, GO:0006099), regulation of translation (FDR of 1.6×10^{-2}, GO:0006412), response to toxic substance (FDR of 1.6×10^{-2}, GO:0009636), peroxidase activity (FDR of 1.8×10^{-2}, GO:0004601), antioxidant activity (FDR of 1.8×10^{-2}, GO:0016209), and ion binding (FDR of 1.8×10^{-2}, GO:0043167) (Table 4).

Table 4: Top 5 (according to FDR) enriched GO Terms pathways for the *Tenacibaculum maritimum* proteins identified in the skin mucus of bath-challenged European sea bass (*Dicentrarchus labrax*).

Category	Term ID	Term description	Observed gene count	Background gene count	FDR
GO Component	GO:0005622	Intracellular anatomical structure	60	931	1.9×10^{-6}
GO Component	GO:0005737	Cytoplasm	54	835	1.1×10^{-5}
GO Component	GO:0110165	Cellular anatomical entity	86	2020	5.5×10^{-3}
GO Component	GO:0061695	Transferase complex, transferring phosphorus-containing groups	4	12	3.9×10^{-2}
GO Process	GO:0006099	Tricarboxylic acid cycle	6	10	7.9×10^{-3}
GO Process	GO:0009987	Cellular process	76	1576	7.9×10^{-3}
GO Process	GO:0006412	Translation	13	96	1.6×10^{-2}
GO Process	GO:0006518	Peptide metabolic process	14	111	1.6×10^{-2}
GO Process	GO:0009636	Response to toxic substance	8	35	1.6×10^{-2}
GO Function	GO:0005488	Binding	57	1004	4.0×10^{-3}
GO Function	GO:0004601	Peroxidase activity	5	9	1.8×10^{-2}
GO Function	GO:0016209	Antioxidant activity	7	26	1.8×10^{-2}
GO Function	GO:0043167	Ion binding	39	633	1.8×10^{-2}
GO Function	GO:0097159	Organic cyclic compound binding	42	724	2.5×10^{-2}

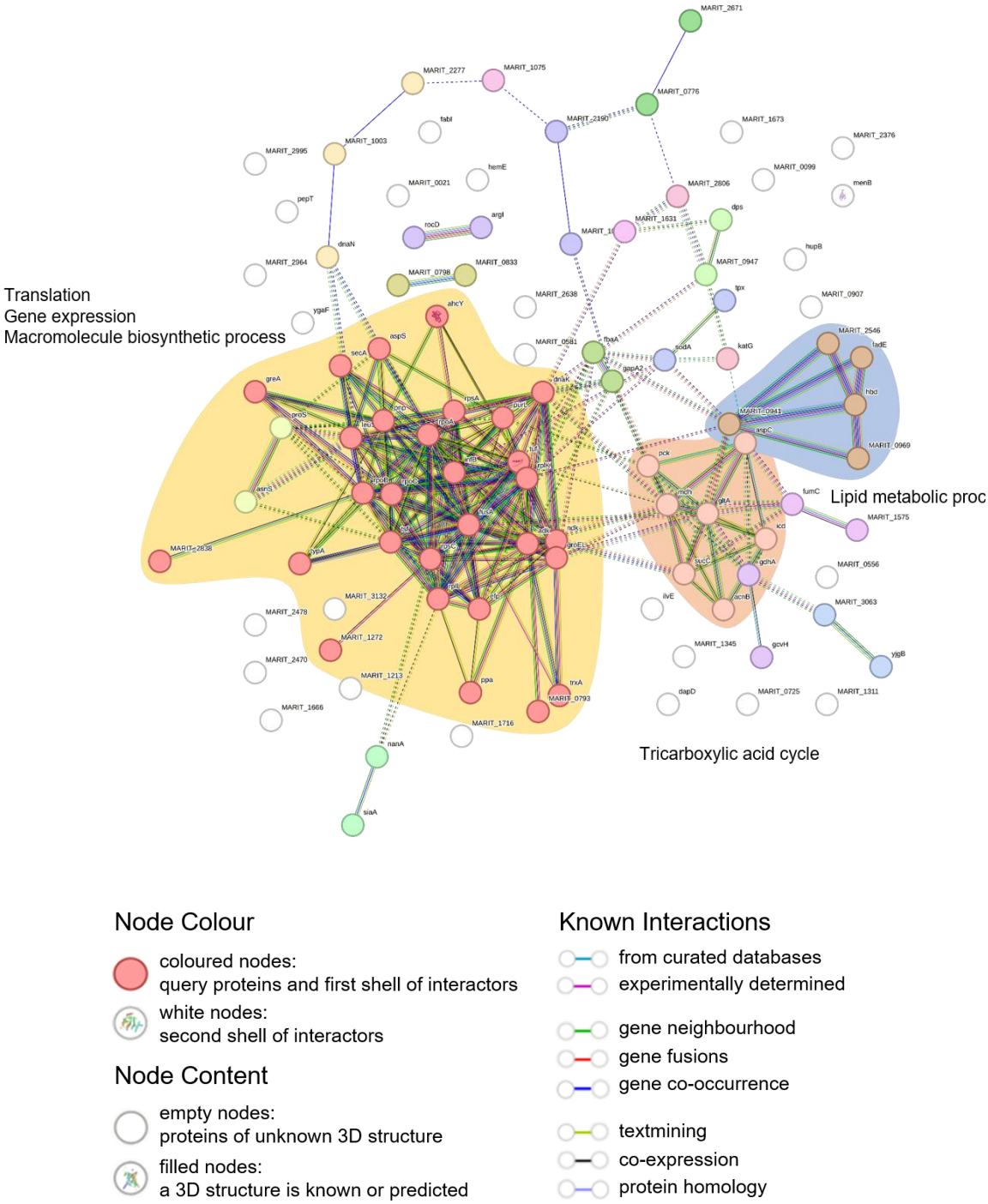

Figure 2: STRING protein-protein interaction network of proteins identified in the skin mucus of European sea bass (*Dicentrarchus labrax*) after bath challenge with *Tenacibaculum maritimum*. Each node (sphere) represents a protein produced by a single, protein-coding gene locus. The edges (connecting lines) represent protein-protein associations, which are specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other. The quantity of edges relates to the strength of the interaction relationship between proteins.

The main PPIs (edges) were centred among three main protein clusters: one associated with translation, gene expression, and macromolecule biosynthetic process (< 1.0×10^{-16} PPIs enrichment) (i.e., proteins such as chaperones, transcription, and elongation factors, proteins for translocation, ribosomal subunit proteins, etc.), other associated with the tricarboxylic acid cycle and carbon metabolism (< 1.0×10^{-16} PPIs enrichment) (i.e., proteins like succinate-CoA ligase, aspartate aminotransferase, malate dehydrogenase, etc.), and the last related to lipid metabolic process (i.e., proteins like fatty acid oxidation enzyme, acyl-CoA dehydrogenases, and 3-hydroxybutyryl-CoA dehydrogenase) (6.09×10^{-9} PPIs enrichment) (Fig. 2). Other two clusters were identified but presented no significant interactions (interaction involving low number of proteins).

5.4. Discussion

Since early studies, *T. maritimum*'s ECPs have prompted interest due to their proteolytic activity and ability to induce host tissue extensive damage, necrosis, and mortality (Baxa et al., 1988; Van Gelderen et al., 2009). It has been proposed that the combined activity of extracellular proteases secreted by *T. maritimum*'s ECPs, may promote the survival of this pathogen under hostile environmental conditions, in which it faces nutrient deprivation and encounters several antibacterial defense mechanisms (Pérez-Pascual et al., 2017). To thrive in such conditions, a pathogen must be able to survive and express the correct virulence factors within the right time through diverse regulatory schemes that include gene expression regulation (Ignatov & Johansson, 2017).

The proteomic analysis of the ECPs produced *in vitro* by *T. maritimum*'s strain ACC13.1 revealed the secretion, from 24-48 h of growth, of multiple proteins that may correspond to potential virulence factors. The identification of proteins presents in some of the most abundant bands excised from SDS-PAGE gels showed the presence of many proteins with a conserved C-terminal sorting domain (CTD) likely involved in T9SS-mediated secretion. The T9SS was identified as a secretion system present in the Cytophaga-Flavobacterium-Bacteroidetes cluster, able to secrete several effector proteins, like adhesins, proteases, chitin, cellulases, and other surface layer proteins to the extracellular medium and cell surface (Gorasia et al., 2020; Veith et al., 2015). The proteins secreted by T9SS possess an N-terminal signal peptide that allows transport across the inner membrane by the Sec system. Once in the periplasm, the proteins destined for T9SS secretion have a CTD, which is a specific secretion signal that targets them to the T9SS machinery in the outer membrane (Gorasia et al., 2020; Veith et al., 2013).

It has been shown that the T9SS is responsible for the secretion of important virulence factors in several members of the phylum Bacteroidetes, including the fish pathogens

Flavobacterium psychrophilum and *F. columnare* (Barbier et al., 2020; Li et al., 2017), and *Porphyromonas gingivalis*, the causative agent of chronic periodontitis in humans and animals (Mei et al., 2020). (Sato et al., 2010, 2013; Veith et al., 2013).

Among the identified *T. maritimum* proteins identified in *in vitro* produced ECPs and in the mucus of infected fish are the T9SS-secreted M14 carboxypeptidase, the M12B and M43 family metalloproteases and the multimodular sialidase. These proteins were also identified in the ECPs used in the *in vivo* challenge described in Chapter 2. Escribano et al. (2023) also reported the presence of a carboxypeptidase M14 in both insoluble (OMVs) and soluble ECP fractions. Bacterial metalloproteases are metal-containing proteases whose primary function is to degrade environmental proteins and peptides to obtain nutrients (Wu & Chen, 2011). In addition, many of these peptidases play an important virulence role (Gimza et al., 2021; Marquart, 2021; Zhou et al., 2015), facilitating the degradation of host immune proteins and mediating adhesion (Tokuda et al., 1996). *T. maritimum* and *F. psychrophilum* are closely related species of the Flavobacteriaceae family, able to trigger diseases with similar gross pathology (Knupp & Loch, 2023; Kumanan et al., 2024). *F. psychrophilum* possesses extracellular proteolytic enzymes, Fpp1 (M12 metalloproteases family) and Fpp2 (M43 cytophagalysin family), that are responsible for extracellular proteolytic activity, bacterial motility, and colony spreading (Pérez-Pascual et al., 2011) and are able to digest components of the extracellular matrix and muscle proteins (Secades et al., 2001, 2003). However, the involvement of such proteins in pathogenesis remains to be determined since deletion of its encoding gene did not affect virulence for rainbow trout (*Oncorhynchus mykiss*) (Pérez-Pascual et al., 2011). Nevertheless, considering the predicted activities of *T. maritimum* M43 and M12B family metalloproteases present in infected sea bass mucus, it would be interesting to investigate their participation in the initial phases of host colonization and to fully uncover their virulence roles.

Another protein identified in the *in vitro*-produced ECPs used in Chapter 2 and in the epidermis mucus of challenged European sea bass was a heme-binding lipoprotein from the HmuY-family, predicted to be involved in heme uptake (Pérez-Pascual et al., 2017). This protein was also identified in Escribano et al. (2023) in OMVs and soluble ECPs fractions. Iron acquisition has been associated with virulence in several pathogens belonging to the Bacteroidetes Phylum, including *F. psychrophilum* and *F. columnare* (Conrad et al., 2022; Zhu et al., 2022), and heme is a vital iron source for many Gram-negative bacterial pathogens (Conrad et al., 2022; Skaar, 2010). For example, *F. psychrophilum* uses a heme acquisition system that involves a HmuY-like protein (HfpY), and the deletion of *hfpY* led to a decrease in virulence for rainbow trout fry (Zhu et al., 2022). Thus, the role of the *T. maritimum* heme-binding lipoprotein HmuY-family identified in the present work deserves to be explored.

Amongst the proteins identified in the *in vivo* trial and *in vitro* produced ECPs were several outer membrane proteins, such as the SusC/RagA family TonB-dependent receptor and OmpH and OmpA, which were also reported in the Escribano et al. (2023) study. Outer membrane proteins of Gram-negative bacteria have an essential role in mediating antibacterial resistance and bacterial virulence, affecting the pathogenicity of bacteria (Mishra et al., 2020). Recently, a study developed by Escribano et al. (2024) used OMVs from *T. maritimum* strain SP9.1, which are known to be enriched in outer membrane proteins such as TonB-dependent transporters and T9SS components, as encapsulated multi-antigen vaccines, and obtained a significant protection against *T. maritimum* infection (RPS = 70 %). Another protein identified in the skin mucus of challenged fish was the SecA translocase subunit. This protein is an essential component of the Sec translocon that, in pathogenic species, is involved in the secretion of virulence factors and toxins required for bacterial viability and virulence (Ambroziak et al., 2021). Studies have demonstrated that SecA inhibition leads to antimicrobial effects, like growth inhibition and attenuated secretion of virulence factors (Cui et al., 2013; Jin et al., 2015, 2021).

The profile of the *T. maritimum* proteins present in the skin mucus of infected sea bass suggests that these bacteria were under potential oxidative stress, possibly generated by the host defense mechanisms. Among the identified proteins related to stress tolerance and adaptation is the cold shock protein (*Csp*, MARIT_2838), which was only identified in the mucus of infected fish. Csps, also known as RNA-binding proteins (RBPs), were initially discovered in bacteria in response to a downshift in temperature (Jones & Inouye, 1994), but are increasingly recognized as players in bacterial growth at suboptimal and optimal temperatures (Graumann et al., 1997; Moon et al., 2023) and in bacterial virulence (Muchaamba et al., 2021; Tomlinson et al., 2022). It can be speculated that *T. maritimum* Csp performs similar roles to those seen for other bacteria, counteracting the stress conditions encountered in the host during infection, thereby ensuring survival and proliferation. Besides Csp, other proteins were also identified only in the *in vivo* trial, like thioredoxin peroxidase, thiol peroxidase, superoxide dismutase, and peroxidase. These proteins are important mediators of bacterial oxidative stress response (Alharbi et al., 2019; Ma et al., 2022; Song et al., 2016). Indeed, it was previously shown that the *Edwardsiella piscicida* thioredoxin system plays a decisive role in its motility, flagella formation, bacterial resistance against host serum, bacterial survival and replication in phagocytes and bacterial dissemination in host immune tissues (He et al., 2023). The deletion of the coding gene for a superoxide dismutase in *E. tarda* resulted in a significant decrease in bacterial resistance to macrophage-mediated killing, and simultaneously, the respiratory burst of Japanese flounder head-kidney macrophages was enhanced (Cheng et al., 2010). Another study revealed that the oxidative stress response of pathogenic *Leptospira interrogans* is

regulated by two peroxide stress regulators that are able to interplay and control virulence and pathogenicity, possibly by transcriptional control (Zavala-Alvarado et al., 2021). Moreover, a study that compared the transcriptome of *F. psychrophilum*-resistant versus *F. psychrophilum*-susceptible rainbow trout genetic lines showed an under-representation of bacterial genes related to oxidative stress response, like thiol peroxidase; this suggested that these bacteria in resistant fish may be more vulnerable to oxidative stress, as the production of oxidants is a commonly used host defense strategy against pathogens (Chapagain et al., 2023). The presence of such proteins in the skin mucus of challenged fish may also contribute to *T. maritimum*'s resistance to oxidative stress and survival in fish mucosa, as seen for other bacterial pathogens (Fang, 2011; Maurya et al., 2021; Ramarao et al., 2000; Treffon et al., 2020).

In addition to the putative virulence factors and oxidative stress mediators, the mucus from infected fish also contained an array of *T. maritimum* proteins associated with the TCA cycle, translation, and gene expression. Additionally, proteins like succinate-CoA ligase, a fumarate hydratase, a 2-oxoglutarate-dependent ethylene/succinate-forming enzyme (related to TCA cycle), and transcription elongation factor GreA among others (related to translation and gene expression), were exclusively found in the mucus samples. Since bacteria form their structures using several macromolecules (e.g., proteins, phospholipids, and nucleic acids) essential to their integrity and pathogenicity (Ozma et al., 2022), energy production, translation, and other macromolecule metabolic processes play crucial roles in host invasion and colonization.

The TCA cycle is central for energy generation and for the production of carbon-based precursor molecules needed for the biosynthesis of amino acids, nucleotides, cofactors, and several mediators required for various pathways in the cell (Kwong et al., 2017). The TCA cycle, which encompasses some of the identified *T. maritimum* proteins (e.g., succinate-CoA ligase, isocitrate dehydrogenase, etc.), has been linked to the control of cellular behaviors and virulence factors in bacteria like *Agrobacterium tumefaciens*, *Escherichia coli*, *E. tarda*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Staphylococcus aureus* (Ding et al., 2014; Kuo et al., 2018; Suksomtip et al., 2005; Takeuchi et al., 2009; Wang et al., 2021). Several studies revealed the important role of the TCA cycle in the regulation of bacterial virulence factors (Dacheux et al., 2002; Hotinger et al., 2021; Mercado-Lubo et al., 2008, 2009; Yimga et al., 2006). The modulation of such virulence factors may be done through the metabolic inhibition of biosynthetic enzyme activity or the regulation (induction/suppression) of virulence gene transcription by the TCA cycle (Somerville & Proctor, 2009). In the case of *T. maritimum*, the dynamic of the metabolic activity (e.g., carbon metabolism) during infection and its role in virulence activation remain undisclosed and deserve to be investigated. HU is one of the most

abundant nucleoid-associated proteins in bacterial cells and is responsible for the regulation of many genes involved in growth, replication, motility, metabolism, and virulence (Stojkova et al., 2019). For example, *Vibrio parahaemolyticus* cytotoxicity is regulated in a growth-dependent fashion by the HU proteins through the regulation of a number of virulence factors (Phan et al., 2015). This protein can also influence virulence gene expression in other pathogenic bacteria, including *Salmonella enterica* serovar *Typhimurium*, *Francisella tularensis*, and *P. gingivalis* (Davey et al., 2013; Mangan et al., 2011; Stojkova et al., 2018). In *E. coli*, this protein controls 8% of genes across the genome that are associated with the adaptation of the cell to the hostile environment of the host cell or with stress response (Oberto et al., 2009). Transcription Elongation Factor GreA can also affects the expression of virulence genes, and its deletion can lead to impaired bacterial invasion, growth retardation, and poor survival under adverse stress (Cui et al., 2018; Feng et al., 2020).
The comparison between the excised bands, the total ECPs (Chapter 2), and *T. maritimum* proteins present in the skin mucus of bath-challenged European sea bass allowed the identification of several proteins that have the potential to be *in vivo*-expressed putative virulence factors. Future studies should be undertaken to study their specific roles in pathogenicity and their suitability as potential antigens to be applied in prophylactic strategies.

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CHAPTER 6

General discussion, final considerations and future perspectives

6.1. General Discussion

6.1.1. Mucosal innate immunity against *T. maritimum*: determining the battle outcome

Fishes are continuously in contact with a microbial-rich environment that allows the exposure of every epithelial barrier of their body to potential pathogens (Salinas, 2015). Many of these pathogens can infect a host through mucosal surfaces, which denotes the importance of the mucosal immune response in both infection and its regulation (Conforto et al., 2021). The understanding of such immune defense mechanisms can be critical for preventing and controlling potential outbreaks, in addition to providing a foundation for the development of effective prophylactic methods.

Using bath challenge - an inoculation pathway that mimics the natural infection route of *T. maritimum*, and consequently, the outbreaks in aquaculture settings - the work presented in **Chapters 3** and **4** of the thesis offered new insights into the mucosal immune response occurring at the gills, skin and posterior intestine of European sea bass. The data presented in **Chapter 3** revealed that a pro-inflammatory response with similar kinetics was triggered at these tissues upon challenge with *T. maritimum*. Nevertheless, the gills seemed to respond faster against *T. maritimum*, which raises the hypothesis that this organ could be a possible entry route for this pathogen. Since gills are physically delicate and permeable, they are susceptible to damage from external factors, which include invasion and colonization by bacteria (Herrero et al., 2018). Apart from the marked increase in expression of interleukin-1 beta (*il1β*), hepcidin (*hamp1*), an antimicrobial peptide related to iron withholding, also displayed a comparable rise in all analyzed organs. Iron starvation is a common strategy used by the host to suppress infection (Ullah & Lang, 2023). The fast expression of hepcidin upon bath infection with *T. maritimum* suggests its important role in the mucosal defense against this pathogen, which has been previously shown to require iron for growth (Avendaño-Herrera et al., 2005; Pérez-Pascual et al., 2017). In addition, the bath challenge also led to an increase in the expression of *il8*, which is known to mediate neutrophil attraction to sites of injury (Fousek et al., 2021), and *mmp9*, which causes ECM breakdown (possibly necessary for cell recruitment) (Tomlin & Piccinini, 2018) in all analyzed organs, further revealing a transversal pro-inflammatory response against *T. maritimum*. Despite the fast response at 6 h post-challenge regarding the molecular immune markers, at 24 h post-challenge, fish showed symptomatology compatible with tenacibaculosis, including ulcers in the skin and frayed fins. Immunohistochemistry analysis showed immunoreactivity to *T. maritimum* only in the skin, revealing an extensive and fast progression of this pathogen across the dermis. Altogether, the findings presented in

Chapter 3 offered a glimpse of the complex molecular mechanisms used by the host to counteract tenacibaculosis. In **Chapter 4**, a transcriptomic approach revealed an increased expression of genes related to eicosanoid metabolism and other inflammatory mediators, acute phase response, iron withholding, and tissue remodelling in the skin of challenged fish. Moreover, bacterial exposure resulted in down-regulation of genes involved in wound healing. Genes coding for some types of collagens and ECM structural proteins were downregulated, whereas genes coding for proteinases responsible for the degradation of the ECM and respective growth factors were up-regulated. The down-regulation of some types of collagens can occur during the re-epithelization process when associated with damage to the skin (Costa & Power, 2018). In gilthead sea bream, this process, independent of inflammation, resulted in a significant decrease of collagen (e.g., collagen type I, V, and X) in regenerating skin from 6 h to a maximum of 3 days' post-lesion induction (Costa & Power, 2018).

In addition to the transcriptomic analysis of skin from bath-challenged fish, a proteomic analysis of the skin mucus was also performed (**Chapter 4**), validating the existence of an inflammatory response in the skin, as well as an increase of proteins associated with complement activation, and wound healing. Several complement proteins were increased in the skin mucus of bath-challenged fish, which indicates a response against *T. maritimum*. Nevertheless, studies developed by Mabrok et al. (2016) and Magariños et al. (1995) revealed that the skin mucus from Senegalese sole (*Solea senegalensis*) and European sea do not display bactericidal activity against *T. maritimum*. This may suggest that these fish species do not contain adequate compounds with potent bactericidal activity to eliminate *T. maritimum* in skin mucosa (Mabrok et al., 2016; Magariños et al., 1995). Despite the existence of an inflammatory response in challenged fish, some animals were not able to control the infection induced by *T. maritimum*, resulting in mortality. Possibly, and according to the previously mentioned studies, *T. maritimum* may possess strategies to control/neutralize host immune response, resulting in the overcoming of mucus antimicrobial activities. The proteomic analysis also uncovered the existence of an innate humoral response in the skin mucus of European sea bass bath-challenged with *T. maritimum*, involving the modulation of proteins that participate in pathogen recognition and elimination. Interestingly, the tight junction (TJ) claudin I was downregulated in the skin and skin mucus of challenged fish. Although a better understanding of TJs functions is necessary, it is speculated that, in mammals, these also participate in wound healing and are needed for effective tissue repair (Shi et al., 2018), besides regulating epithelial permeability (Wibbe & Ebnet, 2023). In teleost, it was already proven that TJ expression could be modulated, leading to a significant decrease or increase in the expression of claudins during viral (Adamek et al., 2013) or bacterial (Deng et al., 2022) infections. The

increased expression of TJs can be related to better intestinal epithelial integrity, which is important to preserve epithelia's physical barrier function against pathogens (Deng et al., 2022). Meanwhile, the decreased expression of TJs can help pathogens gain access to deeper tissue (Adamek et al., 2013; Feng et al., 2022). Therefore, it would be interesting to investigate if the decrease seen in the present study is sustained through time, as well as to understand if *T. maritimum* is directly responsible for modulating claudin expression at early stages of infection (e.g., through virulence factors or possibly post-transcriptional modifications of host proteins).

These results showed that bath challenge with *T. maritimum*, beyond inducing a classical inflammatory response, modulates host wound healing and remodeling processes, indicating that this pathogen triggers a complex local mucosal response in fish skin and mucus.

6.1.2. European sea bass systemic innate immune responses upon *T. maritimum* challenges

Although tenacibaculosis has a significant negative impact on the aquaculture industry, the available knowledge about the host's innate immune response against this pathogen at both systemic and mucosal levels remains limited. Previous studies have reported a bland host response at these levels based on assessments of different humoral and cellular immune parameters, such as bactericidal activity, hematological profile, and kinetics of cell migration (Guardiola et al., 2019; Mabrok et al., 2016).

In this work, the immune response of European sea bass against this pathogen was investigated using two different infection methods: bath challenge and intraperitoneal (i.p.) injection, both with live *T. maritimum*-washed cells (**Chapters 2** and **3**). The bath challenge method was used because it mimics the natural infection route, while the i.p. injection is a more controlled method to induce systemic disease.

The haematological analysis of bath-challenged fish demonstrated a decrease in red blood cell concentration and haematocrit at 24 and 48 h when compared to controls, which points to the occurrence of infection-associated haemorrhagic anemia. It can be speculated that the destruction of red blood cells, one of the main sources of haemoglobin, can benefit *T. maritimum* during pathogenesis. However, when intraperitoneally injected, *T. maritimum* did not have the same effect on the haematological parameters, indicating that a different dynamic was established between the pathogen and host upon this infection method. Regarding cell migration kinetics, bath challenge with *T. maritimum* led to an increase in circulating neutrophils at 6, 48, and 72 h post-challenge, while monocytes only increased from 48 h onwards. Similar results were obtained by Guardiola et al. (2019), who found that

the number of circulating neutrophils increased after 48 and 72 h in bath-challenged Senegalese sole. In contrast, thrombocytes and lymphocytes remained quite unaltered in the present study. Again, a different response was observed for i.p. injected fish, where a fast response was seen early after infection (3 h post-challenge), with an abrupt decrease in blood total WBC, lymphocytes, and thrombocytes. In mammals, invasive bacteria can induce thrombocytopenia and lymphopenia, and several pathogens can activate, aggregate, and destroy these cells (Finfer et al., 2023; Yeaman, 2010), resulting in a drop in platelet and lymphocyte counts. Altogether, the results obtained with the two infection methods suggest the occurrence of a faster systemic response following i.p. inoculation. Considering that bath challenge is a more natural way of infection for *T. maritimum*, it may provide time and conditions for bacteria to proliferate in the skin. Through quorum-sensing mechanisms, bacteria can lessen the host's immune responses by postponing the production of virulence factors until sufficient bacteria have accumulated and prepared to counteract host defense mechanisms and establish infection (Deep et al., 2011). In contrast, inoculation of *T. maritimum* into the peritoneal cavity, which is an environment full of resident immune cells and other immune mediators, may facilitate its rapid elimination by the host. Both infection methods resulted in a general lack of response in plasma innate immune and oxidative stress parameters. Only plasma bactericidal activity remained low until 48 h, when it started to increase in bath-challenge fish, possibly due to a later migration of phagocytes. The same occurred for i.p. injected fish, with a greater increase at 48 h post-challenge. Nevertheless, despite the lack of response, viable bacteria were found in the bloodstream and peritoneal exudates of intraperitoneally challenged fish as soon as 3 h post-challenge. Apparently, these bacteria were quickly eliminated by the host since no growth was observed from 24 h post-challenge onwards. Even though the innate humoral and oxidative stress parameters did not show any differences, the analysis of gene expression in the head-kidney of intraperitoneally injected fish indicated the occurrence of an inflammatory response, with a substantially increased expression of several important cytokines (i.e., *il1b*, *il6*, *il8*, *il10*) as soon as 3 h post-challenge. Notably, in a previous bath challenge trial with European sea bass (data not shown), *T. maritimum* triggered a similar modulation of gene expression in the head-kidney, although the response was much weaker compared to fish injected intraperitoneally. For example, *il1b* expression increased approximately 7-fold in the bath challenge, whereas a more than 200-fold increase was observed in fish injected intraperitoneally. This more intense host response may be related to the presence of the bacteria in both the peritoneal cavity and the bloodstream of i.p. injected fish. It is noteworthy that no mortality and no typical symptomatology were observed in European sea bass challenged by i.p. injection, indicating that this is not an effective method to reproduce the disease. On the other hand, a bath exposure of 2 hours at 25ºC

induced the typical clinical signs of tenacibaculosis and led to significant mortality. Altogether, the findings obtained in both studies suggest that the route of entry of *T. maritimum* is decisive for the establishment of the infection and pathology development.

6.1.3. Immunogenic effects of *T. maritimum* extracellular products

Despite several studies indicating that *T. maritimum* ECPs display important biological activities that contribute to the pathogenesis of tenacibaculosis, data on their immune-stimulating activity are scarce. In this work, the systemic response of European sea bass to the i.p. injection of *T. maritimum* ECPs, alone or mixed with bacterial cells, was evaluated (**Chapter 2**).

The i.p. injection of ECPs did not show any differences regarding the cell migration kinetics in the peritoneal cavity. However, the injection of bacterial cells plus ECPs triggered a significant increase in total leukocyte numbers in the peritoneal cavity at 3 h post-challenge when compared with the remaining groups. Blood total leukocyte, lymphocyte, and thrombocyte numbers decreased immediately after the challenge, more accentuated for the fish injected with bacterial cells plus ECPs when compared with the remaining groups. These acute inflammation signs (Ishimine et al., 2013; Schmitz et al., 2016) suggest that bacteria plus ECPs act synergistically and induce stronger chemotactic signals than bacteria alone, possibly resulting in their fast migration to the peritoneal cavity. Nevertheless, despite no response in the peritoneal cells against the ECPs treatment, the gene expression analysis showed modulation of molecular immune markers congruent with an acute inflammatory response. Increased expression of *il1b*, *il6*, *il8*, and *hamp1* in fish challenged with ECPs and bacterial cells plus ECPs suggested a more exacerbated pro-inflammatory response in the head-kidney against both treatments.

The combination of *T. maritimum*'s cells and its ECPs induced an enhanced inflammatory response compared to the remaining treatments, as demonstrated by peritoneal cell migration kinetics and pro-inflammatory molecular biomarkers. Although some studies have approached the immunogenic potential of *T. maritimum*'s ECPs (Escribano et al., 2023; Salati et al., 2005), the antibody titer in European sea bass could also be further assessed in future studies.

6.1.4. *In vitro* and *in vivo* secreted *T. maritimum* extracellular products: potential virulence factors

Recent evidence suggests that the ECPs secreted by *T. maritimum* are one of the main virulence mechanisms used by these bacteria to adhere, invade, and colonize the host

(Escribano et al., 2023; Mabrok et al., 2023; Michnik et al., 2024). Using a proteomic approach (**Chapters 2**, **4**, and **5**), some of the proteins secreted by *T. maritimum* virulent strain ACC13.1 were identified both *in vitro* and *in vivo*. In the *in vitro* secreted ECPs (**Chapters 2** and **5**), several proteins were identified, including outer membrane proteins, lipoproteins, adhesins, and other proteins with CTD signal, which were previously described as probable secreted virulence factors (Pérez-Pascual et al., 2017). Other proteins, such as M12B and M43 family metalloproteases and carboxypeptidase M14, as well as multimodular sialidase, were also present. For instance, carboxypeptidases and metalloproteases are known to be important for the proteolytic processing of other proteinases or adhesins in other phylogenetically close bacteria (Matiuzzi Da Costa et al., 2024; Veith et al., 2004). These proteolytic enzymes may be responsible for the effects (i.e., cytotoxicity, the disintegration of the epithelium, and necrosis) seen *in vitro* (Mabrok, 2016; Michnik et al., 2024) and *in vivo* (Escribano et al., 2023; Van Gelderen et al., 2009) in studies using *T. maritimum* ECPs, which later facilitates host tissue invasion. Many other enzymes with biological relevance were found *in vitro* (e.g., TonB-dependent receptors associated with the membrane). A study by Escribano et al. (2024) used crude *T. maritimum* OMVs (known to be rich in outer membrane proteins such as TonB-dependent transporters) as an encapsulated multi-antigen vaccine. This approach resulted in significant protection against *T. maritimum* infection (RPS = 70%), with fish exhibiting an increase in anti-Tm antibody titers in blood plasma, with fast induction of innate and adaptive immune-related genes (Escribano et al., 2024).

The proteomic analysis of *T. maritimum* proteins present in the mucus of fish bath challenged with *T. maritimum* (**Chapter 5**) led to the identification of several proteins, including the probable M12B and M43 family metalloproteases, both containing a C-terminal secretion signal. Similarly, to the *in vitro* results, proteins related to the membrane were also found, such as the outer membrane protein (OmpH-like) and the OmpA family. Several oxidative-stress-related proteins were identified in the mucus, including probable cold shock protein, superoxide dismutase, thioredoxin-dependent peroxiredoxin, catalase-peroxidase, and thioredoxin. Being expressed *in vivo*, some of these proteins may play an important role in *T. maritimum* survival in the mucosa by counteracting the host's oxidative burst defense strategies.

Nevertheless, the majority of the proteins identified in the mucus were intracellular proteins related to translation processes, the tricarboxylic acid cycle (TCA cycle), and other carbon-related metabolism. The TCA cycle has been implicated in the control of cellular behaviors (e.g., response to stress defense) and virulence factors in several pathogenic bacteria (Bücker et al., 2014; Dieppedale et al., 2013; Somerville & Proctor, 2009). To establish an effective infection, *T. maritimum* must scavenge for nutrients, as well as coordinate its

metabolism to ensure its proliferation and expression of virulence factors. The crossroads between these hub pathways are unknown, so future studies could also focus on the relationship between the cell metabolic state and virulence triggering.

6.2. Future perspectives

T. maritimum is a complex pathogen that uses an array of virulence factors. In recent years, important information was provided by the complete sequence of its genome and the proteomic characterization of the ECPs secreted *in vitro* and by the here-reported identification of proteins present *in vivo* in infected fish. Nevertheless, studies involving genetic manipulation and the use of isogenic mutants are necessary to explore and confirm the role of such proteins in *T. maritimum* pathogenicity. A complete picture of the virulence arsenal of this pathogen can only be achieved through the identification and characterization of crucial factors operating at the different infection phases, as well as the characterization of the mechanisms involved in their regulation. This would be a big step forward towards the development of prophylactic and therapeutic interventions to control tenacibaculosis.

In the present thesis, a broader perspective of the mucosal response against tenacibaculosis is presented, highlighting the expression profile of several biomarker genes and proteins related to inflammation (e.g., interleukin 1 beta, hepcidin, haptoglobin) and wound healing (e.g., metallopeptidases 9 and 13). To provide a deeper insight into the host response against *T. maritimum* and identify relevant mediators/pathways associated with resistance or susceptibility to this pathogen, it would be interesting to perform transcriptomic and proteomic analyses of the mucosal and systemic tissues of bath-infected fish (individually) and to correlate the gene expression and protein profiles with disease outcome (i.e., dead/ survival). This could allow the identification of disease and resistance biomarkers, potentially useful to design interventions to prevent disease or minimize its severity. Interestingly, intraperitoneally injection of *T. maritimum*'s ECP in European sea bass did not induce mortality or gross pathology, contrary to what has been reported for other species, such as Atlantic salmon. Although this suggests a host-specific response against the pathogen, it cannot be excluded that strain-related differences also contribute to these apparently contrasting findings. Therefore, *in vivo* trials testing the same ECPs preparations in different fish species could clarify if the toxicity of the ECPs, and hence their pathogenic role, varies amongst different hosts. This could have relevant implications for considering the use of ECPs as vaccine antigens.

6.3. References

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Appendix

APPENDIX I

Tenacibaculum maritimum can boost inflammation in *Dicentrarchus labrax* upon peritoneal injection but cannot trigger tenacibaculosis disease

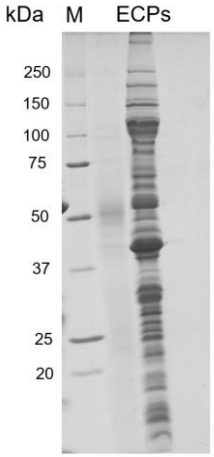

Figure S1: Coomassie-blue stained SDS-PAGE gel of the cell-free concentrated *Tenacibaculum maritimum* ECPs used to challenge European sea bass (*Dicentrarchus labrax*) by intraperitoneal injection (i.p.). The lane contains protein equivalent to 1 mL of concentrated ECPs. M- Molecular weight marker. Numbers on the left indicate the molecular weight of the markers (Precision Plus Protein™ Unstained Protein Standards, Bio-Rad), in kDa.

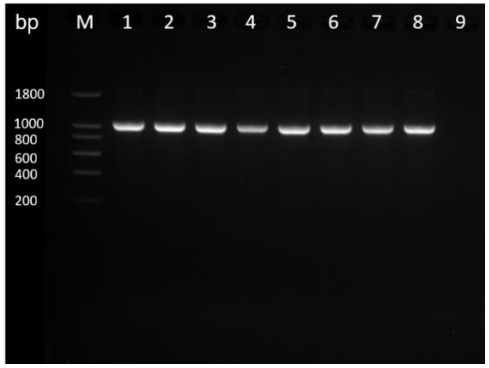

Figure S2: PCR products obtained with MAR1 and MAR2 using bacterial genomic DNA extracted from randomly selected colonies obtained from peritoneal exudates and blood samples of challenged fish (Lanes 1-7); Lane 8- positive control - *Tenacibaculum maritimum* strain ACC13.1; Lane 9- negative control - sterile distilled water); M: NZYDNA Ladder I (NZYTech, Lisbon, Portugal) (200 to 1800 bp). Numbers on the left indicate the size of the marker, in bp.

Table S1: *Tenacibaculum maritimum*'s proteins identified by NanoLC-MS/MS in concentrated ECPs used to challenge European sea bass (*Dicentrarchus labrax*) by i.p. injection. The table shows hits with coverage above 30%, unique peptides superior to 3 and SEQUEST HT score greater than 100.

Accession	Name	Gene names	Coverage (%)	Unique Peptides	MW (kDa)	calc. pI	Score Sequest HT	Gene Ontology (GO)	Go Term
Potential virulence factors									
A0A2H1ECB7	Exo-alpha-sialidase	*siaA* MARIT_2686	55	103	245.5	5.4	2551.5	Carbohydrate metabolic process	GO:0005975
A0A2H1EER9	Probable M43 family metalloprotease containing a C-terminal secretion signal	MARIT_3130	40	15	95.6	5.3	2140.4	Proteolysis	GO:0006508
A0A2H1ECV9	Probable M14 family carboxypeptidase containing a C-terminal secretion signal	MARIT_2507	53	47	147.6	6.6	1914.9	Proteolysis	GO:0006508
A0A2H1EB05	Probable S8 family protease containing a C-terminal secretion signal	MARIT_2055	45	22	59.6	8.6	360.2	Proteolysis	GO:0006508
A0A2H1E5T3	Secreted subtilase family protein, peptidase S8	MARIT_0203	40	20	59.2	6.7	102.6	Proteolysis	GO:0006508
Adhesins									
A0A2H1E8R3	Adhesin SprB	*sprB* MARIT_1321	35	105	625.5	4.5	1308.0	-	-
A0A2H1E9U3	Adhesin SprC	*sprC* MARIT_1318	44	28	80.1	4.9	213.8	-	-
Iron metabolism									

A0A2H1E9M2	Iron regulated protein Imelysin family lipoprotein	*irpA1* MARIT_1664	60	25	41.2	5.0	482.0	-	-
A0A2H1ECT1	Heme binding lipoprotein HmuY-family	MARIT_2477	60	14	27.2	5.6	218.5	-	-
A0A2H1E921	Heme binding lipoprotein HmuY-family	MARIT_1313	60	17	36.5	6.4	145.6	-	-
				Lipoproteins					
A0A2H1EBV5	Probable lipoprotein	MARIT_2470	58	40	46.3	7.9	3747.4	-	-
A0A2H1ED88	Probable lipoprotein	MARIT_3005	67	32	67.8	4.8	2761.2	-	-
A0A2H1E7K5	Probable lipoprotein	MARIT_0907	86	35	48.9	4.9	2115.2	-	-
A0A2H1E9Q6	Probable lipoprotein	MARIT_1705	60	15	44.6	8.1	1327.2	-	-
A0A2H1EDA9	Probable lipoprotein	MARIT_3027	55	18	29.7	5.9	824.8	-	-
A0A2H1E7J0	Probable lipoprotein	MARIT_0452	75	18	32.3	5.2	797.2	-	-
A0A2H1E833	Probable lipoprotein	MARIT_1058	57	15	30.7	5.1	634.4	-	-

A0A2H1E743	Probable lipoprotein	MARIT_0551	55	13	21.8	8.7	253.2	-	-
A0A2H1EA50	Probable lipoprotein	MARIT_1731	37	12	39.6	5.0	193.5	-	-
A0A2H1E5I3	Lipoprotein	MARIT_0099	61	19	32.0	5.0	191.4	-	-
A0A2H1E6T8	Probable lipoprotein	MARIT_0183	59	20	47.7	6.4	184.6	-	-
A0A2H1E6P1	Flagellar motor/Chemotaxis (MotB)-related lipoprotein	MARIT_0403	59	15	31.3	9.4	105.2	Membrane	GO:0016020
				Oxidative stress metabolism					
A0A2H1EB32	Superoxide dismutase 2	*sodC* MARIT_1821	40	6	18.0	6.9	533.4	Copper ion binding; Superoxide dismutase activity	GO:0005507; GO:0004784
A0A2H1EDI6	Superoxide dismutase	*sodA* MARIT_3105	56	7	22.4	5.3	262.5	Metal ion binding; Superoxide dismutase activity	GO:0046872; GO:0004784
A0A2H1EAC5	Metallo-dependent phosphatase containing a C-terminal secretion signal	MARIT_1816	33	22	102.3	5.2	212.4	Hydrolase activity	GO:0016787
A0A2H1EC90	Thioredoxin	*trxA* MARIT_2619	79	7	11.5	4.9	208.2	Protein-disulfide reductase activity	GO:0015035
A0A2H1E827	Alkyl hydroperoxide reductase C (Peroxiredoxin) (Thioredoxin peroxidase)	MARIT_0947	72	11	23.6	4.8	117.9	Peroxiredoxin activity	GO:0051920

Outer membrane proteins and TonB-related proteins									
A0A2H1EBZ4	SusC/RagA family TonB-dependent receptor	MARIT_2376	47	47	113.5	5.6	459.5	Cell outer membrane	GO:0009279
A0A2H1ED77	OmpA family protein	MARIT_2995	57	23	48.7	5.1	329.7	Cell adhesion; monoatomic ion transport	GO:0007155; GO:0006811
A0A2H1E6S9	Putative outer membrane protein	MARIT_0582	68	27	53.5	6.1	308.8	-	-
A0A2H1E9H4	Outer membrane protein beta-barrel domain-containing protein	MARIT_1482	31	4	17.1	8.1	189.0	-	-
A0A2H1E9P7	Outer membrane protein beta-barrel domain-containing protein	MARIT_1564	56	13	22.4	9.0	181.3	-	-
A0A2H1E5Y7	TonB-dependent outer membrane receptor	MARIT_0270	40	28	103.0	8.5	173.0	Cell outer membrane	GO:0009279
A0A2H1E6W5	TonB-dependent outer membrane receptor	MARIT_0214	47	33	102.7	6.5	168.7	Cell outer membrane	GO:0009280
A0A2H1EAX6	TonB-dependent outer membrane receptor	MARIT_1756	36	33	116.1	5.5	168.4	Cell outer membrane	GO:0009281
A0A2H1E9Y1	TonB-dependent outer membrane receptor	MARIT_1457	45	30	100.0	5.4	155.2	Cell outer membrane	GO:0009282
A0A2H1E930	TonB-dependent outer membrane receptor	MARIT_1458	34	25	101.0	6.4	152.3	Cell outer membrane	GO:0009283

A0A2H1EDN0	TonB-dependent outer membrane receptor	MARIT_3004	39	32	104.5	8.8	127.3	Cell outer membrane	GO:0009284
A0A2H1E607	TonB-dependent outer membrane receptor	MARIT_0268	34	27	104.6	6.2	122.8	Cell outer membrane	GO:0009285
A0A2H1E6Q0	Outer membrane protein beta-barrel domain-containing protein	MARIT_0557	44	7	21.9	9.3	112.2	-	-
A0A2H1E9V9	Outer membrane protein beta-barrel domain-containing protein	MARIT_1624	60	12	30.7	7.2	109.2	-	-
Secretion system-related proteins									
A0A2H1E9D1	Secretion system C-terminal sorting domain-containing protein	MARIT_1397	43	8	37.0	5.5	222.9	-	-
A0A2H1E7X7	Gliding motility lipoprotein GldJ	*gldJ* MARIT_0896	35	22	65.3	9.3	162.6	-	-
A0A2H1E7N1	Gliding motility lipoprotein GldK	*gldK* MARIT_0754	36	16	52.8	6.5	146.9	-	-
A0A2H1E7A0	Gliding motility protein GldN	*gldN* MARIT_0757	61	18	33.5	5.3	125.3	-	-
A0A2H1E7X8	Por secretion system protein porV	*porV* MARIT_0894	55	14	40.0	5.2	119.8	-	-

Table S2: Haematological parameters of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or *Tenacibaculum maritimum*'s ECPs (ECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols represent significant differences between the control group (undisturbed) and the remaining groups (Student's *t*-test; $p \leq 0.05$). WBC - white blood cells; RBC - red blood cells; Ht - haematocrit; Hg - haemoglobin; MCV - mean corpuscular volume; MCH - mean corpuscular haemoglobin; MCHC - mean corpuscular haemoglobin concentration.

Parameters	Control	Mock-challenged				ECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
WBC (×10^4/µL)	5.10±0.19#	4.25±0.30	1.16±0.19*****	1.89±0.17*****	1.26±0.12*****	3.48±0.30*****	1.28±0.20*****	1.75±0.17*****	1.24±0.17*
RBC (×10^6/µL)	2.28±0.11#	2.69±0.10*****	2.49±0.09	2.74±0.12*****	2.30±0.13	2.47±0.07	2.26±0.09	2.73±0.12	2.24±0.13
Ht (%)	25.64±1.22#	28.17±0.58	29.00±1.17	25.50±1.38*****	31.00±1.39**a**	28.58±0.70	26.27±1.14	27.08±1.38	26.58±0.74**b**
Hg (g/dL)	2.66±0.14	2.65±0.05	2.82±0.12	2.75±0.12	2.87±0.12	2.65±0.06	2.58±0.04	2.99±0.12	2.76±0.09
MCV (µm^3)	113.53±5.33#	106.32±4.18	117.90±5.74	93.32±4.06*****	137.57±6.51*****	116.79±4.54	116.74±4.90	104.23±4.06	124.27±8.81
MCH (pg/cell)	11.80±0.60#	10.00±0.41*****	11.54±0.69	10.15±0.45*****	12.76±0.57	10.84±0.42	11.62±0.45	11.70±0.45	13.05±1.15
MCHC (g/100 mL)	10.93±0.40#	9.42±0.21*****	9.27±0.33*****	10.35±0.30	9.35±0.35*****	9.33±0.26*****	10.00±0.37	11.13±0.28	10.45±0.40

Student's *t*-test (*p*-values)

Parameters	Mock-challenged x ECPs			
	3 h	6 h	24 h	48 h
WBC (×10^4/µL)	0.097	0.523	0.429	0.625
RBC (×10^6/µL)	0.085	0.130	0.537	0.789
Ht (%)	0.650	0.110	0.347	0.010
Hg (g/dL)	0.935	0.056	0.123	0.458
MCV (µm^3)	0.104	0.881	0.221	0.237
MCH (pg/cell)	0.166	0.925	0.229	0.824

MCHC (g/100 mL)	0.782	0.169	0.154	0.050

Table S3: Absolute values (× 10^4/µL) of peripheral blood leukocytes (neutrophils, monocytes, lymphocytes and thrombocytes) of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or *Tenacibaculum maritimum*'s ECPs (ECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols represent significant differences between the control group (undisturbed) and the remaining groups (Student's *t*-test; $p \leq 0.05$).

Parameters	**Control**	**Mock-challenged**				**ECPs**			
	0 h	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
Neutrophils (×10^4/µL)	0.27±0.06#	0.15±0.03**b**	0.08±0.01**b***	0.22±0.04	0.05±0.01**b***	0.39±0.04**a***	0.19±0.03**a**	0.27±0.02	0.12±0.03**a**
Monocytes (×10^4/µL)	0.11±0.01#	0.05±0.01*	0.04±0.01**a***	0.06±0.01	0.03±0.00	0.06±0.01	0.01±0.00**b***	0.05±0.02*	0.04±0.01
Lymphocytes (×10^4/µL)	1.34±0.08#	0.83±0.08*	0.23±0.01*	0.49±0.07*	0.26±0.04*	0.64±0.08*	0.20±0.03*	0.37±0.05*	0.29±0.04*
Thrombocytes (×10^4/µL)	3.39±0.14#	3.21±0.27	0.80±0.14*	1.12±0.14*	0.93±0.09*	2.39±0.20*	0.87±0.16*	1.06±0.15*	0.80±0.11*

***Student's* t-test (*p*-values)**

Parameters	**Mock-challenged x ECPs**			
	3 h	**6 h**	**24 h**	**48 h**
Neutrophils (x10^4/µL)	<.001	0.004	0.232	0.011
Monocytes (x10^4/µL)	0.557	<.001	0.657	0.488
Lymphocytes (x10^4/µL)	0.055	0.631	0.118	0.569
Thrombocytes (x10^4/µL)	0.060	0.556	0.792	0.217

Table S4: Haematological parameters of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or 5.5 × 10^5 CFU *Tenacibaculum maritimum* without ECPs (BWO) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, *p*-value ≤ 0.05). Different symbols represent significant differences between the control group (undisturbed) and the different treatment groups (Student's *t*-test; $p \leq 0.05$). WBC - white blood cells; RBC - red blood cells; Ht - haematocrit; Hg - haemoglobin; MCV - mean corpuscular volume; MCH - mean corpuscular haemoglobin; MCHC - mean corpuscular haemoglobin concentration.

Parameters	Control	Mock-challenged				BWO			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
WBC (×10^4/µL)	5.10±0.19	4.25±0.30	1.16±0.19	1.89±0.17	1.26±0.12	2.17±0.30	1.57±0.15	2.58±0.30	1.31±0.12
RBC (×10^6/µL)	2.28±0.11	2.69±0.10	2.49±0.09	2.74±0.12	2.30±0.13	2.50±0.14	2.76±0.17	2.78±0.14	2.45±0.20
Ht (%)	25.64±1.22	28.17±0.58	29.00±1.17	25.50±1.38	31.00±1.39	27.17±1.02	27.36±1.09	25.92±1.33	28.75±1.23
Hg (g/dL)	2.66±0.14	2.65±0.05	2.82±0.12	2.75±0.12	2.87±0.12	2.50±0.04	2.69±0.13	3.06±0.15	2.94±0.11
MCV (µm^3)	113.53±5.33	106.32±4.18	117.90±5.74	93.32±4.06	137.57±6.51	111.49±5.26	103.40±4.40	97.66±8.76	109.54±7.75
MCH (pg/cell)	11.80±0.60	10.00±0.41	11.54±0.69	10.15±0.45	12.76±0.57	10.40±0.72	10.11±0.68	11.28±0.78	13.23±1.65
MCHC (g/100 mL)	10.93±0.40	9.42±0.21	9.27±0.33	10.35±0.30	9.35±0.35	9.34±0.40	9.97±0.62	12.35±1.13	10.44±0.59

Parameters	Control	BECPs			
	0 h	3 h	6 h	24 h	48 h
WBC (×10^4/µL)	5.10±0.19	1.41±0.21	0.97±0.11	1.73±0.17	1.34±0.17
RBC (×10^6/µL)	2.28±0.11	2.13±0.10	2.20±0.13	2.52±0.18	2.12±0.10

Ht (%)	25.64±1.22	26.11±1.10	23.17±0.96	3.07±0.98	23.67±1.18
Hg (g/dL)	2.66±0.14	2.56±0.04	2.51±0.06	3.07±0.16	2.84±0.08
MCV (μm^3)	113.53±5.33	122.13±11.31	109.10±8.30	92.91±6.55	114.87±8.19
MCH (pg/cell)	11.80±0.60	11.79±0.86	11.83±0.74	11.98±0.57	13.76±0.71
MCHC (g/100 mL)	10.93±0.40	9.93±0.30	10.98±0.36	13.89±0.67	12.37±0.71

One-Way ANOVA

Parameters	**Time**				**Treatment**		
	3 h	**6 h**	**24 h**	**48 h**	**Mock**	**BWO**	**BECPs**
WBC ($\times 10^4$/µL)	-	-	-	-	-	-	-
RBC ($\times 10^6$/µL)	AB	AB	A	B	a	a	b
Ht (%)	AB	AB	A	B	a	a	b
Hg (g/dL)	A	AB	C	BC	-	-	-
MCV (μm^3)	A	A	B	A	-	-	-
MCH (pg/cell)	A	A	A	B	-	-	-
MCHC (g/100 mL)	A	A	B	A	a	a	b

2-Way ANOVA

	Time x Treatment											
Parameters	**Mock-challenged**				**BWO**				**BECPs**			
	3 h	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
WBC ($\times 10^4$/µL)	Aa	Bb	C	B	Ab	Ba	A	B	ABc	Ab	B	AB
RBC ($\times 10^6$/µL)	-	-	-	-	-	-	-	-	-	-	-	-
Ht (%)	-	-	-	-	-	-	-	-	-	-	-	-
Hg (g/dL)	-	-	-	-	-	-	-	-	-	-	-	-
MCV (μm^3)	-	-	-	-	-	-	-	-	-	-	-	-
MCH (pg/cell)	-	-	-	-	-	-	-	-	-	-	-	-
MCHC (g/100 mL)	-	-	-	-	-	-	-	-	-	-	-	-

2-Way ANOVA (*p*-values)

Parameters	**Time**	**Treatment**	**Time x Treatment**
WBC (×10^4/µL)	<0.001	<0.001	<0.001
RBC (×10^6/µL)	0.006	<0.001	0.671
Ht (%)	0.004	<0.001	0.290
Hg (g/dL)	<0.001	0.801	0.086
MCV (µm^3)	<0.001	0.239	0.081
MCH (pg/cell)	0.001	0.059	0.759
MCHC (g/100 mL)	<0.001	<0.001	0.475

***Student's* t-test**

Parameters	**Control**	**Mock-challenged**				**BWO**				**BECPs**			
	0 h	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
WBC (×10^4/µL)	#	-	*	*	*	*	*	*	*	*	*	*	*
RBC (×10^6/µL)	#	*	-	*	-	-	-	*	-	-	-	*	-
Ht (%)	#	-	-	-	*	-	-	-	-	-	-	*	-
Hg (g/dL)	-	-	-	-	-	-	-	-	-	-	-	-	-
MCV (µm^3)	#	-	-	*	*	-	-	-	-	-	-	*	-
MCH (pg/cell)	#	*	-	*	-	-	-	-	-	-	-	-	*
MCHC (g/100 mL)	#	*	-	-	*	*	-	-	-	-	-	*	-

Table S5: Absolute values (× 10^4/µL) of peripheral blood leukocytes (neutrophils, monocytes, lymphocytes and thrombocytes) of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or 5.5 × 10^5 CFU *Tenacibaculum maritimum* without ECPs (BWO) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, *p*-value ≤ 0.05). Different symbols represent significant differences between the control group (undisturbed) and the different treatment groups (Student's *t*-test; $p \leq 0.05$).

Parameters	Control	Mock-challenged				BWO			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Neutrophils (×10^4/µL)	0.27±0.06	0.15±0.03	0.08±0.01	0.22±0.04	0.05±0.01	0.19±0.03	0.33±0.04	0.34±0.07	0.05±0.01
Monocytes (×10^4/µL)	0.11±0.01	0.05±0.01	0.04±0.01	0.06±0.01	0.03±0.00	0.06±0.01	0.02±0.01	0.09±0.02	0.03±0.00
Lymphocytes (×10^4/µL)	1.34±0.08	0.83±0.08	0.23±0.01	0.49±0.07	0.26±0.04	0.33±0.04	0.23±0.03	0.54±0.07	0.36±0.06
Thrombocytes (×10^4/µL)	3.39±0.14	3.21±0.27	0.80±0.14	1.12±0.14	0.93±0.09	1.58±0.24	0.98±0.10	1.62±0.19	0.87±0.07

Parameters	Control	BECPs			
	0 h	3 h	6 h	24 h	48 h
Neutrophils (×10^4/µL)	0.27±0.06	0.12±0.02	0.12±0.02	0.23±0.03	0.05±0.02
Monocytes (×10^4/µL)	0.11±0.01	0.02±0.01	0.01±0.00	0.05±0.01	0.04±0.01
Lymphocytes (×10^4/µL)	1.34±0.08	0.21±0.03	0.15±0.02	0.30±0.05	0.31±0.05
Thrombocytes (×10^4/µL)	3.39±0.14	1.06±0.16	0.69±0.08	1.15±0.11	0.78±0.13

2-Way ANOVA

Parameters	Time x Treatment											
	Mock-challenged				BWO				BECPs			
	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Neutrophils (×10^4/µL)	AB	ACb	B	C	A	aB	AB	C	A	Ab	B	C
Monocytes (×10^4/µL)	ABa	ABa	A	B	Aa	Bab	A	B	Ab	Ab	B	B
Lymphocytes (×10^4/µL)	Aa	Bab	Ca	B	Ab	Aa	Ba	AB	ABc	Ab	Bb	B
Thrombocytes (×10^4/µL)	Aa	Bab	C	BC	ABb	BCa	A	C	ACb	Bb	C	AB

2-Way ANOVA (*p*-values)

Parameters	Time	Treatment	Time x Treatment
Neutrophils (×10^4/µL)	<0.001	<0.001	0.008
Monocytes (×10^4/µL)	<0.001	0.003	0.002
Lymphocytes (×10^4/µL)	<0.001	<0.001	<0.001
Thrombocytes (×10^4/µL)	<0.001	0.002	<0.001

***Student's* t-test**

Parameters	Control	Mock-challenged				BWO				BECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Neutrophils (×10^4/µL)	#	-	*	-	*	-	-	-	*	*	*	-	*
Monocytes (×10^4/µL)	#	*	*	-	*	-	*	-	*	*	*	*	*
Lymphocytes (×10^4/µL)	#	*	*	-	*	*	*	-	*	*	*	-	*
Thrombocytes (×10^4/µL)	#	-	*	*	*	*	*	*	*	*	*	*	*

Table S6: Immune parameters (antiprotease (%) and proteases activities (%), peroxidase (units/mL), lysozyme (units/mL), bactericidal activity (%) and nitrite concentration (µM)) of plasma of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or *Tenacibaculum maritimum*'s ECPs (ECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols represent significant differences between the control group (undisturbed) and the remaining groups (Student's *t*-test; $p \leq 0.05$). NO - nitrite

Parameters	Control	Mock-challenged				ECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Lysozyme (µg/mL)	10.53±0.72#	7.46±0.57*****	8.26±0.70*****	7.97±1.31	6.81±0.96*****	6.28±0.59*****	8.02±0.77*****	6.99±0.35*****	4.93±0.76*****
Antiprotease activity (%)	97.83±0.36	98.21±0.20	97.28±0.26	97.34±0.24**b**	97.23±0.24	98.16±0.24	97.40±0.26	98.14±0.17**a**	96.68±0.27*****
Protease activity (%)	8.71±0.23	8.68±0.19**b**	8.76±0.23**b**	8.33±0.20	8.15±0.17	9.84±0.32**a**	9.80±0.28**a**	8.06±0.19*****	7.89±0.12*****
Peroxidase activity (U/mL)	38.92±6.62	27.23±2.15	30.91±3.19	72.33±7.82*****	61.70±7.77	23.85±3.32	29.87±2.06	61.71±6.14*****	82.31±11.94*****
Bactericidal activity (%)	35.84±6.08#	45.54±5.48	49.19±3.45	15.67±4.23**b***	27.05±2.25	54.86±2.32*****	50.72±3.61*****	45.22±4.91**a**	23.67±2.45
NO (µM)	0.54±0.06	0.41±0.03**b**	0.52±0.07	0.57±0.10	0.48±0.04**b**	0.63±0.07**a**	0.53±0.04	0.57±0.04	0.68±0.05**a**

Student's *t*-test (*p*-values)

Parameters	Mock-challenged x ECPs			
	3 h	6 h	24 h	48 h
Lysozyme (µg/mL)	0.166	0.819	0.460	0.140
Antiprotease activity (%)	0.874	0.746	0.015	0.135
Protease activity (%)	0.005	0.009	0.163	0.217
Peroxidase activity (U/mL)	0.412	0.787	0.533	0.247
Bactericidal activity (%)	0.121	0.763	<.001	0.330
NO (µM)	0.011	0.896	0.997	0.022

Table S7: Immune parameters (antiprotease (%) and proteases activities (%), peroxidase (units/mL), lysozyme (units/mL), bactericidal activity (%) and nitrite concentration (µM)) of plasma of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or 5.5 × 10^5 CFU *Tenacibaculum maritimum* without ECPs (BWO) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, *p*-value ≤ 0.05). Different symbols represent significant differences between the control group (undisturbed) and the different treatment groups (Student's *t*-test; $p \leq 0.05$). NO - nitrite

Parameters	Control	Mock-challenged				BWO			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Lysozyme (µg/mL)	10.53±0.72	7.46±0.57	8.26±0.70	7.97±1.31	6.81±0.96	6.74±0.61	7.70±0.38	6.33±0.83	4.97±0.47
Antiprotease activity (%)	97.83±0.36	98.21±0.20	97.28±0.26	97.34±0.24	97.23±0.24	97.41±0.34	96.74±0.26	97.36±0.27	97.65±0.17
Protease activity (%)	8.71±0.23	8.68±0.19	8.76±0.23	8.62±0.34	8.15±0.17	8.61±0.17	9.30±0.34	8.51±0.16	8.64±0.39
Peroxidase activity (U/mL)	38.92±6.62	27.23±2.15	30.91±3.19	72.33±7.82	61.70±7.77	24.22±1.99	23.96±2.35	76.22±14.72	86.70±15.44
Bactericidal activity (%)	35.84±6.08	45.54±5.48	49.19±3.45	15.67±4.23	27.05±2.25	49.71±2.68	53.47±1.77	35.96±4.77	45.90±4.89
NO (µM)	0.54±0.06	0.41±0.03	0.52±0.07	0.57±0.10	0.48±0.04	0.50±0.10	0.56±0.07	0.46±0.08	0.74±0.09

Parameters	Control	BECPs				
	0 h	3 h	3 h	6 h	24 h	48 h
Lysozyme (µg/mL)	10.53±0.72	7.46±0.57	6.38±0.74	7.04±0.58	6.94±0.91	6.16±0.87
Antiprotease activity (%)	97.83±0.36	98.21±0.20	97.61±0.17	96.88±0.39	97.16±0.27	97.41±0.23
Protease activity (%)	8.71±0.23	8.68±0.19	9.23±0.39	8.51±0.16	79.49±0.18	7.90±0.28
Peroxidase activity (U/mL)	38.92±6.62	27.23±2.15	27.86±2.68	33.76±8.83	79.49±14.83	98.54±13.90

Bactericidal activity (%)	35.84±6.08	45.54±5.48	47.10±2.99	48.99±1.51	31.88±0.90	52.59±4.24
NO (µM)	0.54±0.06	0.41±0.03	0.51±0.06	0.56±0.05	0.73±0.07	0.60±0.09

One-Way ANOVA

Parameters	Time				Treatment		
	3 h	**6 h**	**24 h**	**48 h**	**Mock**	**BWO**	**BECPs**
Lysozyme (µg/mL)	-	-	-	-	-	-	-
Antiprotease activity (%)	A	B	AB	AB	-	-	-
Protease activity (%)	AB	A	BC	C	-	-	-
Peroxidase activity (U/mL)	A	A	B	B	-	-	-
Bactericidal activity (%)	-	-	-	-	-	-	-
NO (µM)	-	-	-	-	-	-	-

2-Way ANOVA

Parameters	Time x Treatment											
	Mock-challenged				**BWO**				**BECPs**			
	3 h	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
Lysozyme (µg/mL)	-	-	-	-	-	-	-	-	-	-	-	-
Antiprotease activity (%)	-	-	-	-	-	-	-	-	-	-	-	-
Protease activity (%)	-	-	-	-	-	-	-	-	-	-	-	-
Peroxidase activity (U/mL)	-	-	-	-	-	-	-	-	-	-	-	-
Bactericidal activity (%)	A	A	Bb	Cb	A	A	Ba	ABa	A	A	Ba	Aa
NO (µM)	-	-	-	-	-	-	-	-	-	-	-	-

2-Way ANOVA (*p*-values)

Parameters	Time	Treatment	Time x Treatment
Lysozyme (µg/mL)	0.059	0.072	0.911
Antiprotease activity (%)	0.004	0.403	0.249
Protease activity (%)	0.002	0.118	0.114
Peroxidase activity (U/mL)	<.001	0.610	0.714
Bactericidal activity (%)	<.001	<.001	0.004
NO (µM)	0.057	0.160	0.134

Student's *t*-test

Parameters	Control	Mock-challenged				BWO				BECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
Lysozyme (µg/mL)	#	*	*	-	*	*	*	*	*	*	*	*	*
Antiprotease activity (%)	#	-	-	-	-	-	*	-	-	-	-	-	-
Protease activity (%)	#	-	-	-	-	-	-	-	-	-	-	*	*
Peroxidase activity (U/mL)	#	-	-	*	*	-	*	*	*	-	-	*	*
Bactericidal activity (%)	#	-	-	*	-	-	*	-	-	-	-	-	*
NO (µM)	#	-	-	-	-	-	-	-	-	-	-	*	-

Table S8: Oxidative stress biomarkers activity of the liver of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or *Tenacibaculum maritimum*'s ECPs (ECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols represent significant differences between the control group (undisturbed) and the remaining groups (Student's *t*-test; $p \leq 0.05$).

Parameters	Control	Mock-challenged				ECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
U SOD/mg prot	22.18±1.59#	18.05±1.74**b**	27.30±0.67**a***	27.10±1.94	27.11±1.88	27.07±1.04**a***	19.50±1.69**b**	26.28±1.94	25.56±2.64
CAT U/mg	106.61±4.05#	107.28±4.94	98.70±3.07**b**	92.20±6.03*	92.86±5.89	108.99±3.58	110.32±3.59**a**	87.70±6.03*	97.62±4.55

LPO (nmol/g wt)	119.35±18.75	87.12±6.10**b**	90.45±4.14	94.27±6.42	101.07±5.65	131.94±18.24**a**	109.92±12.29	110.49±6.42	129.09±13.80
GSH/GSSG ratio	83.30±9.62#	151.78±19.90**a**	82.89±10.09	23.08±2.99*****	32.53±6.57*****	53.49±12.01**b***	95.44±27.20	33.15±2.99*****	26.58±4.57*****
GSH	2017.35±138.57#	2253.12±56.08**a**	1819.64±94.05	1611.51±112.88	2044.23±267.24	1963.25±124.28**b**	1524.29±121.53*****	1414.77±112.88*****	2419.85±169.55
GSSG	27.53±4.11#	24.18±9.38**b**	24.44±2.93	72.91±8.76*****	72.27±10.56*****	57.63±10.31**a***	34.40±12.79	72.36±8.76*****	105.12±14.21*****

Student's *t*-test (*p*-values)

Parameters	Mock-challenged x ECPs			
	3 h	**6 h**	**24 h**	**48 h**
U SOD/mg prot	<.001	<.001	0.735	0.638
CAT U/mg	0.781	0.024	0.528	0.529
LPO (nmol/g wt)	0.036	0.103	0.210	0.163
GSH/GSSG ratio	0.005	0.394	0.845	0.596
GSH	0.040	0.040	0.456	0.369
GSSG	0.003	0.565	0.618	0.126

Table S9: Oxidative stress biomarkers activity of the liver of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or 5.5 × 10^5 CFU *Tenacibaculum maritimum* without ECPs (BWO) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs). Data are expressed as mean ± SEM (n=12 per treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, *p*-value ≤ 0.05). Different symbols represent significant differences between the control group (undisturbed) and the different treatment groups (Student's *t*-test; $p \leq 0.05$).

Parameters	**Control**	**Mock-challenged**				**BWO**			
	0 h	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
U SOD/mg prot	22.18±1.59	18.05±1.74	27.30±0.67	27.10±1.94	27.11±1.88	27.49±1.48	24.44±2.28	28.77±2.12	30.91±1.25
CAT U/mg	106.61±4.05	107.28±4.94	98.70±3.07	92.20±6.03	92.86±5.89	105.69±5.28	111.51±5.60	86.29±2.90	90.21±2.62

LPO (nmol/g wt)	119.35±18.75	87.12±6.10	90.45±4.14	94.27±6.42	101.07±5.65	104.48±11.37	96.98±6.02	123.86±12.99	121.19±17.61
GSH/GSSG ratio	83.30±9.62	151.78±19.90	82.89±10.09	23.08±2.99	32.53±6.57	113.23±37.90	103.06±25.61	17.87±7.08	28.29±3.80
GSH	2017.35±138.57	2253.12±56.08	1819.64±94.05	1611.51±112.88	2044.23±267.24	1915.60±126.41	1806.95±135.93	843.17±82.49	2303.20±154.43
GSSG	27.53±4.11	24.18±9.38	24.44±2.93	72.91±8.76	72.27±10.56	30.85±6.04	31.74±6.54	60.28±6.28	90.58±12.03

Parameters	**Control**	**BECPs**			
	0 h	**3 h**	**6 h**	**24 h**	**48 h**
U SOD/mg prot	22.18±1.59	26.82±2.10	31.26±2.07	27.62±2.38	29.21±2.23
CAT U/mg	106.61±4.05	100.39±9.23	99.74±3.33	89.68±3.20	80.56±1.95
LPO (nmol/g wt)	119.35±18.75	120.60±12.56	111.68±12.38	139.66±15.84	145.45±10.02
GSH/GSSG ratio	83.30±9.62	97.87±35.53	39.88±8.51	14.82±4.55	33.42±12.09
GSH	2017.35±138.57	1396.82±141.92	1489.66±114.11	816.78±75.68	2441.18±143.48
GSSG	27.53±4.11	25.84±5.65	59.06±13.41	87.68±15.13	107.60±13.68

One-Way ANOVA

Parameters	**Time**				**Treatment**		
	3 h	**6 h**	**24 h**	**48 h**	**Mock**	**BWO**	**BECPs**
U SOD/mg prot	-	-	-	-	-	-	-
CAT U/mg	A	A	B	B	-	-	-
LPO (nmol/g wt)	-	-	-	-	b	ab	a
GSH/GSSG ratio	A	A	B	C	b	ab	a
GSH	-	-	-	-	-	-	-
GSSG	A	A	B	B	-	-	-

2-Way ANOVA

Time x Treatment												
Parameters	Mock-challenged				BWO				BECPs			
	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
U SOD/mg prot	Aa	Bab	B	B	Ab	Aa	AB	B	b	b	-	*
CAT U/mg	-	-	-	-	-	-	-	-	-	-	-	-
LPO (nmol/g wt)	-	-	-	-	-	-	-	-	-	-	-	-
GSH/GSSG ratio	-	-	-	-	-	-	-	-	-	-	-	-
GSH	ACa	BC	Ca	ABb	ABa	B	Cb	Aab	Ab	A	Bb	Ca
GSSG	-	-	-	-	-	-	-	-	-	-	-	-

2-Way ANOVA (*p*-values)

Parameters	Time	Treatment	Time x Treatment
U SOD/mg prot	0.014	0.015	0.025
CAT U/mg	<0.001	0.413	0.156
LPO (nmol/g wt)	0.090	<.001	0.751
GSH/GSSG ratio	<.001	<.001	0.240
GSH	<.001	<.001	<.001
GSSG	<.001	0.075	0.236

Student's *t*-test

Parameters	Control	Mock-challenged				BWO				BECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
U SOD/mg prot	#	-	*		*	*	-	*	-	-	*	-	*
CAT U/mg	#	-	-	*	-	-	-	*	*	-	-	*	*
LPO (nmol/g wt)	#	-	-		-	-	-	-	-	-	-	-	*
GSH/GSSG ratio	#	*	-	*	-	-	-	*	*	-	*	*	*
GSH	#	-	-	*	-	-	-	*	-	*	*	*	*
GSSG	#	-	-	*	-	-	-	*	*	-	*	*	*

Table S10: Quantitative expression of *il34*, *cxcr4*, *mmp9*, *mcsfr*, *mif*, *casp1*, *mhcII* and *hsp70* for head-kidney of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or *Tenacibaculum maritimum*'s ECPs (ECPs). Data are expressed as mean ± SEM (n=9 per treatment). Different lowercase letters stand for significant differences between treatments among time points and different symbols represent significant differences between the control group (undisturbed) and the remaining groups (Student's *t*-test; $p \leq 0.05$).

Parameters	Control	Mock-challenged				ECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
il34	1.05±0.12#	0.42±0.04*****	0.50±0.05*****	0.77±0.07**a**	1.17±0.06**a**	0.38±0.10*****	0.49±0.06*****	0.44±0.08**b***	0.53±0.03**b***
cxcr4	1.02±0.07#	1.17±0.07**a**	1.05±0.09**a**	1.07±0.10	1.05±0.10	0.78±0.05**b***	0.62±0.05**b***	0.91±0.07	0.85±0.04
mmp9	1.07±0.14#	3.33±0.99*****	5.32±0.58**a***	3.56±0.30**a***	2.55±0.25**a***	2.36±0.23*****	2.30±0.27**b***	1.48±0.38**b**	0.26±0.02**b***
mcsfr	1.01±0.06#	0.96±0.10	1.17±0.16	0.87±0.10	0.71±0.08**b***	0.88±0.07*****	1.38±0.08*****	0.97±0.08	1.02±0.09**a**
mif	1.09±0.17#	0.93±0.11	1.01±0.09	1.22±0.11**b**	0.79±0.08**b**	0.85±0.08	0.96±0.10	1.73±0.19**a***	1.99±0.21**a***
casp1	1.15±0.25	0.90±0.90	1.06±0.11	1.10±0.11	0.92±0.12	0.91±0.12	1.22±0.19	1.06±0.17	0.86±0.05
mhcII	1.03±0.09#	0.90±0.07	0.87±0.87	0.68±0.09*****	0.59±0.07*****	0.93±0.13	0.77±0.09*****	0.58±0.04*****	0.55±0.03*****
hsp70	1.01±0.06	1.06±0.09	1.08±0.06	1.02±0.08	1.01±0.10	0.92±0.07	1.10±0.08	1.21±0.12	1.14±0.11

Student's *t*-test (*p*-values)

Parameters	Mock-challenged x ECPs			
	3 h	6 h	24 h	48 h
il34	0.361	0.905	0.005	<.001
cxcr4	<.001	<.001	0.268	0.092
mmp9	0.439	<.001	<.001	<.001
mcsfr	0.587	0.144	0.311	0.028
mif	0.642	0.645	0.030	<.001
casp1	0.944	0.621	0.641	0.949

mhcII	0.944	0.760	0.430	0.948
hsp70	0.199	0.933	0.220	0.407

Table S11: Quantitative expression of *il34*, *cxcr4*, *mmp9*, *mcsfr*, *mif*, *casp1*, *mhcII* and *hsp70* for head-kidney of European sea bass (*Dicentrarchus labrax*) i.p. challenged with MB (Mock) or 5.5 × 10^5 CFU *Tenacibaculum maritimum* without ECPs (BWO) or 5.5 × 10^5 CFU *T. maritimum* with ECPs (BECPs). Data are expressed as mean ± SEM (n=9 per treatment). Different lowercase letters stand for significant differences in treatments among each time point, while different capital letters indicate differences in time among the same treatment (Two-Way ANOVA for interaction between factors, followed by Tukey's HSD or LSD for multiple comparisons, *p*-value ≤ 0.05). Different symbols represent significant differences between the control group (undisturbed) and the different treatment groups (Student's *t*-test; $p \leq 0.05$).

Parameters	**Control**	**Mock-challenged**				**BWO**			
	0 h	**3 h**	**6 h**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**48 h**
il34	1.05±0.12	0.42±0.04	0.50±0.05	0.77±0.07	1.17±0.06	0.51±0.05	0.32±0.06	0.68±0.10	1.05±0.08
cxcr4	1.02±0.07	1.17±0.07	1.05±0.09	1.07±0.10	1.05±0.10	0.85±0.08	0.78±0.05	1.07±0.09	1.04±0.07
mmp9	1.07±0.14	3.33±0.99	5.32±0.58	3.56±0.30	2.55±0.25	4.79±0.72	5.80±0.57	2.24±0.19	1.39±0.11
mcsfr	1.01±0.06	0.96±0.10	1.17±0.16	0.87±0.10	0.71±0.08	0.96±0.09	1.30±0.09	0.80±0.12	0.87±0.07
mif	1.09±0.17	0.93±0.11	1.01±0.09	1.22±0.11	0.79±0.08	0.95±0.12	0.86±0.09	1.44±0.17	0.74±0.07
casp1	1.15±0.25	0.90±0.90	1.06±0.11	1.10±0.11	0.92±0.12	0.94±0.10	1.32±0.18	1.13±0.18	0.85±0.07
mhcII	1.03±0.09	0.90±0.07	0.87±0.87	0.68±0.09	0.59±0.07	1.10±0.13	1.03±0.15	0.69±0.09	0.73±0.09
hsp70	1.01±0.06	1.06±0.09	1.08±0.06	1.02±0.08	1.01±0.10	1.07±0.12	1.15±0.08	0.91±0.09	0.84±0.07

(*continued*)

Parameters	Control	BECPs			
	0 h	3 h	6 h	24 h	48 h
il34	1.05±0.12	0.46±0.05	0.58±0.04	0.42±0.06	0.59±0.06
cxcr4	1.02±0.07	0.70±0.06	0.73±0.05	0.75±0.05	0.73±0.07
mmp9	1.07±0.14	2.37±0.32	2.20±0.30	0.93±0.15	0.87±0.13
mcsfr	1.01±0.06	1.15±0.08	1.49±0.13	0.74±0.05	0.90±0.09
mif	1.09±0.17	0.82±0.08	1.23±0.09	1.46±0.20	1.34±0.11
casp1	1.15±0.25	0.79±0.08	1.37±0.18	0.96±0.09	0.93±0.04
mhcII	1.03±0.09	0.84±0.08	0.64±0.08	0.53±0.02	0.61±0.04
hsp70	1.01±0.06	1.05±0.09	1.26±0.10	1.04±0.11	1.02±0.09

One-Way ANOVA

Parameters	Time				Treatment		
	3 h	6 h	24 h	48 h	Mock	BWO	BECPs
il34	-	-	-	-	-	-	-
cxcr4	-	-	-	-	b	b	a
mmp9	-	-	-	-	-	-	-
mcsfr	A	C	B	B	-	-	-
mif	-	-	-	-	-	-	-
casp1	B	A	AB	B	-	-	-
mhcII	B	AB	A	A	ab	b	a
hsp70	AB	A	AB	B	-	-	-

2-Way ANOVA

Time x Treatment												
Parameters	Mock-challenged				BWO				BECPs			
	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
il34	A	Ab	aB	aC	A	aB	Aa	aC	AB	Ab	Bb	Ab
cxcr4	-	-	-	-	-	-	-	-	-	-	-	-
mmp9	Ab	aB	Ab	aA	aA	aA	aB	bC	Ab	Ab	Bc	Bc
mcsfr	-	-	-	-	-	-	-	-	-	-	-	-
mif	AB	ABab	A	bB	A	Ab	B	Ab	A	aB	B	aB
casp1	-	-	-	-	-	-	-	-	-	-	-	-
mhcII	-	-	-	-	-	-	-	-	-	-	-	-
hsp70	-	-	-	-	-	-	-	-	-	-	-	-

2-Way ANOVA (*p*-values)

Parameters	Time	Treatment	Time x Treatment
il34	<.001	0.002	<.001
cxcr4	0.344	<.001	0.202
mmp9	<.001	<.001	<.001
mcsfr	<.001	0.130	0.466
mif	<.001	0.023	0.016
casp1	0.002	0.857	0.633
mhcII	<.001	0.013	0.692
hsp70	0.021	0.271	0.775

Student's *t*-test

Parameters	Control	Mock-challenged				BWO				BECPs			
	0 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h	3 h	6 h	24 h	48 h
il34	#	*	*	-	-	*	*	*	-	*	*	*	*
cxcr4	#	-	-	-	-	-	*	-	-	*	*	*	*
mmp9	#	*	*	*	*	*	*	*	-	*	*	-	-
mcsfr	#	-	-	-	-	-	*	-	-	-	*	*	-
mif	#	-	-	-	-	-	-	-	-	-	-	-	-
casp1	#	-	-	-	-	-	-	-	-	-	-	-	-
mhcII	#	-	-	-	*	-	-	*	*	-	*	*	*
hsp70	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX II

Early innate immune responses in European sea bass (*Dicentrarchus labrax* L.) following *Tenacibaculum maritimum* infection

Appendix II

Table S1: Haematological parameters (red blood cells (RBC × 10^6/µL), white blood cells (WBC × 10^4/µL), haematocrit (Ht %), haemoglobin (Hg, g/dL), mean corpuscular volume (MCV µm^3), mean corpuscular haemoglobin (MCH, pg/cell) and mean corpuscular haemoglobin concentration (MCHC, g/100 mL)) of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 × 10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

	Control	**Mock-challenged**				**Challenged**			
	0 h	**6 h**	**24 h**	**48 h**	**72 h**	**6 h**	**24 h**	**48 h**	**72 h**
RBC (×10^6/µL)	2.47± 0.06^{Aa}	2.53±0.07^{A*}	2.49±0.10^{A*}	2.03±0.14^{B*}	2.60±0.13^{A}	1.85±0.07^{b*}	2.10±0.08^{b*}	1.58±0.04^{c*}	2.34±0.07^{a}
WBC (×10^4/µL)	2.48±0.22^{Bb}	2.72±0.34^{AB}	2.93±0.20^{AB*}	2.70±0.16^{AB}	3.66±0.32^{A}	2.05±0.26^{b}	2.23±0.20^{b*}	2.47±0.22^{b}	4.20±0.31^{a}
Ht (%)	32.17±0.64^{Bab}	35.58±0.94^{B*}	36.75±1.73^{AB*}	35.90±1.68^{AB*}	41.27±1.28^{A*}	30.58±1.21^{ab*}	29.50±1.63^{ab*}	28.92±1.00^{b*}	33.36±0.75^{a*}
Hg (g/dL)	1.44±0.05^{B}	1.61±0.07^{AB*}	1.56±0.06^{AB}	1.37±0.09^{B}	1.84±0.09^{A}	1.37±0.06*	1.66±0.07	1.46±0.08	1.55±0.12
MCV (µm^3)	130.63±2.73^{c}	141.54±4.39*	148.48±5.85	169.23±12.58	161.63±11.05	167.37±9.11^{ab*}	142.22±8.99^{abc}	168.56±3.97^{a}	147.51±5.35^{bc}
MCH (pg/cell)	5.87±0.26^{Bc}	6.38±0.25^{AB*}	6.33±0.26^{AB*}	6.31±0.36^{AB*}	7.29±0.36^{A}	7.40±0.28^{b*}	7.94±0.35^{ab*}	9.15±2.76^{a*}	6.61±0.45^{bc}
MCHC (g/100 mL)	4.50±0.20^{b}	4.54±0.19	4.32±0.22*	4.06±0.25*	4.61±0.39	4.50±0.20^{b}	5.70±0.26^{a*}	5.14±0.36^{ab*}	4.57±0.39^{ab}

Table S2: Absolute values (× 10^4/µL) of peripheral blood leukocytes (neutrophils, monocytes, lymphocytes and thrombocytes) of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 × 10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

	Control	Mock-challenged				Challenged			
	0 h	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
Neutrophils (×10^4/µL)	0.03 ± 0.01^{Cc}	0.18 ± 0.03^{A}	$0.14\pm0.03^{AB*}$	$0.06\pm0.02^{BC*}$	$0.05\pm0.01^{BC*}$	0.22 ± 0.03^{b}	$0.05\pm0.01^{c*}$	$0.15\pm0.03^{b*}$	$0.57\pm0.06^{a*}$
Monocytes (×10^4/µL)	0.01 ± 0.00^{b}	0.02 ± 0.00	0.02 ± 0.01	$0.01\pm0.00^{*}$	$0.02\pm0.01^{*}$	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	$0.07\pm0.02^{a*}$	$0.07\pm0.02^{a*}$
Lymphocytes (×10^4/µL)	1.05 ± 0.12^{Bb}	1.33 ± 0.13^{AB}	$1.70\pm0.13^{A*}$	1.04 ± 0.14^{B}	1.83 ± 0.25^{A}	1.39 ± 0.17^{ab}	$1.26\pm0.14^{b*}$	0.86 ± 0.10^{b}	1.83 ± 0.25^{a}
Thrombocytes (×10^4/µL)	1.37 ± 0.17^{a}	$1.19\pm0.23^{*}$	1.07 ± 0.12	1.58 ± 0.16	1.44 ± 0.23	$0.51\pm0.10^{b*}$	0.91 ± 0.10^{ab}	1.3 ± 0.19^{a}	1.23 ± 0.15^{a}

Table S3: Immune parameters (antiprotease (%) and proteases activities (%), peroxidase (units/mL), lysozyme (units/mL), bactericidal activity (%) and nitrite concentration (µM)) of plasma of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 × 10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

	Control	Mock-challenged				Challenged			
	0 h	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
Antiprotease (%)	96.31 ± 0.28^{Bb}	97.38 ± 0.47^{AB}	97.77 ± 0.28^{A}	$96.83\pm0.15^{AB*}$	$96.91\pm0.30^{AB*}$	96.82 ± 0.42^{b}	90.87 ± 4.22^{b}	$98.00\pm0.21^{a*}$	$97.96\pm0.19^{a*}$
Protease (%)	10.46 ± 0.69^{ab}	10.12 ± 0.67	11.28 ± 0.73	$9.07\pm0.46^{*}$	$11.11\pm0.57^{*}$	8.69 ± 0.27^{bc}	12.52 ± 0.90^{a}	$7.76\pm0.22^{c*}$	$9.16\pm0.61^{bc*}$

Peroxidase (units/mL)	4.08±0.62^{Bb}	2.58±0.31^{B}	3.56±0.55^{B}	9.93±1.28^{A}	6.23±1.31^{AB}	3.85±0.95^{b}	5.27±0.95^{ab}	8.80±1.34^{a}	8.51±1.48^{ab}
Lysozyme (units/mL)	7.64±0.87^{ABa}	11.09±0.81^{A*}	9.13±1.00^{AB*}	8.10±1.19^{AB*}	6.31±0.77^{B*}	7.04±0.65^{a*}	4.35±0.57^{b*}	3.09±0.42^{b*}	4.13±0.45^{b*}
Bactericidal act. (%)	26.13±2.49^{Bab}	24.74±2.05^{B*}	24.45±1.98^{B*}	15.03±1.94^{A}	31.82±2.65^{AB*}	16.66±2.03^{bc*}	9.80±2.09^{c*}	14.36±1.68^{c}	31.82±2.65^{a*}
NO (µM)	0.11±0.01^{Bc}	0.10±0.01^{B}	0.13±0.01^{B}	0.23±0.03^{A}	0.18±0.02^{A}	0.08±0.01^{d}	0.13±0.02^{bcd}	0.24±0.03^{a}	0.17±0.01^{ab}

Table S4: Oxidative stress biomarkers (catalase activity (CAT), superoxide dismutase activity (SOD), lipid peroxidation (LPO), glutathione-S-transferase (GST), reduced: oxidized glutathione ratio (GSH/GSSG ratio), reduced (GSH) and oxidized glutathione (GSSG) activity of liver of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 × 10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

	Control	**Mock-challenged**				**Challenged**			
	0 h	**6 h**	**24 h**	**48 h**	**72 h**	**6 h**	**24 h**	**48 h**	**72 h**
CAT (U/mg protein)	217.45±4.40^{Aa}	173.54±14.75^{AB}	156.38±5.79^{B}	154.03±10.53^{B}	137.37±6.44^{B}	185.67±12.98^{a}	139.30±9.93^{b}	132.33±11.38^{b}	139.55±9.70^{b}
SOD (U/mg protein)	9.51±0.55B^{c}	12.51±1.08^{AB*}	13.41±0.87^{A}	15.71±0.81^{A*}	14.13±0.92A*	17.67±1.47a*	14.64±4.54^{ab}	12.57±0.71b*	11.64±0.51bc*
LPO (nmol/g wet tissue)	33.82±2.27	38.43±3.30*	36.53±4.12	50.11±5.67*	43.78±5.63	29.12±1.80*	33.36±1.91	28.98±2.56*	43.67±4.71
GST (nmol/mg protein)	194.90±8.93^{ABa}	196.29±10.01^{AB}	212.64±8.66A*	169.51±9.51^{B}	162.14±12.09^{B}	180.17±9.57^{a}	181.29±9.35^{a*}	167.10±6.86^{ab}	130.71±9.64^{b}
GSH/GSSG ratio	45.29±5.38^{B}	37.42±4.56^{B}	44.10±4.73^{B}	82.56±14.33^{A}	40.15±3.42^{B}	39.45±4.69	54.19±4.80	60.94±13.59	42.54±3.20
GSH (µM)	6039.70±214.87^{Aa}	5038.26±285.31^{AB}	5320.99±328.45^{AB}	5146.05±365.65^{AB}	4373.64±272.44^{B}	5133.91±450.02^{ab}	5230.67±439.80^{ab}	6129.85±504.89^{ab}	4642.90±196.25^{b}
GSSG (µM)	145.71±17.17^{A}	138.74±9.72^{A}	114.06±12.05^{A}	65.20±8.36B*	107.82±6.33^{A}	106.84±15.22	89.20±7.58	89.20±23.01*	109.77±9.22

Table S5: Quantitative expression of *tlr2*, *tlr9*, *nod1*, *nod2*, *nf-κB*, *stat3*, *bcl2-like*, *il-6* and *tnfα* for gills of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5×10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

	Control	**Mock-challenged**				**Challenged**			
Genes	**0 h**	**6 h**	**24 h**	**48 h**	**72 h**	**6 h**	**24 h**	**48 h**	**72 h**
tlr2	1.04 ± 0.09^{Bbc}	$1.74\pm0.17^{A*}$	$1.71\pm0.10^{A*}$	$1.86\pm0.19^{A*}$	$2.05\pm0.09^{A*}$	$1.00\pm0.14^{bc*}$	$0.64\pm0.14^{c*}$	$1.28\pm0.12^{ab*}$	$2.05\pm0.09^{a*}$
tlr9	1.01 ± 0.05^{Ba}	$1.59\pm0.13^{A*}$	$1.07\pm0.06^{B*}$	$1.12\pm0.05^{B*}$	$1.37\pm0.08^{A*}$	$0.83\pm0.09^{ab*}$	$0.61\pm0.07^{c*}$	$0.68\pm0.04^{bc*}$	$0.81\pm0.08^{ab*}$
nod1	1.02 ± 0.06^{Bb}	$1.11\pm0.07^{AB*}$	0.99 ± 0.09^{B}	$1.13\pm0.06^{AB*}$	$1.40\pm0.09^{A*}$	$1.57\pm0.13^{a*}$	1.03 ± 0.09^{b}	$0.88\pm0.07^{b*}$	$0.86\pm0.06^{b*}$
nod2	1.03 ± 0.08^{a}	$0.95\pm0.04^{*}$	$1.00\pm0.07^{*}$	$1.00\pm0.04^{*}$	$1.11\pm0.08^{*}$	$0.72\pm0.11^{bc*}$	$0.61\pm0.05^{c*}$	$0.71\pm0.02^{bc*}$	$0.82\pm0.06^{ab*}$
nf-κB	1.01 ± 0.04^{ABa}	$1.01\pm0.05^{AB*}$	0.88 ± 0.06^{B}	$0.99\pm0.05^{AB*}$	$1.11\pm0.06^{A*}$	$1.45\pm0.16^{a*}$	0.74 ± 0.05^{b}	$0.68\pm0.03^{b*}$	$0.78\pm0.05^{b*}$
stat3	1.02 ± 0.06^{Ab}	$0.99\pm0.05^{AB*}$	$0.80\pm0.06^{B*}$	1.01 ± 0.06^{A}	1.04 ± 0.05^{A}	$2.22\pm0.19^{a*}$	$1.25\pm0.06^{b*}$	1.08 ± 0.08^{b}	1.15 ± 0.08^{b}
bcl2-like	1.01 ± 0.05^{ABa}	0.98 ± 0.04^{AB}	0.87 ± 0.06^{B}	$0.90\pm0.03^{B*}$	$1.12\pm0.06^{A*}$	0.87 ± 0.06^{ab}	0.77 ± 0.06^{b}	$0.69\pm0.03^{b*}$	$0.69\pm0.04^{b*}$
il-6	1.03 ± 0.08^{ABa}	1.17 ± 0.08^{A}	0.89 ± 0.08^{B}	$0.86\pm0.03^{B*}$	$0.95\pm0.06^{AB*}$	1.15 ± 0.10^{a}	1.12 ± 0.17^{ab}	$0.62\pm0.03^{c*}$	$0.62\pm0.03^{c*}$
tnfα	1.04 ± 0.09^{Bab}	$1.43\pm0.11^{B*}$	$1.28\pm0.13^{B*}$	$1.85\pm0.16^{A*}$	$2.28\pm0.19^{A*}$	$1.71\pm0.27^{a*}$	$0.86\pm0.18^{b*}$	$1.02\pm0.11^{ab*}$	$0.82\pm0.09^{b*}$

Table S6: Quantitative expression of *tlr2*, *tlr9*, *nod1*, *nod2*, *nf-κB*, *stat3*, *bcl2-like*, *il-6* and *tnfα* for skin of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5 × 10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

Genes	Control	Mock-challenged				Challenged			
	0 h	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
tlr2	1.10 ± 0.14^{Bb}	1.09 ± 0.13^{B}	$1.62\pm 0.12^{A*}$	1.75 ± 0.14^{A}	1.75 ± 0.18^{A}	0.84 ± 0.13^{c}	$0.46\pm 0.12^{c*}$	1.56 ± 0.24^{ab}	2.27 ± 0.23^{a}
tlr9	1.05±0.10	$1.20\pm 0.09^{*}$	$1.11\pm 0.15^{*}$	0.82± 0.08	0.98± 0.08	$0.89\pm 0.08^{*}$	$0.72\pm 0.08^{*}$	0.81± 0.13	1.06± 0.13
nod1	1.10 ± 0.15^{Aab}	0.93 ± 0.10^{AB}	$0.70\pm 0.07^{ABC*}$	0.59 ± 0.06^{C}	0.63 ± 0.06^{BC}	1.27 ± 0.18^{a}	$1.20\pm 0.16^{a*}$	0.71 ± 0.07^{bc}	0.68 ± 0.06^{c}
nod2	1.13 ± 0.20^{Bb}	$1.26\pm 0.15^{B*}$	$2.43\pm 0.21^{A*}$	2.00 ± 0.17^{A}	2.11 ± 0.21^{A}	$2.10\pm 0.22^{a*}$	$1.16\pm 0.24^{bc*}$	1.64 ± 0.12^{ac}	2.10 ± 0.31^{a}
nf-κB	1.01 ± 0.05^{Ab}	$0.78\pm 0.05^{B*}$	$0.62\pm 0.04^{B*}$	0.70 ± 0.04^{B}	0.70 ± 0.04^{B}	$1.29\pm 0.08^{ab*}$	$1.38\pm 0.18^{ab*}$	0.75 ± 0.03^{d}	0.90 ± 0.11^{cd}
stat3	1.06 ± 0.10^{Aab}	$0.84\pm 0.07^{AB*}$	$0.78\pm 0.07^{AB*}$	$0.61\pm 0.05^{B*}$	0.71 ± 0.06^{B}	$1.20\pm 0.06^{a*}$	$1.35\pm 0.09^{a*}$	$0.92\pm 0.08^{bc*}$	0.83 ± 0.07^{c}
bcl2-like	1.02 ± 0.06^{a}	$1.02\pm 0.10^{*}$	$0.82\pm 0.05^{*}$	0.79± 0.03	0.86± 0.05	$0.72\pm 0.04^{bc*}$	$0.58\pm 0.05^{c*}$	0.68 ± 0.05^{bc}	0.81 ± 0.06^{ab}
il-6	1.06 ± 0.12^{Bb}	2.22 ± 0.25^{A}	0.72 ± 0.04^{B}	1.01 ± 0.10^{B}	0.76 ± 0.06^{B}	1.80 ± 0.22^{a}	0.85 ± 0.14^{b}	0.82 ± 0.07^{b}	0.99 ± 0.18^{b}
tnfα	1.11 ± 0.16^{AB}	1.47 ± 0.21^{AB}	$0.94\pm 0.14^{B*}$	1.52 ± 0.16^{A}	1.21 ± 0.10^{AB}	1.49± 0.18	$1.69\pm 0.18^{*}$	1.47± 0.16	1.87± 0.44

Table S7: Quantitative expression of *tlr2*, *tlr9*, *nod1*, *nod2*, *nf-κB*, *stat3*, *bcl2-like*, *il-6* and *tnfα* for posterior-intestine of European sea bass (*Dicentrarchus labrax*) after bacterial bath-challenge with 5×10^5 CFU mL^{-1} *Tenacibaculum maritimum*. Data are expressed as mean ± SEM (n=12 per treatment). Different capital letters in the same row stand for differences between control and mock-challenge and lower case letters indicate significant differences between control and challenged groups, while (*) represents statistical differences between mock and challenged fish at each sampling point (One-way ANOVA or Kruskal-Wallis; $p \leq 0.05$).

Genes	Control	Mock-challenged				Challenged			
	0 h	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
tlr2	1.01±0.05^{ab}	1.07±0.06*	1.17±0.07*	1.17±0.09	1.22±0.08	0.81±0.07^{bc*}	0.68±0.08^{c*}	0.99±0.12^{ab}	1.09±0.09^{a}
tlr9	1.02±0.06^{B}	1.55±0.11^{A*}	1.57±0.10^{A*}	1.38±0.19^{AB}	1.43±0.13^{A}	1.07±0.12*	0.94±0.12*	1.01±0.11	1.38±0.15
nod1	1.04±0.09	0.91±0.05	0.99±0.06	1.07±0.07	1.21±0.12	1.00±0.08	1.40±0.23	1.01±0.05	1.03±0.09
nod2	1.05±0.10^{B}	1.59±0.18^{AB*}	1.91±0.17^{A*}	1.07±0.11^{B}	1.51±0.18^{AB}	0.86±0.12*	1.41±0.46*	1.04±0.13	1.44±0.14
nf-κB	1.05±0.10^{Aa}	0.74±0.05^{B*}	0.64±0.04^{B*}	0.59±0.05^{B}	0.60±0.03^{B}	1.07±0.10^{a*}	1.04±0.14^{a*}	0.60±0.06^{b}	0.63±0.03^{b}
stat3	1.02±0.07	0.87±0.05*	0.86±0.04*	1.01±0.09	0.88±0.06	1.12±0.06*	1.19±0.11*	0.92±0.06	0.88±0.06
bcl2-like	1.03±0.07^{Aa}	0.72±0.05^{B}	0.88±0.05^{AB*}	0.73±0.05^{B}	0.74±0.04^{B}	0.79±0.06^{b}	0.69±0.05^{b*}	0.69±0.04^{b}	0.68±0.05^{b}
il-6	1.06±0.12	1.03±0.10	1.02±0.10	0.98±0.09*	0.89±0.07	0.91±0.10	1.13±0.15	0.75±0.05*	0.88±0.08
tnfα	1.06±0.11^{a}	1.15±0.20	1.13±0.10*	1.46±0.19*	1.03±0.08	1.16±0.17^{a}	0.65±0.13b*	0.86±0.12^{ab*}	1.30±0.18^{a}

APPENDIX III

Mucosal transcriptome and proteome analyses in European sea bass (*Dicentrarchus labrax*) following *Tenacibaculum maritimum* bath challenge

Table S1: Summary of Fastp platform report on the analyzed samples after filtering.

	Sample ID	Raw Reads	Clean Reads	Q20(%)	Q30(%)	GC (%)
Mock	TENA_28	45,289,540	45,043,166	98.17	94.47	48.82
	TENA_29	44,557,068	44,322,330	98.15	94.37	48.98
	TENA_30	46,633,094	46,406,638	98.25	94.63	48.70
Challenged	TENA_31	47,590,562	47,341,730	98.16	94.42	48.69
	TENA_32	48,421,018	48,176,634	98.17	94.42	48.43
	TENA_33	44,519,706	44,292,206	98.15	94.40	48.51
	TENA_34	46,977,548	46,735,660	98.11	94.27	48.80
	TENA_35	54,274,336	54,011,326	98.22	94.54	47.92

Figure S1: Principle component clustering between mock-challenged (blue) and challenged (red) fish at 24 h post-challenge for skin samples of European sea bass (*Dicentrarchus labrax*).

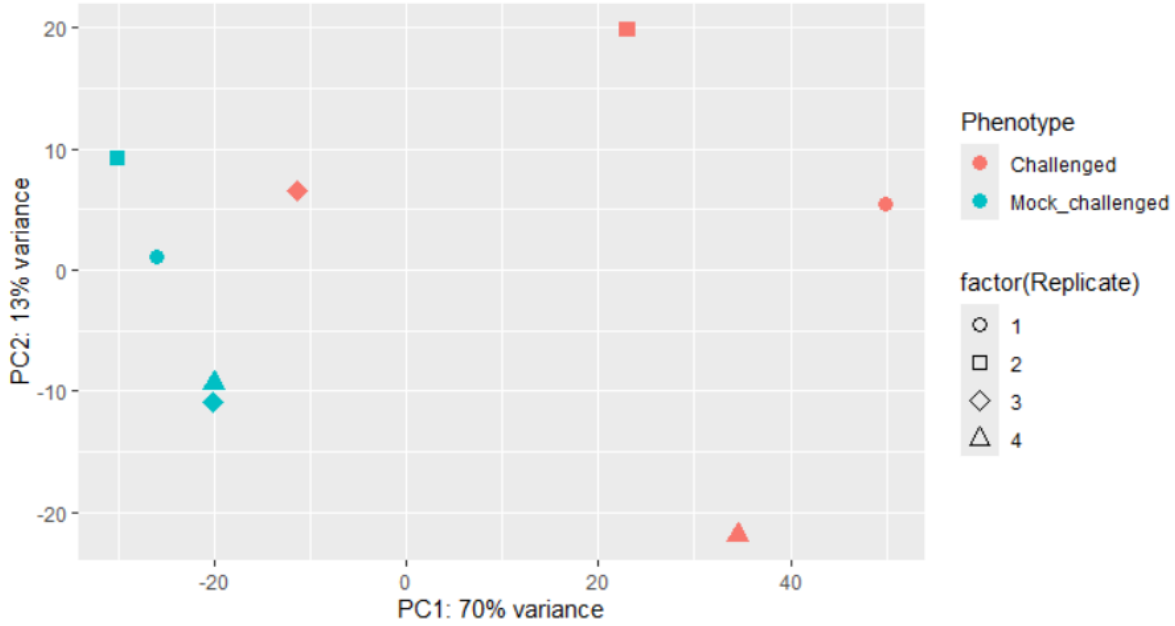

Table S2: List of matched DEGs and DEPs related to innate immune functions in the skin and skin mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Gene ID (Ensemble)	Gene name	Protein name	Log_2 Fold Change		*q*-Value		Regulated	
			DEGs	***DEPs***	***DEGs***	***DEPs***	***DEGs***	***DEPs***
ENSDLAG00005013039	*pinlyp*	Phospholipase A2 inhibitor and Ly6/PLAUR domain-containing protein-like	9.95	6.67	3.3×10^{-12}	3.4×10^{-5}	Up	Up
ENSDLAG00005007722	*alox15b*	Polyunsaturated fatty acid lipoxygenase ALOX15B-like isoform X1	9.03	3.94	5.9×10^{-17}	4.1×10^{-6}	Up	Up

ENSDLAG00005005393	*cd209*	CD209 antigen-like protein C isoform X2	8.06	2.75	2.4×10^{-18}	1.0×10^{-4}	Up	Up
ENSDLAG00005022256	*hp*	Haptoglobin	7.57	4.63	4.3×10^{-10}	2.2×10^{-6}	Up	Up
ENSDLAG00005021347	*il-1b*	Interleukin 1 beta	7.38	2.43	7.5×10^{-12}	1.1×10^{-4}	Up	Up
ENSDLAG00005008130	*mmp13a*	Matrix metallopeptidase 13	6.55	2.76	2.3×10^{-8}	2.1×10^{-4}	Up	Up
ENSDLAG00005024758	*c7b*	Complement component 7b	6.49	3.69	7.7×10^{-10}	8.2×10^{-4}	Up	Up
ENSDLAG00005018682	*mmp9*	Matrix metallopeptidase 9	4.77	2.25	7.7×10^{-10}	2.7×10^{-4}	Up	Up
ENSDLAG00005028846	*ptx3*	Pentraxin-related protein PTX3	2.75	5.56	2.3×10^{-6}	6.0×10^{-6}	Up	Up
ENSDLAG00005016361	*capn9*	Calpain-9	2.42	1.80	5.1×10^{-4}	8.8×10^{-4}	Up	Up
ENSDLAG00005021944	*tfr1b*	Transferrin receptor 1b	2.35	1.73	6.1×10^{-7}	2.7×10^{-2}	Up	Up
ENSDLAG00005029008	-	C-factor	1.63	-1.73	3.1×10^{-2}	4.8×10^{-2}	Up	Down

Table S3: List of matched DEGs and DEPs related to extracellular matrix (ECM) in the skin and mucus of European sea bass (*Dicentrarchus labrax*) bath-challenged with *Tenacibaculum maritimum* (Log_2 Fold Change ≥ 1.5; *q*-value ≤ 0.05).

Gene ID (Ensemble)	Gene name	Protein name	Log_2 Fold Change		*q*-Value		Regulated	
			DEGs	***DEPs***	***DEGs***	***DEPs***	***DEGs***	***DEPs***
ENSDLAG00005016526	*mfap4*	Microfibril-associated glycoprotein 4-like	8.40	2.89	3.7×10^{-15}	1.5×10^{-5}	Up	Up
ENSDLAG00005025809	*adamts*	A Disintegrin and metalloproteinase with thrombospondin motifs 13	3.58	1.77	6.1×10^{-4}	3.7×10^{-3}	Up	Up
ENSDLAG00005006625	*thbs1*	Thrombospondin-1	2.22	4.90	2.0×10^{-3}	6.9×10^{-4}	Up	Up
ENSDLAG00005018655	*lamb3*	Laminin subunit beta-3	1.95	1.73	1.4×10^{-2}	8.3×10^{-3}	Up	Up
ENSDLAG00005018287	*icam5*	Intercellular adhesion molecule 5 isoform X3	1.83	1.98	4.4×10^{-5}	8.3×10^{-5}	Up	Up
ENSDLAG00005022480	*emilin2a*	Elastin microfibril interfacer 2a	1.88	1.95	1.3×10^{-3}	1.5×10^{-3}	Up	Up
ENSDLAG00005020511	efemp2a	EGF containing fibulin extracellular matrix protein 2a	1.60	3.28	4.8×10^{-3}	2.8×10^{-4}	Up	Up
ENSDLAG00005030080	*timp2*	TIMP metallopeptidase inhibitor 2	1.52	3.46	4.8×10^{-3}	1.2×10^{-3}	Up	Up
ENSDLAG00005005020	*col18a1*	Collagen alpha-1(XVIII) chain isoform X6	-1.53	1.53	1.8×10^{-2}	3.0×10^{-2}	Down	Up
ENSDLAG00005026355	*cldni*	Claudin I	-1.64	-1.83	1.4×10^{-2}	1.1×10^{-2}	Down	Down

Figure S2: Principle component clustering between mock-challenged fish (blue) and challenged fish (red) at 24 h post-challenge for mucus samples of European sea bass (*Dicentrarchus labrax*).