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THE ROLE OF MICROALGAE AS FUNCTIONAL ADDITIVES AND GENETIC ENGINEERING: INNOVATIVE APPROACHES TO EUROPEAN SEA BASS HEALTH

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Abstract

During the last few decades, the aquaculture industry has been experiencing massive development, leading to the growing demand for farmed fish as a source of protein. This growth creates challenges, such as a higher incidence of pathogens and diseases, that threaten the health of the fish and the productivity. Among those, the bacterium *Tenacibaculum maritimum*, etiological agent of the disease known as tenacibaculosis, is one of the most concerning problems today, causing high mortality rates and major economic losses in aquaculture facilities. Therefore, it is necessary to explore innovative and sustainable approaches to prevent this growing problem. Present dissertation aimed to study the impact of two microalgae species on the European sea bass (*Dicentrarchus labrax*) immune response using different approaches.

In the first chapter, the effects of four diets supplemented with the microalga *Euglena gracilis* were tested on the immunological response of juvenile sea bass following infection with *T. maritimum*. For this study, juvenile sea bass were placed in recirculation tanks and fed with a control diet (CTR) and four diets (G1, G2, J1 and J2) containing different extracts of *E. gracilis.* These diets were prepared by adding to the control diet different proportions of *E. gracilis*, subjected to high-pressure homogenization (HPH) or presented in different fractions.

The animals were bath infected with an inoculum of *T. maritimum (*1 x 10^4 CFU/mL*)*, and samples were taken in two different time points: before infection (Non-Challenged), and 24 h post infection (Infected). Hematological indicators, immune humoral parameters and oxidative stress biomarker were analyzed. G1 diet, which consist of adding 0.5% of *E. gracilis* subjected to HPH to the CTR diet, showed to be the formulation with the best immunogenic potential for improving the immune response of sea bass. This claim can be sustained by the increase of plasma anti-protease activity (which can be an indicator of an improvement in immune response), and by the pattern observed in peripheral leukocytes mobilization, which can be an indication of a more readily response to pathogens.

In the second chapter of the work, a vector was developed for expressing a target immunogenic protein (antigen) from *T. maritimum*, known as sphingomyelinase. This vector was developed with the aim to genetically transform the chloroplast of the microalgae *Tetraselmis suecica*.

Geneious Prime® software was used to design the expression vector, including promotors (bi-directional 5' *rbcl* / 5' *atp*B) and terminator (URL) sequences, and also right and

left flanking regions (LFR and RFR) for the precise integration of the construct into the plastome of *T. suecica* by homologous recombination. Moreover, sphingomyelinase gene cassette coding sequence was optimized from both codon usage and open reading frame (ORF) perspectives. Importantly, in this case a turbo green fluorescent protein (tGFP) was used as selection marker of the transformants of *T. suecica* instead an antibiotic resistance gene, which is considered an environmentally friendly approach. The complete sequence of the generated recombinant vector was corroborated by both sequencing and restriction enzyme digestion analyses. Such results showed that insertion of the gene of interest (sphingomyelinase) cassette into the plasmid was successfully achieved. This recombinant expression vector will be used in future research to generate, by chloroplast transformation, a genetically modified strain of *T. suecica* that will be used as a platform for both expression and oral delivery of sphingomyelinase antigen to European sea bass. This promising synthetic biology approach represents one of the potential applications of microalgae to create biotechnological solutions for sustainable aquaculture purposes.

This work has shown that both microalgae used (*E. gracilis* and *T. suecica*) represent potential innovative and effective solutions for improving fish health, productivity and sustainability in the aquaculture sector. Subsequently, more studies targeting other pathogens should be carried out, while the future deployment and benefits of these microalgae should be further explored to better understand their influence on improving the fish immune response to bacterial infection.

Keywords: Aquaculture; European sea bass; Tenacibaculosis; Functional diets; *Euglena gracilis*; Vaccination*; Tetraselmis suecica*; *Tenacibaculum maritimum*.

Resumo

Durante as últimas décadas, a indústria da aquacultura tem vindo a registar um enorme desenvolvimento, conduzindo a um aumento da procura de peixes de viveiro como fonte de proteína. Este crescimento cria desafios, tais como a maior incidência de agentes patogénicos e doenças, que ameaçam a saúde dos peixes e a produtividade das empresas. Entre estes, a bactéria *Tenacibaculum maritimum*, agente etiológico da doença tenacibaculose, é um dos problemas mais preocupantes atualmente, causando elevadas taxas de mortalidade e grandes perdas económicas nas instalações de aquacultura. Assim, é necessário explorar abordagens inovadoras e sustentáveis para prevenir este problema crescente. A presente dissertação teve como objetivo estudar o impacto de duas espécies de microalgas na resposta imunitária do robalo (*Dicentrarchus labrax)* utilizando duas abordagens distintas.

No primeiro capítulo, foram testados os efeitos de quatro dietas suplementadas com microalga *Euglena gracilis* na resposta imunológica de juvenis de robalo após a infeção com *T. maritimum.* Para este estudo, os juvenis de robalo foram colocados em tanques de recirculação e alimentados com uma dieta controlo (CTR) e quatro dietas (G1, G2, J1 e J2) compostas por diferentes extratos de *E. gracilis.* Estas dietas foram preparadas através da adição à dieta controlo diferentes proporções de *E. gracilis*, submetidas a homogeneização a alta pressão (HPH) ou apresentadas em diferentes frações.

Os animais foram infetados no banho com um inóculo de *T. maritimum* (1 x 10^4 CFU/mL), e as amostras foram recolhidas em dois momentos diferentes: antes da infeção (Não Infetados) e 24h após a infeção (Infetados). Foram analisados indicadores hematológicos, parâmetros humorais imunitários e biomarcadores de stress oxidativo. A dieta G1, que consiste na adição de 0.5% de *E. gracilis* submetida a HPH à dieta CTR, mostrou ser a formulação com melhor potencial imunogénico para melhorar a resposta imunitária do robalo. Esta afirmação pode ser sustentada pelo aumento da atividade anti-protease plasmática (que pode ser um indicador de uma melhoria da resposta imunitária), e pelo padrão observado na mobilização de leucócitos periféricos, que pode ser um indicador de uma resposta mais rápida aos agentes patogénicos.

No segundo capítulo do trabalho, foi desenvolvido um vetor para expressar uma proteína imunogénica alvo (antigénio) de *T. maritimum*, conhecida como esfingomielinase. Este vetor foi desenvolvido com o objetivo de transformar geneticamente o cloroplasto da microalga *Tetraselmis suecica.*

O software Geneious Prime® foi utilizado para desenhar o vetor de expressão, incluindo as sequências do promotor (5' rbcl / 5' atpB bidirecional) e do terminador (URL), bem como as regiões flanqueadoras direita e esquerda (LFR e RFR) para a integração precisa da construção no plastoma da *T. suecica* por recombinação homóloga. Além disso, a sequência codificante da esfingomielinase foi otimizada tanto do ponto de vista da utilização de codões como da estrutura de leitura dos codões (ORF). É importante notar que, neste trabalho, foi utilizada uma proteína de fluorescência verde turbo (tGFP), como marcador de seleção dos transformantes de *T. suecica*, em vez de um gene de resistência a antibióticos, o que é considerado uma abordagem ecológica. A sequência completa do vetor recombinante desenvolvido foi corroborada por análises de sequenciação e de digestão com enzimas de restrição. Estes resultados mostraram que a inserção da *cassete* do gene de interesse (esfingomielinase) no plasmídeo foi conseguida com êxito. Este vetor de expressão recombinante será utilizado em investigação futura para gerar, por tranformaçao de cloroplastos, uma estirpe geneticamente modificada de *T. suecica* que será utilizada como plataforma para expressão e a administração oral do antigénio da esfingomielinase ao robalo europeu. Esta promissora abordagem de biologia sintética representa umas das potenciais aplicações das microalgas para criar soluções biotecnológicas para fins de aquacultura sustentável.

Este trabalho demostrou que ambas as microalgas utlizadas *(E. gracilis e T. suecica)* representam potenciais soluções inovadoras e eficazes para melhorar a saúde, a produtividade e a sustentabilidade dos peixes no sector da aquacultura. Simultaneamente devem ser realizados mais estudos dirigidos a outros agentes patogénicos, ao mesmo tempo a futura utilização e os benefícios destas microalgas devem ser mais explorados para compreender melhor a sua influência na melhoria da resposta imunitária dos peixes à infeção bacteriana.

Palavras-chave: Aquacultura; Robalo europeu; Tenacibaculose; Dietas funcionais; *Euglena gracilis*; Vacinação; *Tetraselmis suecica*; *Tenacibaculum maritimum*

Contents

Abbreviations list

AMPs - Antimicrobial Peptides

A. tumefaciens - *Agrobacterium tumefaciens*

aadA - Spectinomycin/streptomycin resistance

aphA6 - Kanamycin/amikacin resistance

BOGA - Biotério animais aquáticos

BSA - Bovine serum albumin

CAT - Catalase

cat - Chloramphenicol acetyltransferase resistance gene

CFU - Colony-forming unit

CTR - Control

cDNA - Complementary DNA

DNA - Deoxyribonucleic acid

DTPA - Diethylenetriaminepentaacetic acid

ereB - Erythromycin resistance gene

tGFP - Turbo green fluorescent protein

GSH - Reduced glutathione

GSSG - Oxidized glutathione

Hb - Hemoglobin

HPH- High-pressure homogenize

Ht – Hematocrit

IHN - Infectious Hematopoietic Necrosis

IPNV - Infectious Pancreatic Necrosis Virus

LFR - Left flanking region

LPO - Lipid peroxidation

MCH - Mean corpuscular hemoglobin

MHCH - Mean corpuscular hemoglobin concentration

mRNA - Messenger ribonucleic acid

MCV - Mean corpuscular volume

MDA - Malondialdehyde

NIH - National Institutes of Health

NCCs - Non-specific cytotoxic cells

ORF - Open reading frame

PAMPs - Pathogen-associated molecular patterns

PCR - Polymerase chain reaction

PEG - Polyethylene glycol

PRR - Pattern recognition receptor

PUFA - Polyunsaturated fatty acid

RBC - Red blood cell

RFR - Right flanking region

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RT- PCR- Reverse transcription PCR

SD - Standard deviation

SOD - Superoxide dismutase

T9SS - Type IX secretion system

TBE - Tris-Borate-EDTA

TCA - Trichloroacetic acid

TLRs -Toll-like receptors

Tm - Melting temperature

UV - Ultraviolet

VP2 - Viral capsid protein 2

WBC - White blood cell

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Aim

The aim of this study is related to the use of different microalgae as potential prophylactic strategies to improve fish health in aquaculture. Chapter I describes the study of the oxidative stress and innate immune responses of sea bass in the first days of infection with *Tenacibaculum maritimum*, using a diet supplemented with compounds from the microalga *Euglena gracilis*.

Chapter II was focused on the development of a vector, using a combination of bioinformatics and laboratory procedures, capable of expressing an antigen protein (sphingomyelinase) of the bacterium *T. maritimum* to be used in future research for the chloroplast transformation of *Tetraselmis suecica* by biolistic.

Objetivo

O objetivo deste estudo está relacionado com a utilização de diferentes microalgas como potenciais estratégias profiláticas para melhorar a saúde dos peixes em aquacultura. O capítulo I descreve o estudo do stress oxidativo e das respostas imunitárias inatas do robalo nos primeiros dias de infeção com *Tenacibaculum maritimum*, utilizando uma dieta suplementada com compostos da microalga *Euglena gracilis.*

O capítulo II centrou-se no desenvolvimento de um vetor, recorrendo a uma combinação de bioinformática e procedimentos laboratoriais, capaz de expressar uma proteína antigénica (esfingomielinase) da bactéria *T. maritimum.* Este vetor tem como finalidade ser utilizado em futuras investigações para a transformação cloroplástica de *Tetraselmis suecica* por biolística.

1. Introduction

1.1. Global aquaculture and its challenges

The aquaculture industry, especially fish production, has a significant economic importance for different nations around the world (Charoonnart et al., 2018). In 2020, global aquaculture revenues reached 87 billion USD, which also indicates the trend of its future development. It is estimated that by the year 2032, world production of aquatic animals will reach 205 million tonnes, of which 111 million tonnes will come from aquaculture **(Fig. 1)** (FAO, 2024). The income from aquaculture exceeds the income from capture fisheries, which has remained constant, mainly due to over-exploitation (FAO, 2020). Also, as described by Ma et al. (2020), aquaculture is projected to contribute around 62% of the fish consumed by the global population by the year 2030. In addition, Ma et al. (2020) also states that aquaculture is expected to maintain its prominent position in the global fish supply and show sustainable growth beyond 2030.

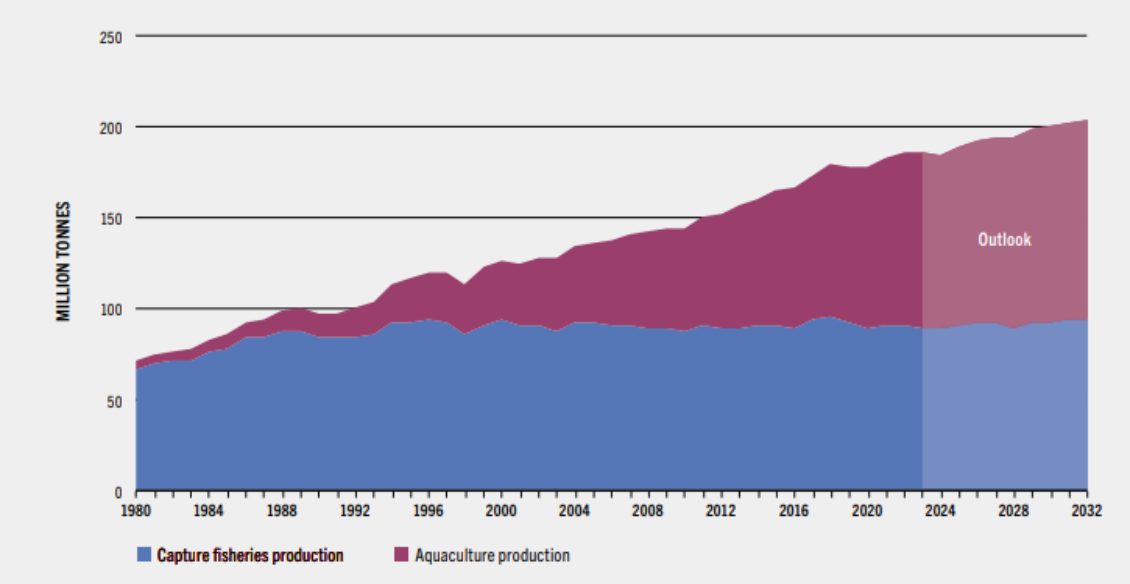

Figure 1. World fisheries and aquaculture production of aquatic animals, 1980–2032. Source: FAO estimates 2024.

European sea bass (*Dicentrarchus labrax*) (Linnaeus, 1758) **(Fig. 2)** is a fish species found in the coastal shallows, usually at depths of less than 100 m (FAO, 2009). It is found in areas ranging from the northeast Atlantic Ocean to the Mediterranean and the Black Sea (Vandeputte et al., 2019). Besides salmon, sea bass was the pioneer among marine species, to be cultivated industrially in Europe (Bagni, 2005). Intensive farming of sea bass in the Mediterranean started as a relatively new industry at the very end of the 1980s. It experienced fast growth in the 1990s and has expanded consistently over the following two decades (Muniesa et al., 2020). Over the last 15 years, there has been an increasing demand in Europe for this species because of its nutritional benefits, tasty flavor, attractive aroma, and excellent quality (Fuentes et al., 2010). In general, sea bass is a resistant species, but it is still subject to a wide range of diseases under farming conditions. These diseases have a serious impact on commercial production and can slow down the expansion of the sector in some countries (Bagni, 2005).

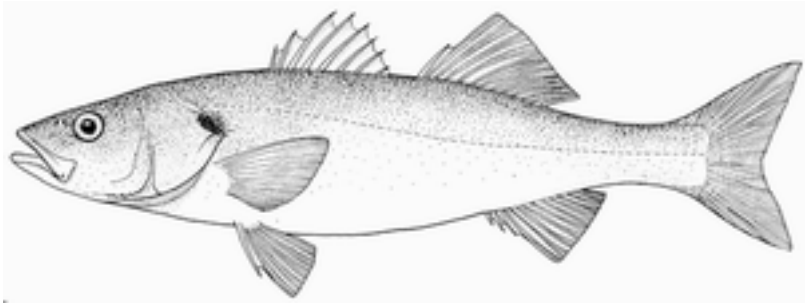

Figure 2. *Dicentrarchus labrax* (Linnaeus, 1758). Source: FAO 2009

The rapid expansion of aquaculture has led to several negative consequences for both the environment and human health (Cabello, 2006). Pathogens continue to represent a serious risk to biodiversity, as well as to animal production and human health (Daszak et al., 2000). Although there have been several innovative approaches to treatment, fish diseases continue to represent a serious economic challenge in commercial aquaculture worldwide (Ma et al., 2019). Main principal diseases in aquaculture are mostly caused by viruses, a variety of protists and bacteria (Lafferty et al., 2015). Approximately 75% of the reported diseases affecting European sea bass in the Mediterranean are caused by bacterial infections (Monzón-Atienza et al., 2023). Indeed, approximately 10% of total fish losses in aquaculture result from disease, and more than half of these deaths are particularly caused by bacterial pathogens (Freund et al., 1990). *Vibrio* spp., *Photobacterium* spp. and

Tenacibaculum spp. have been reported as the most frequent pathogens affecting European sea bass (Muniesa et al., 2020).

Over the last few decades, the global marine aquaculture sector has seen a general emergence of tenacibaculosis. Tenacibaculosis is a relatively unexplored pathology, mainly caused by *Tenacibaculum* spp., that affects many commercially important aquaculture species and produces large economic losses (Avendaño-Herrera et al., 2020; Flores-Kossack et al., 2020; Mabrok et al., 2023). Most of the research on *Tenacibaculum* spp. relates tenacibaculosis to infections caused by *Tenacibaculum maritimum* (Avendaño-Herrera et al., 2006). *T. maritimum*, a fish pathogen found all over the world, is known for its lethal impact on different marine fish species, including both wild and farmed fish (Le Luyer et al., 2021). It has been observed that, although tenacibaculosis outbreaks have had a significant impact on the aquaculture sector, knowledge of the virulence mechanisms used by *T. maritimum* is quite scarce, as indicated by Avendaño-Herrera et al. (2006).

1.2. *Tenacibaculum maritimum*

T. maritimum, previously *Flexibacter maritimus*, is a bacterium that belongs to the Flavobacteriaceae family in the phylum Bacteroidetes (Suzuki et al., 2001). *T. maritimum* has a slender, filamentous shape and has been classified as Gram-negative **(Fig. 3)** (Avendaño-Herrera et al., 2020). It generally ranges from 2 to 30 µm in length and has a diameter of approximately 0.5 µm. Similar to the other members of the Flavobacteriaceae, this bacterium has the capacity to move through surfaces by means of gliding motility, a method that does not depend on pili or flagella for the active movement process (Pérez-Pascual et al., 2017). This bacterium grows in moderate temperature ranges, particularly between 15 - 34ºC (Avendaño-Herrera et al., 2006). This pathogen has successfully spread to different aquaculture areas, reaching Europe through the Mediterranean coast of France, affecting various types of farmed marine fish, including European sea bass (Pepin & Emery, 1993; Pérez-Pascual et al., 2017). *T. maritimum* causes a disease called marine flexibacteriosis or tenacibaculosis, which results in ulcerative disease in a large number of marine fish worldwide (Avendaño-Herrera et al., 2005). This pathology has been known by various names, such as "slippery bacterial disease of marine fish", “eroded mouth syndrome” and “black spot necrosis”, all referring to the diseases causes by *T. maritimum* (Toranzo et al., 2005).

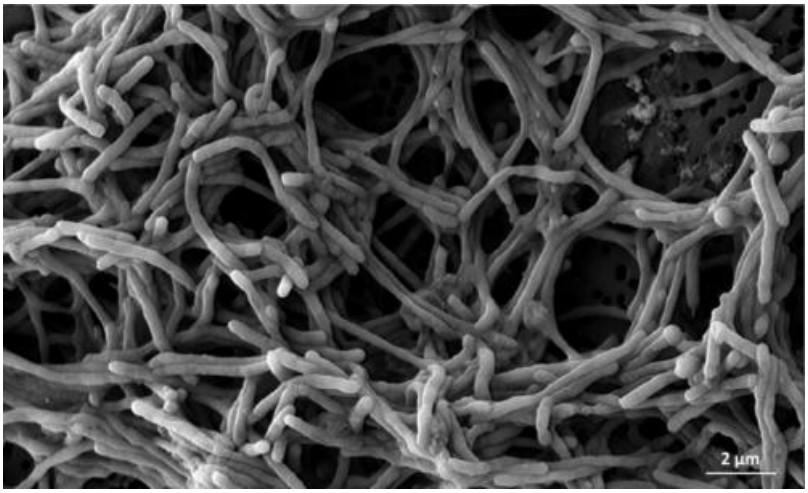

Figure 3. Scanning electron microscopy of *T. maritimum*. Source: Mabrok et al. (2023) Front. Cell. Infect. Micro-biol. 12:1068000.

The diagnosis of tenacibaculosis is mainly based on observing clinical signs in dead fish, and then trying to isolate the pathogen and identify the bacterial species responsible. However, this process can be controversial and sometimes difficult to execute (Nowlan et al., 2020). Methods such as PCR and advanced RT-PCR have recently been used to confirm the diagnosis more accurately (Mabrok, 2016).

Fish affected by *T. maritimum* show a spread of the pathogen in their external tissues, causing pronounced skin lesions and ultimately a rapid mortality (Avendaño-Herrera et al., 2006). Moreover, moribund fish affected by tenacibaculosis show signs such as discoloration of the liver (bleeding or pale), small spot hemorrhages on the jaw, fins and underbody, anemia, and erratic swimming behavior (López et al., 2010). This pathogen forms an optimal biofilm that attracts other microorganism or organic materials. When it established in the gills, it affects the fish's ability to breath properly (Ferreira et al., 2023; Kitatsuji et al., 1996). Additionally, skin lesions may lead to co-infection with other opportunistic pathogens such as *Vibrio spp.* (Pérez-Pascual et al., 2017). Juvenile fish are more vulnerable to infection with *T. maritimum* as the damage to the affected tissues can progress rapidly from the initial stages to advanced ulcerative lesions in a relatively short period of time, which is usually just a few days (Bernardet et al., 1994).

Additionally, the bacterium resilient nature of attaching itself strongly to the mucus of the skin of various fish species, and its capacity to resist their natural antibacterial defenses, have been pointed out as potential factors that contribute to its virulence (Magarinos et al., 1995). Moreover, it is well-recognized that the ability to obtain iron during an infection has

a crucial role to play in the pathogenicity of many bacteria, as it is crucial for the proliferation and multiplication of the pathogen (Avendaño-Herrera et al., 2005). In a study conducted by Avendaño-Herrera et al. (2005), it was demonstrated for the first time that *T. maritimum* has two distinct mechanisms for obtaining iron: one involves the creation of siderophores, while the other permits the direct binding and utilization of heme groups as a source of iron.

Despite the serious impact of this pathogen, there are few commercially available vaccines for *T. maritimum*, one of which has been developed for turbot, targeting a specific strain known as LPV1.7, serotype O2 (Mabrok et al., 2023). In this case, the immunity effect accomplished was the only 6 months (Miccoli et al., 2019).

1.3. Virulence factors of *T. maritimum*

Virulence is defined as the ability of a microorganism to successfully evade the host's immune defenses, in order to propagate and reproduce, leading, eventually, to the manifestation of a disease (Casadevall and Pirofski, 1999). Determination of virulence factors in bacterial pathogens is essential for understanding their pathogenicity and developing specific therapeutic strategies.

The complete genome sequence of *T. maritimum* has revealed important information about specific genes associated with virulence. These genes are responsible for the synthesis of exopolysaccharides, the presence of a type IX secretion system (T9SS), iron absorption mechanisms, adhesion proteins, hemolysins, proteases, and glycoside hydrolases. This extensive genomic analysis allows a detailed understanding of the genetic factors that contributes to the pathogenicity of *T. maritimum* (Pérez-Pascual et al., 2017). The pathogenesis of *T. maritimum* is significantly influenced by its ability to adhere to living (biotic) and non-living (abiotic) surfaces, exhibiting gliding motility, producing hemagglutination and extracellular products. These several mechanisms are believed to be crucial in the pathogenic mechanisms used by *T. maritimum* (Escribano et al., 2023; Mabrok et al., 2023).

Recently discovered, T9SS has been suggested as the principal virulence mechanism of *T. maritimum*, as it is believed to promote the colonization of fish tissues by bacteria from the phylum Bacteroidetes (McBride, 2019). T9SS is responsible for improving the gliding motility of bacteria, increasing their ability to adhere to living and non-living surfaces by supporting the formation of biofilms, and by serving as a translocation mechanism of virulent factors to extracellular medium (Eckroat et al., 2021).

Another virulence mechanism of *T. maritimum* that has been investigated involves its ability to compete with the host for iron. While iron is abundantly present in the environment, its availability is restricted when a host is infected. Most iron is present intracellularly, stored in the iron storage protein ferritin or bound to heme as a cofactor in hemoglobin or myoglobin (Defoirdt, 2014). Avendaño-Herrera et al. (2005) suggested the existence of two different iron acquisition systems in various serotypes of *T. maritimum*. One system consists of the production of siderophores, while the other enables the direct use of heme groups as sources of iron through binding. Many pathogenic bacteria use siderophores, which are small iron-binding components that are released into the environment (Cherayil, 2011). Siderophores play a crucial role in allowing bacteria to effectively compete with iron-binding proteins produced by the host (Defoirdt, 2014).

In addition, different lytic proteins were identified in the secretome of *T. maritimum*. Among the highly abundant extracellular product proteins, a diverse range of hydrolytic enzymes have been identified, including sphingomyelinase (Sph), chondroitinase (ClsA), sialidase (SiaA), ceramidase (Cer), and collagenase (Col). Sphingomyelinase is described as one of the virulence mechanisms of *T. maritimum*, being a highly potent cytotoxin for the host (Oda et al., 2010). Its function can result in tissue damage and lead to the progression of disease (Mabrok et al., 2023). These enzymes have been suggested as potential virulence factors in *T. maritimum* (Escribano et al., 2023; Pérez-Pascual et al., 2017). Importantly, the majority of these predicted toxins reported in *T. maritimum,* are not present in the genomes of the other species (Escribano et al., 2023).

1.4. Fish immune system

Fish are a group of vertebrates that emerged during the Devonian period after adaptive radiation, establishing themselves as the most diverse and abundant category of vertebrates (Rauta et al., 2012). Despite some differences, the immune system of fish is physiologically very similar to that of higher vertebrates (Uribe et al., 2011). This diverse group of animals is at an important evolutionary point where the innate immune system and the adaptive immune system diverged (Rauta et al., 2012). The immune system protects the organism from disease by recognizing and eliminating pathogens, and also by maintaining homeostasis during development, growth and after an inflammatory reaction or tissue attack (Magnadottir, 2010).

The immune system of fish is composed by two intrinsically related immune mechanisms: i) innate or non-specific; and ii) adaptive or specific (Mokhtar et al., 2023). The innate immune system is the earliest defense mechanism, characterized by its non-specific nature. It is also reactive to external molecules, simultaneously inducible and constitutive, permitting quick reactions in a short time (Tort et al., 2003). It is generally sub-classified into three components: a) the epithelial/mucosal barrier, b) the humoral, and c) the cellular components (Magnadottir, 2010; Rodrigues et al., 2020; Uribe et al., 2011).

In most fish species, mucus extensively coats external surfaces, in particular the skin and gills (Tort et al., 2003); thus, mucus has an important role to play, as it serves as a fundamental platform for the antibacterial mechanisms. In addition, these mucosal barriers, enriched with several immune system effectors, including lysozyme, complement-related proteins, immunoglobulins M (IgM), and mucosal specific immunoglobin T (IgT), antimicrobial peptides, lectins, mycosporine-like amino acids, among others, provide a wide range of defense mechanisms against pathogens (Magnadóttir, 2006). IgM is the most abundant class of immunoglobins, and its strong immune response to infection or vaccination can be detected in plasma (Xu et al., 2016).

The innate immune system is also composed by cellular elements such as phagocytes (macrophages, neutrophils), dendritic cells and non-specific cytotoxic cells (NCCs). Besides these cellular elements, various molecular components contribute to inflammation, including antibacterial peptides and proteins such as the complement, transferrin, ciclooxigenase-2, chemokines, cytokines, and tumor necrosis factor, among others (Magor & Magor, 2011; Uribe et al., 2011).

The recognition process in the innate immune system is dependent on the group of receptors known as pattern recognition receptors (PRRs). These receptors have a broad specificity, and have developed to identify patterns related to pathogens, known as pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway Jr, 1997). Of the many classes of PRRs, the Toll-like receptors (TLRs) are the most extensively studied and better understood (Alvarez-Pellitero, 2008).

There is a diversity of humoral parameters, involved on the immune response, providing a wide range of different functions, from recognizing molecules to developing agents capable of killing bacteria. Neutrophils and macrophages can ingest bacteria and eliminate them primarily through the generation of reactive oxygen species (ROS), in what is known as a respiratory burst. These ROS, including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (O^{Hv}), have strong bactericidal properties (Ellis, 1999).

Antimicrobial Peptides (AMPs) are a type of small molecule that are produced by various types of cells, including cells of the immune system, mucous membranes and neurons. These peptides have the capacity to kill or suppress the growth of microorganisms such as bacteria, viruses and fungi (Mokhtar et al., 2023). There is also a wide variety of lytic enzymes that can cause the lysis of pathogenic cells. They can act individually or in association. In fish, some of the lytic enzymes include complement compounds, lysozyme and AMPs (Ellis, 1999).

The complement is an important component of the immune system that plays a crucial role in linking the innate and adaptive immune responses (Birkemo et al., 2003). Most of the enzymes in the complement system are inactive until they are activated by binding to each other or through enzymatic action (Mokhtar et al., 2023). The complement system has a crucial function in several different aspects of the immune response. It optimizes the phagocytosis process by targeting pathogens for ingestion by immune cells. Additionally, it mediates inflammation, drawing immune cells to areas of infection or injury (Uribe et al., 2011). Also, it can kill pathogens directly by creating pores in their membranes. It also favors the interaction between the innate and adaptive immune systems (Magnadottir, 2010). Lastly, it can help remove immune complexes formed during and following infection (Holland & Lambris, 2002). The complement system has three activation pathways, which depend on the method used. The classical complement pathway is initiated when an antibody binds to the surface of a cell, by acute phase proteins (Petersen et al., 2001) or by direct activation by certain viruses or bacteria (Spiller & Morgan, 1998). The alternative complement pathway (ACP) is activated directly by pathogens, without the need for antibodies (Holland & Lambris, 2002). In turn, the lectin complement pathway occurs when a protein complex made up of lectins that bind to mannose (MBL) attaches to polysaccharides on the bacterial surface - mannans (Holland & Lambris, 2002).

Cytokines are essential signaling molecules, responsible for controlling and mediating both the innate and adaptive immune systems. They also play a critical role in cell differentiation, cell growth and cell survival. There are various sub-categories that come under the cytokine label: interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), colony stimulating factors (CSFs), chemokines, and growth factors (Vilček & Feldmann, 2004; Whyte, 2007).

On the other hand, the adaptive immune response operates through a complex system of specialized cells, proteins, genes and chemical signals. This system allows the body to respond precisely to antigens, antibodies and effector cells with exceptional specificity and efficiency (Uribe et al., 2011). For instance, B lymphocytes have a key role to play

regarding the adaptive immune response. They are responsible for the production of antibodies, also known as immunoglobulins, and for presenting antigens to T lymphocytes, which helps to activate the cellular immune response (Rodrigues et al., 2020). The antibodies produced by B lymphocytes can be categorized into 3 groups: IgM, IgD and IgT/IgZ. IgM is the predominant circulating antibody and can be found in its transmembrane or secreted form (Rauta et al., 2012). Their functions, including opsonization, antibody-dependent cell-mediated cytotoxicity and complement activation, are highly preserved throughout the evolutionary tree (Smith et al., 2019). IgD is usually found only in the transmembrane form, and to date, there is limited information on its function and action mechanisms. However, its structure is known to be highly variable and recent findings suggest a phylogenetic arisen close to IgM (Mokhtar et al., 2023). The IgT/IgZ immunoglobulins has been described mostly on mucosal surfaces. Some studies have demonstrated a considerably higher prevalence of IgT in gut-associated lymphoid tissues compared to plasma (Xu et al., 2016). Additionally, it has been shown that infections by pathogens in the intestine, results in a higher *in situ* level of IgT (Uribe et al., 2011).

T lymphocytes, or T cells, are important components of the adaptive immune response. These cells are generated in the thymus and, similarly to B lymphocytes, they have special receptors linked to the membrane, known as T-cell receptors (TCRs) (Secombes & Wang, 2012). T cell activation is mediated by antigen presenting cells, which differentiate into two principal subpopulations: helper T cells (Th) and cytotoxic T cells. Cytotoxic T cells are able to kill their targets by causing apoptosis, and this can occur in two ways: i) via the secretory pathway, with the release of toxins, and ii) via the non-secretory pathway, that is dependent on receptor binding (Magnadottir, 2010).

The master histocompatibility complex (MHC) is composed by proteins located on the cell membrane, produced by a group of highly differentiated genes among individuals. There are two main classes of MHC: MHC I and MHC II. The polymorphic MHC synthesis is extremely important for the host's capacity to recognize different antigens (Wegner, 2008). MHC I is found on most cells and is responsible for detecting intracellular pathogens and tumors. It delivers peptides obtained from these pathogens to the cell surface to be recognized by cytotoxic T cells (Uribe et al., 2011). MHC II is expressed only by phagocytic cells that can deliver processed peptides to helper T cells. It is also responsible for presenting exogenous antigens, which are caught and processed by phagocytic cells, to T helper cells (Wegner, 2008).

1.5. Vaccination in aquaculture

Effective and advanced aquaculture practices depend on the success of the prevention and treatment of a variety of bacterial parasitic and viral water-borne pathogens. The constant need for effective vaccination strategies results from high stocking densities and increasing production intensity, with the objective of guaranteeing protection, minimizing losses, and reducing dependence on antibiotics throughout the production cycle (Villumsen et al., 2017). Although antibiotics or chemotherapeutic agents can be used to treat diseases, there are significant drawbacks, including challenges related to drug resistance and safety problems (Sneeringer et al., 2019). While certain bacterial diseases respond effectively to vaccines and antibiotics, others are unaffected by antibiotic treatment.

Moreover, the incorrect use of antibiotic has led to an increase in antibiotic-resistant bacteria, which represents a growing risk of transferring not only these resistant strains, but also the residual antibiotics, from aquatic products to humans, causing global concern (Costa et al., 2015). Additionally, the accidentally ingestion of antibiotics through food can lead to allergy and toxicity problems. In some cases, the diagnosis of such problems is difficult due to a lack of prior information about antibiotic ingestion (Alderman & Hastings, 1998; Cabello, 2004). Therefore, this leads to the necessity of the development of new alternatives to the use of antibiotics to control diseases in aquaculture.

Vaccination is a very efficient strategy for preventing a diverse number of bacterial and viral diseases and plays a fundamental role in promoting sustainability in the environmental, social, and economic aspects of global aquaculture industry (Ma et al., 2019). Around the world, vaccination has become a successful and widely applied practice in almost all livestock. Aquaculture sector is no exception, providing new solutions and possibilities for prevention and control of diseases in aquaculture (Bikezina et al., 2021; Ma et al., 2020). However, vaccination represents economic, technical, and regulatory challenges. For instance, the costs of producing vaccines often exceed those of antibiotics (Charoonnart et al., 2018).

While administering vaccines by injection reduces waste and guarantees accurate dosing, the laborious and costly procedures involved in the capture, handling, anesthesia, and injection of fish can lead to injury as defended by Charoonnart et al. (2018). As a result of this problem, oral vaccination has become an important method for delivering vaccines in aquaculture (Radhakrishnan et al., 2023). Oral vaccination is an easy delivery process that reduces stress in fish (Assefa & Abunna, 2018). The principal disadvantage of oral vaccination is the uncertainty about the dose administered which often translates into low

immunization efficiency. This usually results from the possible loss of the vaccine by degradation, both in water before feeding, and in the gastrointestinal tract of fish during the digestion process. Consequently, oral vaccination usually only provides a temporary period of immunity, demanding more frequent vaccination boosts (Charoonnart et al., 2018).

A conventional vaccine contains a substance that acts as an antigen, which is usually an inactive form of the pathogen, or part of it, such as a protein. This element triggers a immune response in the fish, directed against the specific pathogen (Ma et al., 2019). The various types of vaccines include those that are killed, attenuated, Nucleic acid-based, composed of synthetic peptides, use recombinant vectors, involve genetic modification, and those designed as subunit vaccines (Assefa & Abunna, 2018).

1.6. DNA vaccines

The introduction of recombinant DNA technology in the 1970s changed biological research and created the bases for contemporary biotechnology. It started with the creation of artificial circular DNA molecules in laboratory (Cutolo et al., 2022). DNA vaccines are a novel technique of vaccination resulting from the technological and empiric advancements developments in the molecular biology field (Assefa & Abunna, 2018). These vaccines are formed by a specific gene contained in a plasmid. This gene contains instructions to produce a specific immunogenic compound, and when expressed in the host, it causes a strong specific immune reaction (Ma et al., 2019; Reyes et al., 2017).

Extracting extremely pure plasmid DNA from *Escherichia coli* is a fundamental process in several modern molecular biology techniques. The induction of chemical competence followed by transformation is a method frequently used to insert plasmids or different DNA fragments into *Escherichia coli* (Tu, 2008). Common DNA cloning and assembly methods usually involve the step of introducing the generated plasmid into competent *E. coli* strains (DH5α, Top10, etc.), which can accept and maintain the external DNA (Kostylev et al., 2015). Such process is known as bacterial transformation. Some important components responsible for plasmid replication into the bacterial host include a region for replication, and a selectable marker (Glenting & Wessels, 2005). Additionally, the cassette sequence, coding a specific target gene framed by control elements such as the promoters and terminators sequences, which will help the vector to be expressed in prokaryotic and/or eukaryotic cells (Kurath, 2008).

Indeed, it has been proven that DNA vaccines have the capacity to trigger strong responses from both cellular and humoral immune system perspectives (Ma et al., 2019). The first DNA vaccine for aquaculture was developed against the etiologic agent causative of the Infectious Hematopoietic Necrosis (IHN) and was tested specifically on rainbow trout (*Oncorhynchus mykiss*) (Kurath, 2008). In recent years, several studies have been published on the application of DNA vaccines to aquaculture fish. For example, in a study conducted by Reyes et al. (2017), it was demonstrated that the administration of a DNA vaccine targeting a central region of the viral protein (VP2), and delivered orally via feed using a liposomal formulation, effectively induced a strong protective response against Pancreatic Necrosis Virus (IPNV) in Atlantic salmon (*Salmo salar*). Thus, DNA vaccines are a promising approach to the treatment of diseases in aquatic animals (Kurath, 2008).

1.7. Nutritional value of microalgae for aquaculture

Microalgae stand out as a key source of bioactive compounds that are showing promise as functional ingredients in pharmaceuticals formulations (Letsiou et al., 2017). Microalgae are a diverse collection of photosynthetic organisms, which include both eukaryotic organisms and prokaryotic cyanobacteria (Yan et al., 2016). Native microalgae are a rich source of many natural compounds, such as pigments, proteins, vitamins, polyunsaturated fatty acids (PUFAs), and polysaccharides derivatives. Since they constitute the natural diet of aquatic animals, its compositions make them ideal for creating microalgae-based additives (Ma et al., 2020). Frequently used species, such as those belonging to genera *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema,* and *Thalassiosira,* are combined in variable proportions to achieve a well-balanced composition of proteins, lipids, and essential micronutrients (Shah et al., 2018). This important role in the life cycles of several marine invertebrates, has led commercial and experimental fish farms to incorporate microalgae production systems in their operations (Guedes & Malcata, 2012).

The use of microalgae in aquaculture provides many potential benefits in comparison to the production of algae for consumption by humans or for feeding terrestrial animals. These include improved conversion efficiency and the elimination of the need for harvesting, drying and storage, as the algae can be used directly by animals or food chains (Benemann, 1992).

Among the diverse microalgae investigated for producing biomaterials, *Euglena* species are highly attractive. They are easy to cultivate and have the capacity to produce various bioactive compounds (Kottuparambil et al., 2019). The protist genus *Euglena* is a group of freshwater unicellular flagellated organisms that can be photoautotrophic, heterotrophic or photoheterotrophic. In particular, *Euglena gracilis* offers a great and important nutritional value for fish diets. Its use has increased in recent years due to its protein content, and also by the presence of nutrients such as ß-1,3-glucans, essential amino acids, fatty acids, vitamins, and minerals (Dai et al., 2022). Several studies have shown that B-glucans, when administered orally, improves innate and adaptive responses in fish, as well as increases resistance to experimental infection (Skov et al., 2012). β-1,3-glucans are also known as a bioactive compound, which are widely used for the treatment of various diseases in aquaculture (Meena et al., 2013).

1.8. Microalgae as delivery carriers

The utilization of microalgae as vaccine delivery carriers has recently increased in aquaculture industry, offering new alternatives for the prevention and control of diseases in this sector (Ma et al., 2020; Ramos-Vega et al., 2021). Furthermore, the chloroplast of microalgae has many advantages as a platform to produce recombinant proteins and small molecules. These include the cost-effectiveness of the culture, the lack of toxins, and the simplicity of genetic modification (Young & Purton, 2016). Plastids, which are crucial for the expression of vaccine antigens, in microalgae, are vital in the production of therapeutic proteins, being the chloroplast a fundamental component in this process (Dyo & Purton, 2018).

To allow the creation of vaccines from edible microalgae, more research and studies are essential (Jiji et al., 2023). Among the different species of microalgae, the eukaryotic green microalgae *Tetraselmis suecica* is commonly used in aquaculture because of its ability to grow under stressful conditions and its adaptability to diverse environments. Moreover, the selection of *T. suecica* as platform for the production of vaccines is also due to its availability and its known role in increasing the antioxidant response, resistance to disease, and reinforcing the immune response (Gutiérrez et al., 2020; Messina et al., 2019; Rentería-Mexía et al., 2022). The morphological structure of *T. suecica* incorporates a cell wall, which makes the transformation process and the insertion of a plasmid more challenging (Su et al., 2023). Nevertheless, this microalga is suitable for various biotechnology applications, including chloroplast transformation, and protein expression (Gutiérrez et al., 2020).

1.9. Chloroplast genetic engineering

Algae cells function as nature's green factory, specializing in efficient photosynthesis using direct solar energy (Ma et al., 2020). Chloroplasts, known mostly for their important role in photosynthesis, also serve as crucial targets for many essential biosynthetic processes in plants (Day & Goldschmidt-Clermont, 2011). Furthermore, the genetic engineering of chloroplasts is a technology in development with the potential to produce large quantities of recombinant proteins. This technology has the potential for industrial use, for medicinal applications or to improve natural strains of microalgae for new purposes (Wang et al., 2020). Additionally, proteins obtained from microalgae are biocompatible and can be consumed directly by animals without need for isolation and purification. This means avoiding the costs of purification and extraction processes (León-Bañares et al., 2004).

For microalgae to become viable hosts for biotechnological applications, the need to develop molecular tools for genetic manipulations is essential, given that they have three separate membrane-bound organelles (nucleus, mitochondria, and chloroplast) (Kwon et al., 2018). Most of the genes that code for enzymes for metabolic pathways are in the nucleus (Yan et al., 2016). However, using the nuclear genome for the expression of recombinant proteins has disadvantages. For instance, transgenes can be integrated randomly, can favor certain codons, can be affected by their location, and/or can be silenced by epigenetic factors (Kwon et al., 2018). Therefore, these challenges often result in inappropriate or variable expression of the transgenes.

On the other hand, chloroplast transformation has become an ideal platform for transgene expression (Kumar & Ling, 2021). This method has a major advantage: it allows precise integration of transgenes by homologous recombination, in contrast to nuclear transformation of microalgae, which usually leads to unpredictable and aleatory integration events (Doron et al., 2016). The transformation of chloroplast DNA started in 1988 with an experiment on the unicellular green alga *Chlamydomonas reinhardtii* (Boynton et al., 1988). Since then, there have been significant progresses in the methods and techniques used to genetically engineered the chloroplast in different microalgae species (Cutolo et al., 2022).

The genomes of chloroplasts, often designated as "plastomes", are circular polyploid molecules that carry around 100 to 200 genes. Most of these genes encode crucial elements of the photosynthetic machinery and/or the elements needed for transcription and translation within the organelle (Taunt et al., 2018). Chloroplast transformation involves modifying the existing genome or inserting a new foreign gene into the chloroplast (Kumar & Ling, 2021). To start the production of recombinant microalgae, the first step involves

isolating the gene of interest and creating a compatible DNA vector that enable the transfer, insertion, and expression of the transgene in the microalgae (Cabello, 2004).

After the vector has been developed, it is transformed into the chloroplast. The transformation of chloroplast has two main challenges: first, ensuring the introduced DNA efficiently enters the chloroplast membranes; and secondly, establishing the domination of the transformed chloroplasts over other plastids during selection and regeneration process: homoplastic state (Haring & De Block, 1990).

The main approach frequently used for gene delivery is biolistic transformation, known also as microprojectile bombardment (Doron et al., 2016). In this method, the transgenic DNA is introduced into the chloroplast by bombarding a field of microalgae or plant tissue with small particles of gold/tungsten coated with DNA (Dyo & Purton, 2018; Kumar & Ling, 2021). Additionally, polyethylene glycol (PEG) - mediated transformation is the second most popular transformation method, being extensively used in plant bioengineering, is particularly useful for transforming chloroplasts (Low et al., 2018; Yu et al., 2020). In this process, the chloroplast will be co-cultured with PEG, ions, and recombinant DNA. The changes in membrane permeability allow the foreign DNA to penetrate both cytoplasm and chloroplasts (Kofer et al., 1998). Another method is the *Agrobacterium*-mediated transformation, that is based on a bacterium called *Agrobacterium tumefaciens*, which imitates the natural process of plant transformation (Kumar & Ling, 2021). According to Kumar and Ling (2021), in this technique, the pathogen infects the plant and then transfer the desired gene into the plant's chloroplast, without causing damage. Additionally, electroporation is an alternative technique that involves the utilization of pulsed electric fields to increase the permeability of the cell membrane, allowing the introduction of substances into the cell (Yarmush et al., 2014).

Moreover, selectable markers are the principal method of identification and selection of transformed organisms (Kumar & Ling, 2021). Antibiotic resistance genes derived from bacteria, such as *aadA* (for spectinomycin/streptomycin resistance), *aphA6* (for kanamycin/amikacin resistance) and *ereB* (for erythromycin resistance), have been frequently used as selectable markers for chloroplast transformants in genetic engineering of plants (Bateman & Purton, 2000). Even though genetic engineering of chloroplasts in plants has several benefits, currently there are significant problems associated to the use of antibiotic resistance genes as selectable markers (Daniell et al., 2001). Alternatively, the green fluorescent protein (GFP) is a marker commonly used in laboratory transformations. This involves the insertion of GFP gene into the vector as reporter, allowing the transformed chloroplast to show fluorescent characteristics when exposed to darkness on the microscope (Cutolo et al., 2022; Kumar & Ling, 2021; Verma & Daniell, 2007).

1.10. Synthetic biology and bioinformatic analyses

The term “synthetic biology” first appears in 1980, when Barbara Hobom introduced it to characterize bacteria that had been genetically modified using recombinant DNA techniques (Hobom, 1980). These bacteria are living organisms originally biological in nature, but that have been modified through human intervention, making them synthetically modified in the end (Benner & Sismour, 2005). Synthetic biology uses engineering techniques when working with living systems and organisms, with the purpose of generating artificial life or creating replaceable components derived from natural biological components (Heinemann & Panke, 2006). This technology can operate at multiples scales, from proteins to complete organs. In this context, the 20 amino acids can be seen as fundamental components, while protein design algorithms and protein structure databases act as tools for simulations and bioinformatics, resulting in macromolecules that have new biological functions (Serrano, 2007). Synthetic biology has a wide range of uses, including therapeutics, biosensing, as well as the development of biofuels, pharmaceuticals and new innovative biomaterials (Garner, 2021). Particularly in the field of therapeutics, these developments are contributing to understanding disease mechanisms, identifying drug targets and innovating the production and delivery of new therapeutic solutions (Garner, 2021; Khalil & Collins, 2010).

On the other hand, a therapeutic target is a specific molecule, frequently a protein or nucleic acid, associated with a disease and that is capable of being altered by a drug or a therapy to achieve a specific result (Tian et al., 2022). In synthetic biology, the identification of these target molecules is very important particularly in applications such as drug development of innovative treatments (Xie et al., 2020). In aquaculture, the identification of therapeutic targets is focused not only on disease control, but also in the development of sustainable solutions that can include specific pathogens or disease agents (Bondad-Reantaso et al., 2023). Strategies to reach these targets involve multiple approaches, including alternative treatments, vaccines, or the adoption of sustainable feeding options (Schar et al., 2020). Moreover, the term gene therapy refers to the introduction of genomic materials straight into specific cells, with the purpose of producing therapeutic impact by rectifying pre-existing anomalies or by introducing new cell functions (Stone, 2010).

Framed into these paradigms, the introduction of foreign genes (therapeutic targets) into the chloroplast makes it simple to place foreign DNA in specific locations through homologous recombination (Doron et al., 2016). Particle bombardment or biolistic is the main method of direct transfer of genetic material and consists of projecting DNA-coated metallic

particles through the cell wall and membrane (Christou, 1995; Kumar & Ling, 2021). Usually, the metal particles are coated with plasmid DNA and this guarantees that all the components of the vector, including the transgene and the plasmid backbone, have an equally chance of being integrated into the genome (Kohli et al., 2003). Nonetheless, understanding the processes that control the efficiency of protein translation are a major challenge for fields like proteomics, computational biology, and biotechnology (Navon & Pilpel, 2011).

One method that is often applied to improve protein production is codon optimization. This is possible due the redundancy of the genetic code, which allows most amino acids to be represented by multiple synonymous codons which has a significant impact on protein expression levels (Mauro, 2018). Codons which code for the same amino acid are known as synonymous codons (Fu et al., 2020). The codon serves as the fundamental unit connecting nucleic acids that carry genetic information to proteins, acting as the primary link for information transfer within living organisms (Fu et al., 2020). Several software programs use codon optimization algorithms, incorporating different factor, for example, DNA Works (Hoover & Lubkowski, 2002), Optimizer (Puigbo et al., 2007), GeMS (Jayaraj et al., 2005), Gene Designer, Gene Designer Synthetic, as well as services provided by ThermoFisher and Genewi (Fu et al., 2020).

The regulation of gene expression includes a crucial aspect: the control of the translation (Valencia-Sanchez et al., 2006). Translation is the mechanism by which the information provided by a mRNA template is translated to produce an amino acid sequence in a polypeptide (Mauro, 2018) In the translation process, the mRNA is translated on the basis of the genetic code that determines the correspondence between the sequence of DNA and the sequence of amino acids in proteins (Cooper & Adams, 2022). Genetic sequences have been originally recognized by the presence of open reading frames (ORFs), which are potential regions to be translated into proteins (Couso & Patraquim, 2017). In the translation process, the ribosome reads sequentially sets of three nucleotides, which are known as triplets, and the reading frame for a specific coding sequence is determined by the start codon, normally AUG (Wan et al., 2018). Thus, an Open Reading Frame 1 (ORF1) is a DNA segment that is characterized by starting from the first start codon in the sequence and a stop codon (Pinter et al., 2021; Wan et al., 2018).

2. Objectives

Once all this information has been exposed, which serves as a conceptual framework to thoroughly explain the background that justifies and supports this dissertation, the two main objectives of this dissertation are:

1) Assess the effect of dietary *E. gracilis* extracts on the performance, immune response, oxidative stress and disease resistance of European seabass (*D. labrax*) juveniles.

2) The development of a vector to be used for the chloroplast transformation of *T. suecica*. Such genetically modified microalgae would be capable of expressing an antigen (sphingomyelinase) against the bacterial pathogen *T. maritimum*. Therefore, the transgenic *T. suecica* strain generated would be used as platform for oral delivery vaccination strategies.

This proposal combined a research strategy designed to explore the links between functional nutrition and immune plasticity, a complex issue relevant to animal production that is receiving increasing attention. Especially in the early stages of life, the immunity defenses and oral vaccination become of great importance.

In this work, we first find a theoretical framework of the techniques and processes involved throughout the study. An in-depth review of the study of the bacterium *T. maritimum* has also been carried out. The methodology used will be presented, as well as the detailed laboratory procedures. Finally, the results will be presented and discussed.

Chapter I

The effect of different compounds from *E. gracilis* on the performance, immune response, oxidative stress and disease resistance of European seabass (*D. labrax)* juveniles

Various solutions have been applied to control the infectious diseases that have appeared in aquaculture facilities, such as the use of antibiotics (Alderman & Hastings, 1998). However, these solutions have raised serious environmental and food safety concerns, as is the case with the use of antibiotics. It is therefore necessary to create approaches that are safe for human health and the environment and that also promote market acceptance (Nagappan et al., 2021). Microalgae have been studied as a potential bulk additive in feeds for farmed fish (Bahi et al., 2023).

Therefore, this chapter aimed to understand the effect of *E. gracilis* extracts in the diet on the performance, immune response, oxidative stress and disease resistance of juvenile sea bass at 24 h following infection with *T. maritimum.*

1. Materials and Methods

1.1. Experimental design

The research was conducted partly at the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) and the Abel Salazar Institute of Biomedical Sciences (ICBAS). European seabass juveniles, provided by Écloserie de Gravelines (France), were subjected to a two-week quarantine period. This period of acclimatization was closely monitored by CIIMAR's Bioterium of Aquatic Organisms (BOGA), assuring ideal conditions for the health and well-being of the animals before they were used in the experimental trials. The fish were then transferred to ICBAS bioterium facilities, place where the trials were to

be conducted. A group of 675 fish (with an initial average weight 10.7±1.2 g) were individually weighed and then placed in 15 recirculation tanks, each containing 100 L of sea water. Water parameters, such as oxygen saturation (>95%), salinity (35.12 ± 0.07 ppm), and pH (7.37 ± 0.05), were measured and monitored daily. The temperature was maintained at 18.05 ± 0.04 °C, and ammonium and nitrite levels were constant (<0.05 mg/L for NH_4 and 0.5 mg/L for NO_2).

1.2. Experimental diets and feeding trial

Fish were fed *ad libitum,* twice a day, during quarantine and acclimatization for 1 week with a control commercial diet (CTR) to adapt the experimental conditions. After this period, five experimental diets, including the CTR diet, were tested in triplicate, being randomly assigned to each tank. The feeding trial lasted for 7 days. Diets were produced by SPAROS Lda. (Olhão, Portugal), and consists of different proportions of *E. gracilis* subjected to high-pressure homogenization (HPH), or in the form of different fractions following a biorefinery process, that were added to the control diet **(Table 1)**.

The HPH process breaks the cell walls of microalgae and promotes the secretion of their internal components, including β-glucans (Yong et al., 2017). As previously mentioned, β-glucans are known polysaccharides with antioxidant and immunostimulant properties (Skov et al., 2012). The residue that results from the HPH process also contains a quantity of *E. gracilis* proteins. This residue is subjected to an enzymatic hydrolysis process using the enzyme alcalase, which breaks down the proteins into amino acids, facilitating the extraction of the microalgae proteins (Ellaiah et al., 2002).

The G1 and G2 diets consisted of adding 0.5% and 2.5%, respectively, of *E. gracilis* subjected to HPH to the control diet. The J1 and J2 diets consisted of adding different fractions of the microalgae to the control diet. Unlike the G1 and G2 diets, the J1 and J2 diets are not composed of *E. gracillis* subjected to HPH, but of two different extracts. *E. gracilis* was subjected to a hydrolysation process with alcalase treatment and the aqueous fraction was collected (i.e., F4) whereas its residue rich in carbohydrates was also recovered as fraction F5. Therefore, diets J1 and J2 consisted of adding 0.117% and 0.390% of the F5 extract, and 0.033 % and 0.110% of the F4 extract to the control diet, respectively.

Table 1- Composition of the experimental dietary treatments used in the bacterial infection trials on European sea bass.

Diet	Composition
CTR	Commercial diet
G1	*Euglena gracilis* HPH (0.5%)
G2	*Euglena gracilis* HPH (2.5%)
J1	*Euglena gracilis* F5 (0.117%) + F4 (0.033%)
J2	*Euglena gracilis* F5 (0.390%) + F4 (0.110%)

1.3. Bacteria preparation and inoculation procedure

T. maritimum (strain ACC13.1) serotype O3, provided by Professor Alicia E. Toranzo (Department of Microbiology and Parasitology, Faculty of Biology, University of Santiago de Compostela, Spain) and previously isolated from Senegalese sole (*Solea senegalensis*), was cultivated in Erlenmeyer flasks containing 50 mL of marine broth and incubated with constant shaking at 25 °C for 48 h. Subsequently, the flask contents were transferred to 50 mL tubes and centrifuged for 10 min at 3.500 x g. The supernatants from the centrifuged tubes were then discarded and the pellet suspended in marine broth. The bacterial concentration was measured at 600 nm (OD_{600}) and adjusted to 1 x 10^4 CFU/mL.

1.4. Bacterial challenge

After the feeding trial, 25 fish from each tank were infected by bath with a suspension containing 1 x 10^4 CFU/mL for 2 h. To assure that fish mortalities were due to infection process, 10 fish were sham infected with marine broth. Furthermore, isolation of bacteria was performed from moribund fish's head-kidney.

1.5. Sampling methodology

Both infected and non-challenged groups were sampled immediately before infection (Non-Challenged) and 24 h after challenge (Infected). At each sampling point, 5 fish per tank (n=15) were euthanized under an overdose of anesthetic by immersion in 2-phenoxy-ethanol (1500 ppm). Afterwards, blood collection was performed by caudal vein puncture using 1mL heparinized syringes (2500 I.U., Braun; 25 G, 1 mL). Collected blood was transferred to 1.5 mL heparinized tubes and homogenized for future hematological analysis. The remaining blood was centrifuged for 5 min at 14,000 x g and room temperature. After centrifugation, the plasma was collected and stored at -80 ºC. Liver was also extracted and stored in RNALater® (at a ratio of 1/10 p/V), 4 ºC for the first 24 h and then stored at -80 ºC.

1.6. Hematological analysis

Blood from 3 fish per tank (n=9) was sampled from the non-challenged and infected animals. To carry out total white blood cell counts (WBC) and red blood cell counts (RBC), hematocrit (Ht) and hemoglobin (Hb) determination (SPINREACT kit, ref. 1001230, Spain), a small portion of blood was taken before being centrifuged. Subsequently, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were calculated. The formulas used were as follows:

$\underline{\text{MCV } (mm^3)} = (Ht/RBC) \times 10$

$\underline{\text{MCH (pg/cell)}} = (Hb/RBC) \times 10$

$\underline{\text{MCHC (g/100 mL)}} = (Hb/Ht) \times 100$

A drop of heparinized blood was used to make the smears, which were left to air dry. After, the slides were fixed with formaldehyde-ethanol (90% absolute ethanol to 10% 37% formaldehyde) for 1 min (Kaplow 1965). The neutrophils were stained for peroxidase, following the method described by Afonso et al. (1998). The slides were observed under oil immersion (1000 x) after being stained with Wright's dye (Haemacolor, Merck). After, different leukocytes populations were counted until a total of 200 cells were achieved. Leukocytes cell types evaluated were neutrophils, monocytes, lymphocytes and thrombocytes. Finally, the relative counts were later converted in absolute values ($\times 10^4$/mL) of each cell type, using differential leukocyte cell counting results.

1.7. Innate humoral parameters

Innate humoral parameters are components of innate immunity and are present in the blood of fish. They constitute the first protective barrier in the case of infection (Shishido et al., 2012). Humoral parameters, inherent to plasma, were tested for lysozyme, peroxidase, and anti-protease activity. All assays were performed in triplicate and absorbance measurements were performed using the Synergy HT microplate reader available from Biotek.

Lysozyme activity: Lysozyme is an enzyme that plays a role in innate immunity with a high antibacterial effect. The principal function of lysozyme is to destroy bacterial cells by breaking down the peptidoglycans that are present in the bacterial cell wall. This leads to the death of the bacteria (Nawaz et al., 2022).

The assessment of lysozyme activity was performed using the EnzChek® Lysozyme Assay Kit (E-22013, Thermo Fisher Scientific) from Molecular Probes. *Micrococcus lysodeikticus* cells were used as the substrate. The plasma samples were diluted in 0.1 M phosphate buffer solution, pH 7.4, in a ratio of 1:5. The diluted samples were then mixed with 50 µL of substrate working suspension, which was labelled with fluorescent lysozyme from *M. lysodeikticus*. The samples were kept at 37°C for 30 min in the dark and the fluorescence of all the samples was recorded. The maximum absorbance and fluorescence emission of the digested substrate product were recorded at 494 nm and 518 nm, respectively. The activity was quantified in units of activity per mg of protein (U/mg). The quantity of lysozyme present in the plasma was calculated by replacing the absorbance value in the equation of the standard curve.

Peroxidase activity: Peroxidase transform the hydrogen peroxide in water and acts as a barrier to protect the organism from oxidative stress (Nóbrega et al., 2023).

To evaluate the peroxidase activity, it was used the method described by Quade and Roth, (1997) with slight modifications. In 96-well flat-bottom plates, 15 µL of the plasma samples were diluted in 135 µL of Hank's balanced salt solution without calcium and magnesium. After this, to each well was added 20 mM of tetra-methyl-benzidine solution (TMB; Sigma) in ddH_2O (double-distilled water) until reach a final volume of 50 µL, followed by the addition of 50 µL of 5 mM hydrogen peroxide, H_2O_2 (Sigma). To stop the reaction, after 2 min, it was added 50 µL of H_2SO_4 2 N (Sigma), while for the blank, the 15 µL of plasma were replaced with 15 µL of 0.1 mM phosphate buffer at a pH of 7.4. All absorbances were read at 450 nm. Peroxidase activity was estimated based on the assay and expressed in units/mL of plasma based on the change in absorbance of OD by one unit.

Anti-protease activity: As a result of bacterial infections, the immune response in the body also results in an increase in proteases such as trypsin, which break down bacterial proteins. If not regulated, these proteases can cause tissue damage. The organism, under normal circumstances and during infections, releases trypsin inhibitors (such as alpha-1-antitrypsin) to regulate the activity of trypsin and other proteases (Culp & Wright, 2017).

Anti-protease activity protocol was carried out as described by Machado et al., (2015). Plasma aliquots of 5 µL were mixed with 10 µL of trypsin solution (0.5% Sigma). Then, the samples were incubated at room temperature for 10 min. Subsequently, it was added to all samples 125 µL of azocasein (2% Sigma in 60 mM $NaHCO_3$) and 105 µL of PBS (Phosphate-buffered saline) . The resulting mixture was incubated for 1 h. To stop the reaction, 250 µL of 10% TCA was added to all samples. The samples were left to incubate at room temperature for 30 min. The samples were then vortexed and centrifuged at 10,000 x *g* for 5 min. Subsequently 100 µL of the sample supernatant was pipetted into a 96-well plate. Then, 100 µL of NaOH (1N) was added to each well. For the blank, plasma and trypsin were replaced with phosphate-buffered saline, while the reference sample replaced plasma with phosphate-buffered saline. All samples were read at 450 nm. The percentage inhibition of protease activity and trypsin activity is calculated by comparing the absorbance of the samples, the blank and the reference samples, using the following formula:

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

% inhibited trypsin = 100 - % non-inhibited trypsin

1.8. Homogenization procedure

Before carrying out oxidative stress analysis, all liver samples were homogenized. Liver sections were extracted from each animal and placed in individual microcentrifuge tubes. To these tubes, K-phosphate buffer (0.1 M pH 7.4 (K_2HPO_4 + KH_2PO_4, Sigma) in proportion of 1 to 10 was added. The homogenization process was conducted using sonication technique, sample by sample until reaching the visual homogeneity of the sample. An aliquot of 200 µL of the homogenate was created for LPO analysis. The rest of the volume was centrifuged at 10,000 × g for 20 min (4 °C). After centrifugation, aliquots were carefully collected and stored at -80 ºC for later analysis (1 per analyses). All assays were

performed in triplicate, and the absorbance readings were performed using a Synergy HT (Biotek) microplate reader.

1.9. Protein concentration

For the assessment of the activity of certain enzymes it is necessary the precise determination of the total protein concentration in the samples. The colorimetric test was performed using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, catalog numbers 23225 and 23227). First, a total of 10 µL of the samples was pipetted into K phosphate buffer (0.1 M; ph 7.4) at different dilution factors: 1:50, 1:40, 1:30, 1:20 and 1:10. Then, 25 µl of each diluted sample was transferred to a 96-well plate. Subsequently, 200 µL of the reaction buffer was added to each well. For the measure of absorbance, it was used Synergy HT microplate reader (Biotek), ate 562 nm. Bovine serum albumin curve (BSA, starting at 2 mg/mL) was used as a calibration standard.

1.10. Oxidative stress

Oxidative stress parameters measured were total protein, lipid peroxidation (LPO), catalase (CAT), reduced glutathione (GSH) vs oxidized glutathione (GSSG) Ratio, and superoxide dismutase (SOD) activities. A total of 15 fish per treatment were evaluated.

LPO: During bacterial infection, the body produces reactive oxygen species (ROS). This ROS, attack the unsaturated lipids in cell membranes, provoking lipid peroxidation (Vinagre et al., 2012). The assessment of LPO is based on the principle that the oxidative degradation of lipids leads to the formation of malondialdehyde (MDA). The MDA forms a pink substance when reacts with thiobarbituric acid (TBA) (Pérez-Jiménez et al., 2009).

The lipid peroxidation was performed as described by Peixoto et al. (2021), with very few modifications. Initially, 100 µL of cold 10% TCA was added to 200 µL of the homogenized samples. Following this, 500 µL of 0.73% thiobarbituric acid (TBA) solutions containing 2-Tiobarbituric acid at a concentration of 5mM, 60mM Trizma hydrochloride (Tris-HCl) and 0.1mM diethylenetriaminepentaacetic acid (DTPA) were added. The samples were incubated for 60 min at 100 ºC. Centrifugation was carried out for 5 min at 11,500 x g. Finally, 200 µL of the supernatant was transferred to a 96-well plate. The absorbance was read at 535 nm and the results were expressed in nmol TBA/g (w/w).

Catalase activity: During a bacterial infection, as previously mentioned, it occurs an increase of the production of ROS, such as hydrogen peroxide H_2O_2. The catalase is an antioxidant enzyme that has the purpose of catalyzingthe transformation of hydrogen peroxide into water and oxygen, protecting the organism's cells (Bonnichsen et al., 1947).

The assessment of catalase activity was performed following the methodology described by Claiborne et al. (1985). First, all the samples were diluted in K-phosphate buffer, (0.1 M; pH 7.4). to a protein concentration of 0.7 mg/mL. Then, 10 µL of each sample were pipetted into a UV microplate. At each well it was added 140 µL of K-phosphate buffer (0.05 M; pH 7, Sigma). Following this, 150 µL of the reaction mixture containing K-phosphate buffer (0.05 M; pH 7.0) and H_2O_2 (30%, Sigma) was added to each well. The absorbance was read at 240 nm for 2 min, with readings taken at 15 sec intervals. For the blank assay, the samples were replaced with K-phosphate buffer (0.1 M, pH 7.4). Catalase activity was expressed as µmol of substrate broken down per minute per milligram of protein (µmol/min/mg PTN).

GSH/GSSG Ratio: The GSH neutralizes de ROS and protects the cells against the oxidative damage, during an infection. The increase of oxidative stress, which leads to an

increase in ROS, results in the oxidation of GSH into GSSG. To keep the homeostasis and antioxidant defenses, the GSSG is converted again in GSH. So, the relationship between these two molecules is important to check the state of oxidative stress (Zitka et al., 2012).

The GSH/GSSG ratio was determined by microplate assay using the GSH/GSSG (reduced/oxidized glutathione) kit (GT40, Oxford Biomedical Research, Oxford, UK). For GSH, 50 µL of each sample was mixed with 350 µL of cold 5% metaphosphoric acid (MPA). It was centrifuged for 10 min at 1.000 x g at 4 ºC. Separately, 25 µL of the supernatant was added to 1.5 mL of assay buffer. For GSSG analysis, 50 µL of the samples were added to 15 µL of thiol remover and incubated at room temperature for 10 min. Subsequently, 135 µL of 5% MPA was added. The samples were centrifuged at 1,000 x g at 4 ºC for 10 min. Then, 25 µL of the supernatant was added to the test cap. After obtaining the GSH and GSSG samples, 50 µL of each were added to the microplate wells along with 50 µL of DTNB, 50 µL of GR and 50 µL of NADPH. The absorbance was read at 412 nm for 10 min (1 reading every 60 sec). A standard curve was prepared using an assay buffer and a standard stock of GSSG. The GSH/GSSG ratio was calculated by dividing the difference between the concentrations of GSH and GSSG (µM).

SOD activity: SOD is an antioxidant enzyme that catalyzes the dismutation of superoxide anion (O_2-), a reactive oxygen species, into oxygen (O_2) and hydrogen peroxide (H_2O_2), protecting cells from oxidative damage (McCord, 1999). This protocol followed the guidelines described in the protocol by Teixeira et al. (2022), with slight modifications.

Firstly, the concentration of the samples was adjusted to a protein concentration of 0.3 mg/mL using K-phosphate buffer (0.1 M, pH 7.4). Then, 50 µL of the diluted samples were transferred to 96-well flat-bottom plates. To each well was added 200 µL of the reaction solution (0.7 mM xanthine dissolved in 1 mM NaOH, combined with a 0.029 mM cytochrome C solution in 0.05 M Na-phosphate buffer, pH 7.8, containing 1 mM Na-EDTA, 1:10 v/v, Sigma) and 50 µL of xanthine oxidase solution (0.03 U/mL in 0.1 mM Na-EDTA, Sigma). For the blank, K-phosphate buffer (0.1 M, pH 7.4) was used instead of the samples. The progress of the reaction was monitored by measuring the formation of superoxide anion at 550 nm, at 20-sec intervals, for 3 min. SOD activity is indicated in units per mg of total protein (U/mg protein).

1.11. Statistical analysis

For the statistical analysis, the mean and standard deviation were calculated for each diet and time group (Non-Challenged and Infected). All statistical procedures were carried out using the IBM SPSS 29 for WINDOWS program. Firstly, it was performed a screening of the data for the removal of outliers. After, data was analyzed for normality and homogeneity of variance, which are the basic principles for a parametric analysis with ANOVA. Normality was checked using appropriate tests, and homogeneity of variance was assessed using Shapiro Wilk. Initially, two-way ANOVA was used to assess whether the different treatments could affect the parameters evaluated. When two-way ANOVA indicated significant differences between the diets, a one-factor post hoc test was carried out to identify which groups differed from each other. The significance level adopted was $p \leq 0.05$ for all statistical tests.

When the data did not meet the requirements of normality or did not respect the parameters of kurtosis and asymmetry, non-parametric tests were performed. Specifically, the Kruskal-Wallis test was used to compare the diet groups and the interaction between diets and infection time. This non-parametric test is appropriate for data that does not follow a normal distribution and allows differences between multiple groups to be assessed.

2. Results

2.1. Descriptive statistics

The descriptive statistics (mean values and standard deviations) of all evaluated parameters can be seen in the following tables **(Table 2, 3 and 4).** Two groups were evaluated, one non-challenged and another Infected, subjected to 5 different diets.

Table 2. Total white blood cells (WBC; $\times 10^4/\mu L$), red blood cells (RBC; $\times 10^6/\mu L$), Hematocrit (ht; %), Hemoglobin (hb; g/dL), mean corpuscular volume (MCV; μm^3) , mean corpuscular hemoglobin (MCH; pg $cell^{-1}$), and mean corpuscular hemoglobin concentration (MCHC; g 100 ml^{-1}) in sea bass in the different experimental groups (CTR, G1, G2, J1, and J2) in the non-challenged fish (NC) and in the infected fish (INFECTED).

	CTR		G1		G2		J1		J2	
	NC	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**
WBC	3.61 ± 1.14	3.31 ± 1.33	3.93 ± 0.67	2.56 ± 1.03	2.78 ± 1.57	2.56 ± 1.03	3.13 ± 0,80	3.68 ± 1.03	3.16 ± 1.34	2.97 ± 0.85
RBC	1.14 ± 0.29	1.58 ± 0.29	1.30 ± 0.29	1.37 ± 0.23	1.29 ± 0.32	1.47 ± 0.27	1.31 ± 0.24	1.48 ± 0.31	1.24 ± 0.31	1.37 ± 0.40
Ht	16.25 ± 3.15	18.29 ± 2.44	17.88 ± 2.77	19.56 ± 4.50	17.22 ± 4.57	17 ± 3.83	15.22 ± 2.35	18.75 ± 2.61	16.38 ± 2.45	20.50 ± 4.64
Hb	2.17 ± 0.14	2.28 ± 0.23	2.26 ± 0.18	2.26 ± 0.11	2.16 ± 0.12	2.43 ± 0.28	2.06 ± 0.16	2.34 ± 0.14	2.32 ± 0.11	2.35 ± 0.30
MCV	22.73 ± 12.42	35.28 ± 33.94	23.23 ± 13.59	14.15 ± 5.48	18.85 ± 13.19	23.81 ± 11.93	20.59 ± 13.60	23.04 ± 15.16	16.73 ± 7.96	19.33 ± 10.94
MCH	7.63 ± 2.69	9.66 ± 4.93	7.74 ± 3.64	8.30 ± 4.09	8.29 ± 5.21	8.78 ± 4.09	7.99 ± 4.10	9.14 ± 4.62	7.89 ± 4.94	9.60 ± 5.13
MCHC	34,64 ±11.64	57.79 ± 31.48	34.66 ± 9.62	62.85 ± 25.55	65.35 ± 44.65	44.17 ± 16.95	43.61 ± 9.83	47.81 ± 12.82	48.51 ± 26.99	52.24 ± 17.58

*Values are presented as mean ± standard deviation (n = 9)

Table 3- Plasma immune parameters: Anti-protease activity (%), Peroxidase activity (units' mL $^{-1}$) and Lysozyme activity (units' mL $^{-1}$) in seabass in the different experimental groups (CTR, G1, G2, J1, and J2) in the non-challenged fish (NC) and in the infected fish (INFECTED).). Different lowercase letters represent significant differences between different diets in non-challenged animals, while different uppercase letters represent differences between different diets in infected animals. The symbols represent differences in the same diet between different sampling groups, where "#" symbolizes higher activity compared to " * ". Kruskal-Wallis non-parametric test ($p<0.05$).

	CTR		G1		G2		J1		J2	
	NC	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**
Antiprotease	23.47 ± 8.76	38.58 ± 17.47	39.99 ± 21.11^{a}	67.43 ± 14.87^{A}	17.88 ± 5.07^{b}	45.28 ± 21.75	33.17 ± 20.75	29.11 ± 16.60^{B}	34.97 ± 16.81	43.5 ± 18.80
Peroxidase	96.83 ± 13.33	58.92 ± 47.36^{A}	39.99 ± 21.12	5.55 ± 4.30^{B}	94.92 ± 28.95$^{\#}$	21.97 ± 24.49^{*AB}	86.90 ± 21.14	53.90 ± 18.64^{AB}	63.76 ± 14.56	34.08 ± 17.80^{AB}
Lysosyme	133.45 ± 53.57	164.11 ± 73.42	39.99 ± 21.13	140.94 ± 64.14	199.34 ± 89.52	183.11 ± 80.88	137.32 ± 53.93	150.11 ± 46.88	166.69 ± 61.45	137.6 ± 57.12

The values (means ± SD) were calculated by dividing the value of each parameter of the fish challenged at the different times by the average value of the fish sampled (n=15).

Table 4- Hepatic oxidative stress parameters: lipids peroxidation (LPO; nmols TBA g tissue $^{-1}$), catalase activity (CAT; U mg prot^{-1}), superoxide dismutase activity (SOD; U mg prot-1), and reduced glutathione: oxidized glutathione (Ratio), in the different experimental groups (CTR, G1, G2, J1, J2) in the non-challenged fish (NC) and in the infected fish (INFECTED). Different lowercase letters represent significant differences between different diets in non-challenged animals, while different uppercase letters represent differences between different diets in infected animals. The symbols represent differences in the same diet between different sampling groups, where "#" symbolizes higher activity compared to " * ". Kruskal-Wallis non-parametric test ($p<0.05$).

	CTR		G1		G2		J1		J2	
	NC	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**	**NC**	**Infected**
LPO	55.08 ± 6.16^{*b}	68.59 ± 9.87$^{\#B}$	55.71 ± 5.03^{*b}	97.17 ± 37.50$^{\#AB}$	72.78 ± 12.83^{a}	111.77 ± 9.23^{A}	61.29 ± 6.53^{ab}	65.91 ± 10.17^{B}	62.61 ± 9.23^{ab}	64.76 ± 8.37^{B}
CAT	85.73 ± 38.80	74.74 ± 8.46	82.22 ± 33.57	49.74 ± 42.39	104.24 ± 35.11	75.10 ± 24.96	102.58 ± 11.19	80.67 ± 21.29	105.80 ± 22.72	79.12 ± 16.15
SOD	34.42 ± 34.14^{b}	48.24 ± 20.08	45.23 ± 37.19^{ab}	53.70 ± 19.94	39.34 ± 11.68^{ab}	45.46 ± 15.48	59.39 ± 15.38^{a}	59.39 ± 15.37	50.41 ± 21.36^{ab}	45.44 ± 34.24
Ratio	343.70 ± 96.14	144.06 ± 59.18	371.15 ± 116.87$^{\#}$	185.54 ± 82.84*	631.87 ± 348.05$^{\#}$	149.50 ± 105.58*	285.72 ± 266.615	211.62 ± 67.00	266.61 ± 61.64$^{\#}$	139.411 ± 77.90*

The values (means ± SD) were calculated by dividing the value of each parameter of the fish challenged at the different times by the average value of the fish sampled (n=15)

2.2. Hematological analysis

The hematological parameters assessed include WBC, RBC, hematocrit, hemoglobin, MCV, MCH, and MCHC. Results obtained in the Two-way ANOVA test and Kruskal-Wallis non-parametric test, are presented in the **Table 5**, considering the factors: Infection, diet and the interaction of time of infection with diet.

There were no significant changes in MCV, MCH, or MCHC in any of the factors analyzed **(Table 5)**.

In relation to the differences obtained between the infected and non-challenged groups, it was found that the RBC values were lower in the non-challenged animals when compared to the infected group **(Fig. 4A)**. This result is in line with the values observed for hematocrit and hemoglobin, where there were significant differences between the different infection times **(Fig. 4B)**. The hemoglobin and hematocrit values varied at the different infection times, with higher values in the infected group.

Statistically, no significant differences were found in WBC in any of the factors analyzed in relation to hematological parameters **(Table 5)**. Despite the lack of statistically significant results, there was a visually decreasing tendency in WBC values in the infected group **(Table 2).** All the diets showed a decrease in leucocytes, except for the J1 diet. The G1 diet showed the highest tendency to decrease, with the most variation from the non-challenged group (3.93 ± 0.67) (×10^4/µL) to the infected group (2.56 ± 1.03) (×10^4/µL) **(Table 2)**.

Table 5- Evaluation of the role of diet and infection in altering hematological parameters: WBC, RBC, Ht, Hb, MCV, MCH, and MCHC (Two-way ANOVA). ANOVA: n.s.: non-significant differences (p>0.05).

Parameters	Time	Diet	Time x Diet
WBC	n.s	n.s	n.s
RBC	**0.005**	n.s	n.s
Ht	**0.004**	n.s	n.s
Hb	**0.003**	n.s	n.s
MCV	n.s	n.s	n.s
MCH *	n.s	n.s	n.s
MCHC *	n.s	n.s	n.s

* MCH and MCHC were statistically analyzed using Kruskal-Wallis non-parametric test: non-significant differences (p>0.05).

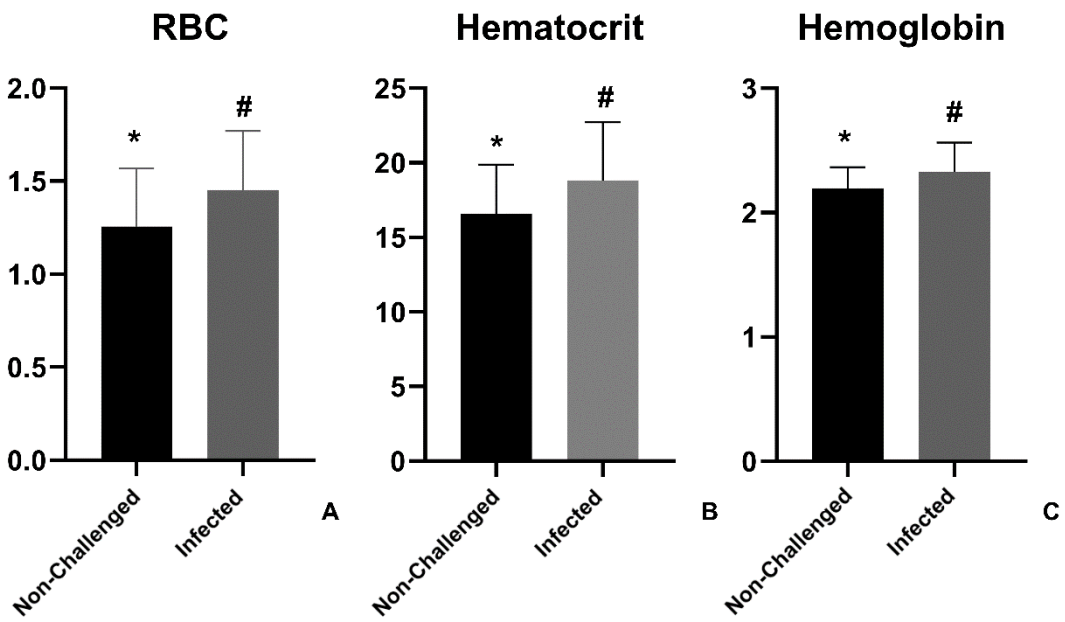

Figure 4. Levels of RBC (x10^6/µL) (A), Hematocrit (%) (B), Hemoglobin (g/dL) (C), at the different sampling times (Non-Challenged and Infected), showing the differences between infection times. Data are expressed as mean ± SD (n=45). The symbols represent differences between different sampling groups, where "#" symbolizes higher activity compared to " * ". Two-way ANOVA. ANOVA: n.s.: non-significant differences ($p>0.05$).

2.3. Innate humoral parameters

Immunological parameters assessed in plasma samples include anti-protease, peroxidase and lysozyme activities. The results are presented in **Table 6**, which shows the significance values obtained in the Kruskal-Wallis test. The results obtained for each of the parameters are detailed below, considering the factors infection, diet and interaction of time of infection with diet.

There were significant differences in peroxidase activity in all the treatment assessed. In relation to infection time, the non-challenged group had higher values than the infected group. Significant differences were also observed between the diets **(Table 6).** The results showed that, in the infected group, the CTR diet had significantly higher peroxidase values compared to the G1 diet **(Fig. 5D)**. In addition, there was a significant difference in the G2 diet at different times of infection, where higher peroxidase values were found in the non-challenged group **(Fig. 5D)**.

Statistical analysis of anti-protease activity revealed significant differences in all treatments **(Table 6)**. The anti-protease activity was found to be significantly higher in the infected group compared to the non-challenged group. In addition, significant differences were detected in the non-challenged group between the G1 and G2 diets ($p=0.041$), with the animals fed with the G1 diet exhibiting higher anti-protease activity **(Fig. 5E)**. Furthermore, in the Infected group, there were significant differences between the G1 and J1 dietary treatments ($p<0.001$) with the G1 diet again showing higher plasma anti-protease values **(Fig. 5E)**.

The analysis of lysozyme activity showed no significant differences in any of the factors evaluated **(Table 6)**.

Table 6- Evaluation of the role of diet and infection in altering immune parameters: Anti-Protease activity (%), Peroxidase activity (units mL^{-1}) and Lysozyme activity (units mL^{-1}) using the Kruskal-Wallis non-parametric test. Kruskal-Wallis n.s.: non-significant differences ($p>0.05$).

Parameters	Time	Diet	Time x Diet
Antiprotease activity	<0.001	<0.001	<0.001
Peroxidase activity	<0.001	0.003	<0.001
Lysozyme activity	n.s	n.s	n.s

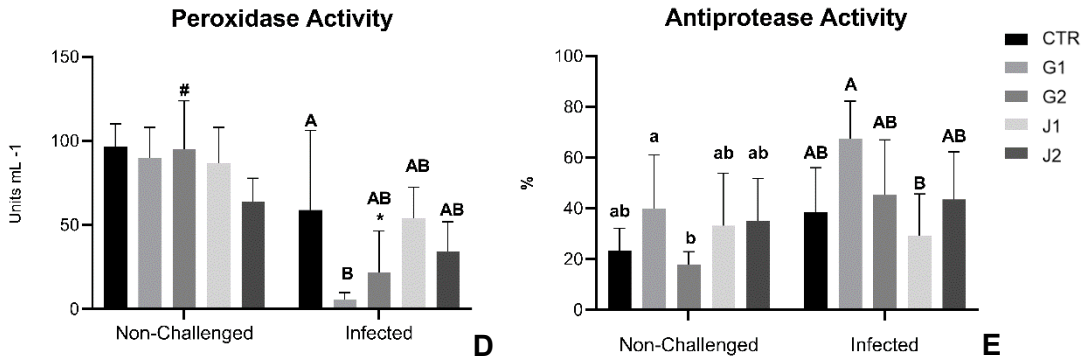

Figure 5. Peroxidase activity (D) and Anti-protease activity (E) at the different sampling times (Non-Challenged and Infected), demonstrating the differences in the diets (CTR, G1, G2, J1, and J2). Data are expressed as mean ± SD (n=5). Different lowercase letters represent significant differences between different diets in non-challenged animals, while different uppercase letters represent differences between different diets in infected animals. The symbols represent differences in the same diet between different sampling groups, where "#" symbolizes higher activity compared to " * ". Kruskal-Wallis non-parametric test ($p<0.005$).

2.4. Oxidative stress

Oxidative stress was evaluated using SOD, CAT, LPO, and the GSH/GSSG ratio. The results of the statistical differences are shown in **Table 7**, followed by the quantitative expression of the different parameters in the various diets and sample groups in **Fig. 6** and **Fig. 7**. The results obtained for each parameter are detailed below, considering the factors infection, diet and interaction of time of infection with diet.

The results obtained in the evaluation of catalase showed significant differences in the infection time factor, revealing significantly higher catalase levels in the non-challenged group compared to the Infected group. In addition, significant differences were found in the diet factor, with the G1 diet showing lower catalase activity compared to the G2, J1 and J2 diets **(Fig. 6)**. No significant interactions were found between time and diet.

In the LPO parameter, there was a significant difference in the time of infection factor. The Infected group presented a significantly higher LPO concentration when compared to the Control group. In addition, there were significant differences in the diet factor and the interaction between time and diet. Specifically, infected animals that were fed with the CTR diet showed lower LPO values than non-challenged animals ($p=0.036$) **(Fig. 7E)**. In the non-challenged group, the G2 diet was found to have significantly higher LPO values than the

CTR diet (p=0.010) and the G1 diet (p=0.010) **(Fig. 7E)**. In the Infected group, it was also found that the G2 diet had higher LPO levels compared to the J2 diet (p=0.002), the J1 diet (p=0.003) and the CTR diet (p=0.034) **(Fig. 7E)**. Finally, the G1 diet showed significantly higher values in the Infected group compared to non-challenged group (p<0.001) **(Fig. 7E)**.

For SOD activity, significant differences were found only in the interaction between infection time and diet. Specifically, in the non-challenged group, J1 showed significantly higher values compared to CTR diet (p=0.008) **(Fig. 7F)**. The factors diet and time of infection showed no significant differences.

The data obtained on the GSH/GSSG ratio showed significant differences in relation to infection time, with the non-challenged group showing a higher ratio compared to the Infected group. There were also significant differences in the interaction between diet and infection time. Specifically, the J2 diet showed a significantly higher ratio in the non-challenged group compared to the Infected group (p=0.044) **(Fig. 7G)**. It was found that the G2 and G1 diets in the non-challenged group had significantly higher values than in the Infected group (p<0.001 and p=0.021, respectively) **(Fig. 7G)**. No significant differences were found between the diets in isolation.

Table 7- Evaluation of the role of diet and infection in altering oxidative stress parameters: LPO (nmols TBA g tissue -1), CAT (U mg prot-1), SOD activity (U mg prot-1), GSH/GSSG ratio) using the non-parametric Kruskal Wallis test: n.s.: non-significant differences (p>0.05).

Parameters	Time	Diet	Time x Diet
LPO	<0.001	<0.001	<0.001
CAT *	<0.001	0.001	n.s
SOD	n.s	n.s	0.011
RATIO	<0.001	n.s	<0.001

*CAT activity was statistically analyzed using the two-way ANOVA test: non-significant differences (p>0.05).

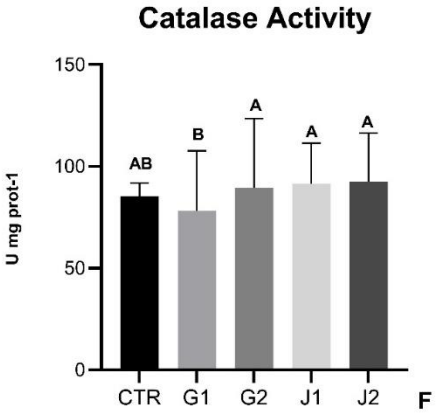

Figure 6. Hepatic catalase activity in the different diets (CTR, G1, G2, J1, and J2). Data are expressed as mean ± SD (n=10). Different capital letters represent differences between the different diets where "A" symbolizes higher activity compared to "B". Two-way ANOVA; Tukey post-hoc test; p≤0.05).

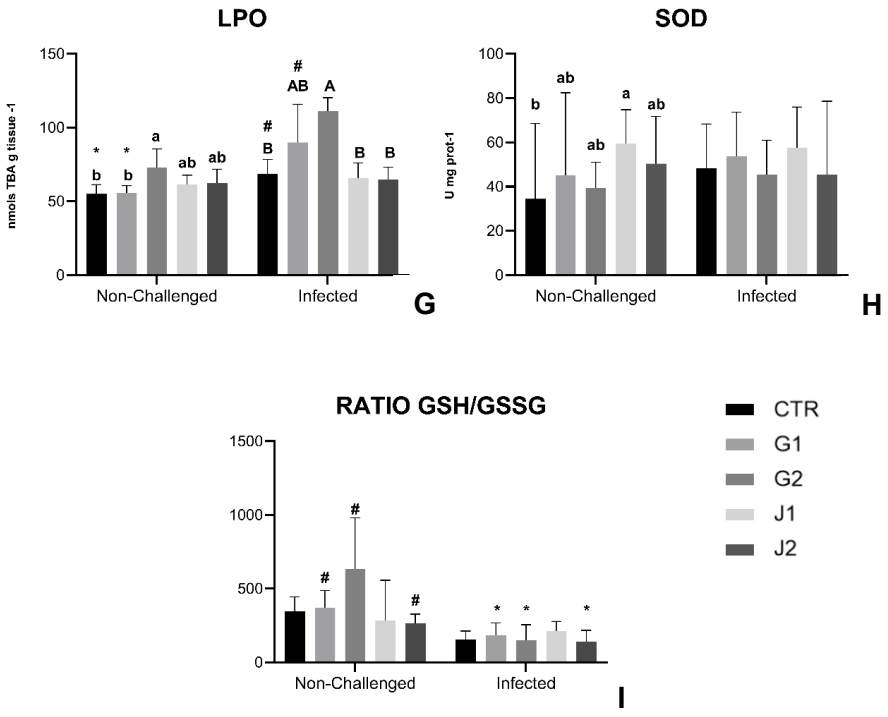

Figure 7. Hepatic LPO (G), SOD (H) and Ratio GSSH/GSG (I) at the different sampling times (non-challenged and Infected), demonstrating the differences in the diets (CTR, G1, G 2, J1, and J2). Data are expressed as mean ± SD (n=5). Different lowercase letters represent significant differences between different diets in non-challenged animals, while different uppercase letters represent differences between different diets in infected animals. The symbols represent differences in the same diet between different sampling groups, where "#" symbolizes higher activity compared to " * ". Kruskal-Wallis non-parametric test (p<0.005).

3. Discussion

In aquaculture industry, a proper nutrition plays an important role in the susceptibility of fish to bacterial infections. Diets over-fortified with specific nutrients can improve fish health and resistance to infection and diseases (Oliva-Teles, 2012). In this chapter, the impact of different diets with different extracts of *E. gracillis* was studied on the immune status of sea bass, and its capacity of response in the presence of a pathogen.

Evaluating hematological parameters is essential to understand the animal's immune response capacity, as well as, to understand the stressing pressure to which the fish are subjected. These indicators provide information on the animal's oxygenation capacity, anemia associated with infection or the host's compensation mechanisms (Docan et al., 2018). No statistically significant changes were found in WBC, MCV, MCH, or MCHC in any of the factor evaluated. These results suggest that neither the diet or the infection were capable to significantly affect the composition and immune response of sea bass (Fazio, 2019), at least under the conditions stablished in the present study. RBC values were higher in the group of infected animals. In accordance with these results, the hematocrit and total hemoglobin values were also significantly different prior and after infection, being higher in the Infected group. The inflammation that happens during a bacterial infection can cause a decrease in the availability of oxygen in the tissues. To compensate this hypoxia state, the organism can increase the production of red blood cells, leading to an increase in hemoglobin and hematocrit levels (Shen et al., 2018). This response happens as an effort of the organism to maintain homeostasis status. In addition, the damage caused by oxidative stress in the tissue and RBC can also be a factor that leads to this response.

Assessing the humoral parameters of the innate immune system during an infection is essential to understand the initial defensive responses of the fish. These studies provide important information on host's ability to neutralize pathogens and maintain homeostasis. Understanding the variations in these parameters is essential to understand animal's response to infection and dietary changes (Magnadóttir, 2006). Also, the evaluation of oxidative stress parameters is essential for assessing the susceptibility of fish to bacterial infections. During an infection, there may be an increase in the intracellular production of ROS, such as superoxide, hydrogen peroxide and hydroxyl radical (Chowdhury & Saikia, 2020). These ROS can cause oxidative damage to cells and tissues (Claiborne, 1985).

As previously mentioned, lysozyme is an important component of the innate immune system that hydrolyzes the peptidoglycan layer of bacterial cell walls (Shishido et al., 2012).

The stability of plasma lysozyme values during the study, despite the infection, suggests that this particular parameter was not altered by external factors such as infection or a change in diet, or it can also be deduced that the specific conditions of this study may not have been sufficient to detect a response at the level of this enzyme. For instance, a bath infection primarily affects mucosal tissues where myeloid cells (i.e., monocytes and neutrophils) migrate from blood to the infection site. Since phagocytes are the main source of lysozyme, it is tempting to speculate that lysozyme levels could have increased in skin mucus and not at systemic level.

In relation to peroxidase activity, the non-challenged group showed higher activity of these enzymes compared to the Infected group. Peroxidase facilitates the reduction of hydrogen peroxide to water and oxygen, which helps in the defense against oxidative stress (Nóbrega et al., 2023). Although the statistical results showed no differences in WBC, when analyzing the descriptive hematological data **(Table 2)**, it can be seen that, with the exception of the J1 diet, all the diets showed a tendency for WBC to decrease in the infected group. G1 diet showed the higher difference in values between non-challenged group and Infected group. These results suggest that the decrease in WBC may be related to the migration of neutrophils from the peripheral blood to the infection site (De Oliveira et al., 2016). Neutrophils are the first line of cellular defense to be recruited to the local of infection (Ellis, 1999). Myeloperoxidase is a type of enzyme present in these cells that produces reactive oxygen species, that will effectively react with the pathogen (Rizo-Téllez et al., 2022). A reduction in WBC values, specifically neutrophils, may suggest that they are migrating to the site of the infection. Therefore, the presence of neutrophils is associated with peroxidase activity (Metzler et al., 2011). This theory is also supported by the evident decrease in peroxidase in the G1 diet, which can be visually seen in **Fig. 5B**. From these results, the G1 diet seems to be promising, as it increases the migration of neutrophils to the site of infection, improving the animal's capacity to fight bacterial infections.

The results obtained for anti-protease activity are in line with the expected immune response, and can also be explained as a compensatory response, and an additional defense, as a result of neutrophil migration. Anti-proteases play an important role in balancing the immune response, by producing trypsin inhibitors, enzyme that degrade bacterial proteins (Culp & Wright, 2017). The results from this study showed that the infected group regulates the production of anti-proteases to protect host tissues from excessive damage during infection. The G1 diet showed higher anti-protease values than the G2 and J1 diets in the non-challenged and infected groups, respectively. This indicates that the G1 diet showed a more robust immune response and was more effective in promoting an immune response against bacterial proteases.

The SOD catalyzes the conversion of superoxide anions into molecular oxygen and hydrogen peroxide (McCord, 1999). It was found that in the non-challenged group, the J1 diet presented significantly higher values compared to the CTR diet. This suggests that in an initial phase, the J1 diet may present a greater capacity to neutralize free radicals, possibly due to the presence of antioxidant components obtained from *E. gracilis*, such as carotenoids (e.g., β-carotene) and β-glucans (Meena et al., 2013; Skov et al., 2012). The J1 diet therefore showed an initial ability to increase SOD activity, potentially improving the animal's ability to deal with oxidative stress associated with infection (Chowdhury & Saikia, 2020).

The results showed that the GSH/GSSG ratio was significantly higher in non-challenged group than in Infected group. As explained previously, during an infection GSH neutralizes ROS. The oxidation of GSH to GSSG, due to increased oxidative stress, leads to a need for GSSG to be converted back into GSH. The ratio between these two molecules indicates the state of oxidative stress in the body (Zitka et al., 2012). It can be concluded from these results that the non-challenged group sustained a more favorable intracellular redox balance, with higher antioxidant capacity and lower oxidative stress. In contrast, the Infected group, as expected, suffered an increase in the oxidation of GSH to GSSG due to the increased oxidative stress caused by the infection. Significant differences were observed between diets and infection time. Diets G1, G2 and J2 showed a significantly higher ratio in the non-challenged group compared to Infected group. This suggests that these diets, probably due to some of the compounds present in *E. gracilis*, may have provided a more favorable pro-oxidant environment in non-infected fish (Skov et al., 2012). Although the statistical results did not reveal significant differences between diets in the non-challenged group, by visualizing the graph it can be clearly seen that diet G2 presents higher values compared to the other diets. This may indicate that diet G2 may have a greater impact on the GSH/GSSG ratio, providing a higher level of free radicals due to non-specific immune activation, and therefore eventually causing some damage to the host. In fact, an increased in hepatic LPO was observed in this group suggesting tissue damage. .

Based on the results obtained, G1 diet seems to show the best results and seems to be the most effective in improving the immune response of sea bass. This diet showed the best results in terms of host protection, as can be seen from the higher anti-protease activity. In addition, the G1 diet suggested an increase in the migration of neutrophils to the site of infection, by reducing the levels of peroxidase and WBC, although there were no statistical differences in WBC.

4. Conclusion

This study investigated the dietary effects of different extracts obtained from *E. gracillis* on the immune response of European sea bass exposed to infection with the bacteria *T. maritimum*.

Analysis of hematological, plasma humoral parameters and hepatic oxidative stress markers provided information on the animals' immune responses and capacity to infection. The results obtained through hematological and biochemical analyses suggest that the G1 diet was the most promising, improving the fish's immune response during a bacterial infection. There was a greater migration of neutrophils to the site of infection in the animals supplemented with this diet. These results were supported by a reduction in peroxidase levels and a tendency for a decrease in the WBC count. There was also a significant increase in anti-protease in the G1 diet, thus, increasing host protection against proteases.

Despite these results, further studies are needed to better understand the impact of Euglena microalgae on the health of sea bass. Experiments with longer feeding periods may be useful to obtain clearer results. It is important to continue exploring the link between the nutritional value of microalgae and the improvement of fish health.

Chapter II

Design of a recombinant vector for the expression of sphingomyelinase in the chloroplast of *Tetraselmis suecica*

The principal aim of this section of the research was to develop a vector, using the plasmid pSM657 (Gutiérrez et al., 2020), harboring the sphingomyelinase gene sequence (codon optimized and in the correct ORF), to produce this recombinant antigen against the bacterium *T. maritimum*. The recombinant vector, denominated pSM658 will be subsequently used in the process of chloroplast transforming the microalga *T. suecica*. The development of this vector involves a combination of conventional laboratory procedures and bioinformatics analyses to guarantee the precise ligation and expression of the gene (GenBank accession: NZ_CP020822.1:1548176-1549225**)** / protein (GenBank accession: WP_136438322.1) sphingomyelinase.

Expression strategy

The experimental procedures were conducted at the Aquatic Animal Health laboratory (A_2S) of the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) of the University of Porto, in Matosinhos. This methodology section has been structured in two distinct parts, comprising both experimental laboratory procedures, and bioinformatic analyses using different tools, particularly the Geneious Prime® 2023.2.1 software.

In this study, the expression vector pSM657 was selected as a tool for the transformation of the *T. suecica* chloroplast (Gutiérrez et al., 2020). This plasmid contains the *cat* (chloramphenicol resistance) and tGFP (turbo green fluorescent protein) genes as selection markers. At this point, the approach was to make a modification to pSM657 plasmid, specifically at the level of *cat* gene sequence. Such modification was carried out in order to replace *cat* with the gene of interest (sphingomyelinase of *T. maritimum*), and therefore, use the tGFP as selection marker, instead an antibiotic resistance strategy for the selection of the transformants of *T. suecica*.

The modification of the vector pSM657 involved a series of molecular biology techniques, including bioinformatics, and a subsequent verification of the integrity of the

recombinant vector. Each step of these procedures was conducted based on standard protocols, guaranteeing the precision and effectiveness of the genetic modifications carried out.

1. Materials and Methods: Target antigen selection and analysis

1.1. Bacterial strain and growth conditions

T. maritimum (strain ACC13.1) serotype O3 used in the present research was isolated from Senegalese sole (Avendaño-Herrera et al., 2005). The strain was provided by Professor Alicia E. Toranzo (Department of Microbiology and Parasitology, Faculty of Biology, University of Santiago de Compostela, Spain), and the stocks were kept frozen at -80 °C until their utilization. Frozen stocks were recovered using agar petri dishes at 25 °C for 48 h. For the inoculum, the bacteria were added to 50 mL of Marine Broth (MB) in a 500 mL Erlenmeyer flask. The mixture was incubated at 25°C and continuously agitated at 180 rpm for 48 h (Mabrok, 2016).

1.2. Target antigen selection and analysis

An important part of this research was focused in identifying potential antigens linked to virulence, with a particular interest in factors connected with pathogenicity and immunogenicity. Following an extensive review of literature, sphingomyelinase phosphodiesterase was identified as a potential factor of virulence and antigen associated with *T. maritimum* (Escribano et al., 2023; Mabrok et al., 2023).

The National Center for Biotechnology information (NCBI), specifically the NIH database, was used to obtain the protein sequence of sphingomyelinase (GenBank accession: WP_136438322.1) and also the complete genome of bacterium *T. maritimum* (GenBank accession: NZ_CP020822.1). The search was carried out on the NCBI GenBank, focusing on the genomic data of the TM- KORJJ chromosome of the *T. maritimum* strain (GenBank accession: NZ_CP020822.1). The complete genome sequence was extracted in FASTA

format to be analyzed by bioinformatics. Geneious Prime (Biomatters) software was used for detailed analysis of the gene sequence and for bioinformatics analysis throughout the process.

1.3. Primer design for sphingomyelinase detection

Primers were designed using the Geneious Prime software, and Primer-Blast provided by NIH, to amplify the region containing the sphingomyelinase sequence of *T. maritimum*. These primers were designed to bind to conserved regions of the gene, permitting selective amplification of the DNA fragment corresponding to the gene of interest during the PCR reaction. Primer selection was based on specific selection parameters. According to the recommendations of the studies carried by You et al. (2008) and Ye et al. (2012), it was possible to ensure the quality of an ideal primer pair for subsequent PCR amplification. According to these authors, the parameters defined for a good pair of primers are the following:

1) Primers should generally be 18-24 bases long. Longer primers can result in non-specific amplification.
2) The G/C content should ideally be between 40% and 60%.
3) The primers should start and end with 1-2 G/C pairs to improve the stability of the primers binding.
4) The melting temperature (Tm) of the primers should be in the range of 50-60ºC, and the Tm of the primer's pairs should be within 5ºC of each other to guarantee identical annealing temperatures.
5) Primers should not contain complementary regions to prevent the formation of primer-dimers or other unspecific products.

1.4. RNA extraction from *T. maritimum*

The bacterial cultures of *T. maritimum* were cultivated under the appropriate conditions until they reached the exponential growth phase. The bacterial cells were collected from the petri dish to a sterile tube aseptically with a sterile loop inside a UV chamber to maintain sterility. RNA extraction from *T. maritimum* was carry out following the protocol of

the Maxwell RSC simplyRNA Cells Kit. The quality and concentration of the extracted RNA were measured using a Thermo Fisher NanoDrop™ spectrophotometer (1020.374 ng/µL). The purified RNA was stored at -80 °C to preserve the integrity of the RNA for later analysis and to ensure long-term stability.

1.5. cDNA synthesis from *T. maritimum* RNA

After the extraction of total RNA from *T. maritimum*, cDNA was synthesized using the NZY First-Strand cDNA Synthesis Kit. This step was fundamental for converting messenger RNA (mRNA)into cDNA. The decision to extract RNA to subsequently synthesize cDNA (instead of directly extracting the genomic DNA of *T. maritimum* for PCR detection) is because in this way can be ensured the detection of the expression of the sphingomyelinase gene in the *T. maritimum* strain used in this study (ACC13.1; serotype O3). The quantity and quality of the synthesized cDNA was measured with Thermo Fisher NanoDrop™ spectrophotometer, providing information on the quantity and purity of the cDNA product. The synthesized cDNA was stored at -20 °C, to preserve its stability for subsequent analyses.

1.6. PCR amplification of *T. maritimum* sphingomyelinase

After the synthesis of cDNA from *T. maritimum* RNA, and the design of specific primers for the amplification of the sphingomyelinase gene, PCR was used to amplify the desired genomic region to confirm its presence. The purpose of this procedure was to confirm the expression of the sphingomyelinase gene in *T. maritimum* (ACC13.1; serotype O3).

Primers (P1F, P1R, P2F and P2R) were rehydrated using nuclease-free water, following the manufacturer's instructions, to reach a concentration of 100 µM (stock solution). The PCR reactions were prepared using the NZYTaq II 2x Green Master Mix Kit from NZYTech. For each PCR reaction, the following components were mixed: 1 µl of Primer F (10 µM), 1 µl of Primer R (10µM), 10 µl of nuclease-free water, 1 µl of the bacterial cDNA template (2424.157 ng/µl) and 12 µl of NZYTaq II 2x Green Master Mix Kit from NZYTech. The total volume of each reaction was 25 µl. The PCR reactions were performed according to the protocol supplied with the NZYTaq II 2x Green Master Mix Kit, using a thermal cycler programmed with the following cycle conditions: Initial denaturation: 95°C for 3 min (1 cycle);

Denaturation: 94°C for 30 s; Annealing: 57°C for for 30 s; Extension: 72°C for 30 s (35 cycles); Final extension: 72°C for 5 min (1 cycle); and hold at 4°C until the tubes were extracted from the thermal cycler.

The PCR result was visualized by electrophoresis using a 1% agarose gel stained with GreenSafe Premium from NZYtech. This was performed using TBE (1x) at 110 V for 60 min, using a NZYDNA Ladder I from NZYtech as a molecular weight marker. The resulting bands were visualized under UV light to confirm the presence of the amplified fragments of the sphingomyelinase gene.

1.7. Purification of PCR product and sequencing analysis

An agarose gel containing the individual bands was visualized under UV light and each band corresponding to the amplified fragments of the sphingomyelinase gene was carefully cut out with a sterile scalpel. The extracted gel bands were subsequently subjected to DNA extraction and purification using a specialized gel extraction Kit (NZYGelpure Kit; NZYtech), following the manufacturer's protocol. The concentration and purity of the amplified fragments were measured using a Thermo Fisher NanoDrop™ spectrophotometer (P1: 26.577 ng/µL; P2: 7.053 ng/µL). After purification, PCR amplicons were submitted to DNA Sanger sequencing analysis for confirmation. The sequencing analysis was conducted by the i3s institute.

2. Development of the recombinant expression vector pSM658

This section describes the process of insertion of sphingomyelinase gene cassette (coding optimized and in correct ORF) into the original pSM657 plasmid, to design the recombinant vector pSM658. Bioinformatics tools were used, including the Geneious Prime software, to ensure adequate expression of the target gene.

Before starting the process of replacing the *cat* gene (antibiotic resistance marker) with the gene of interest (sphingomyelinase), the complete sequence of the pSM657 vector/plasmid was obtained by whole plasmid sequencing analysis (Eurofins). To accomplish the heterologous gene expression in the chloroplast of *T. suecica*, it was essential to use efficient promoters and UTRs. Transformation of the chloroplast is achieved by homologous recombination, which requires two flanking regions (LFR and RFR) to introduce foreign genes into the plastome, guided by a site-specific integration event. This leads to uniform expression of transgenes between transgenic lines, eliminating the position effect. Given the intrinsic characteristics of *T. suecica*, the pSM657 **(Fig. 8)** co-expression plasmid was chosen (Gutiérrez et al., 2020).

This vector also contains the reporter gene tGFP, which was designed to confirm the transcriptional activity (expression) controlled by the divergent dual promoters on the vector pSM657. This divergent double promoter consists of a “back-to-back” promoter region. This region suggests that the *5'rbcL* and *5'atpB* promoters are placed in a way that the 5' end of one promoter is opposite the 3' end of the other promoter, or vice versa. This is fundamental for the divergent transcription of adjacent genes, where the *rbcL* and *atpB* genes are transcribed in opposite directions from these bidirectional promoters (Gutiérrez et al., 2020). This type of promoter is useful in genetic engineering for the co-expression of multiple genes. Thus, the bidirectional promoter *5'rbcL/5'atpB* would control the co-expression of both sphingomyelinase and tGFP genes in the chloroplast of *T. suecica* (Vogl et al., 2018).

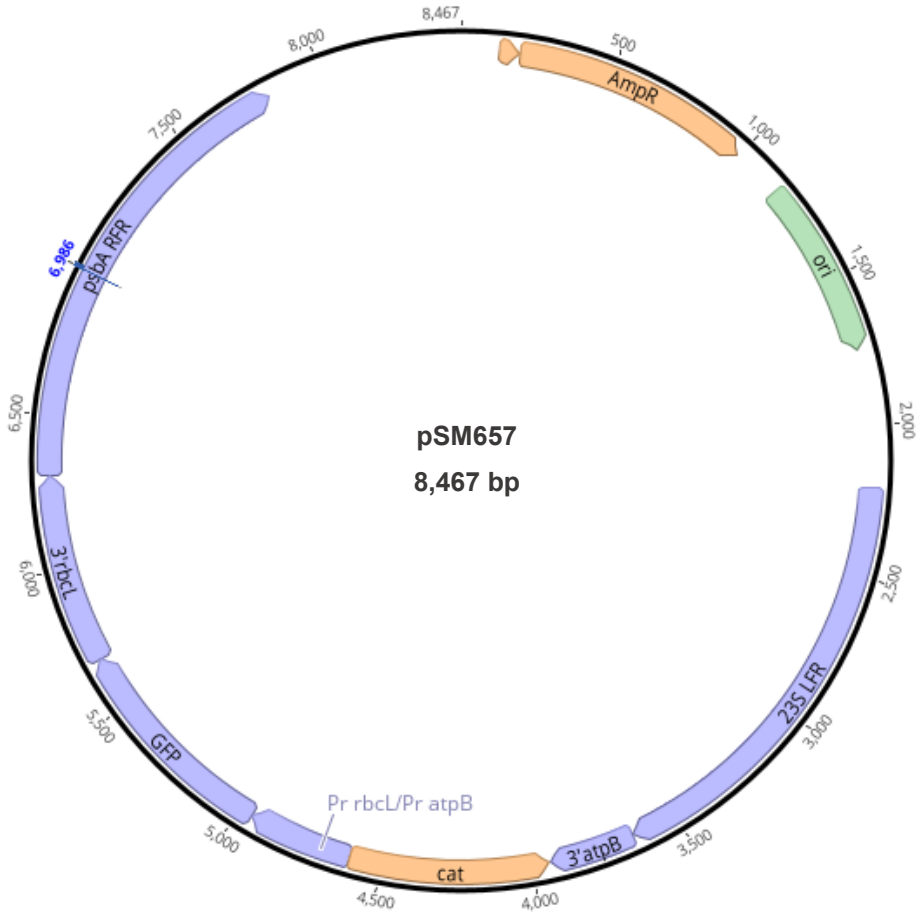

Figure 8. Structure of the original pSM657 vector. The vector is 8,467 base pairs (bp) long and includes key elements such as the cat gene (cat), the ampicillin resistance gene (AmpR), the green fluorescent protein (tGFP) gene, LFR and RFR, specific promoters (dual divergent promoters) and the origin of replication (ori). The image was generated by sequencing the vector and using Geneious Prime software. This vector is derived from the work of Gutiérrez et al. (2020).

2.1. Codon optimization of the expression cassette

To design the expression cassette to be ligated into the pSM657 vector, a fundamental codon optimization step was performed. The genetic coding usage of *T. suecica* was imported from the database available at the Codon Usage Database from the Kazusa DNA Research Institute, guaranteeing an updated and precise analysis of the codon usage in the microalga.

The data given, including the 252 codons of *T. suecica*, was used as a reference for optimizing the codon usage in the sequence of the target protein (sphingomyelinase,

GenBank accession: WP_136438322.1). Therefore, the sphingomyelinase gene sequence has been adjusted to adapt to the algae's expression system, considering the codon preferences of *T. suecica*. Codon optimization was carried out considering factors such as the composition of the algae's preferred codons, preventing problematic sequences and maintaining the functional integrity of the sphingomyelinase. This process was carried out in order to maximizes translation efficiency of the recombinant protein.

Subsequently, a detailed comparison was performed between the original sphingomyelinase sequence (GenBank accession: WP_136438322.1) and the optimized sequence for the heterologous expression in *T. suecica*. This procedure consisted of translating the DNA sequences into amino acid sequences and then aligning the two resulting sequences to assure the correct translation into the sphingomyelinase recombinant protein.

2.2. Bioinformatic analysis for the substitution of antibiotic resistance marker (*cat*) by the sphingomyelinase codon optimized gene sequence

During the design of the recombinant vector pSM658 for sphingomyelinase expression, a crucial consideration was the orientation of the genetic elements within the vector **(Fig. 9)**. Therefore, following the codon optimization, it was necessary to invert the orientation of the optimized sphingomyelinase gene ORF to ensure efficient integration and translation into the chloroplast of *T. suecica*. This was crucial to guarantee that, during transcription and translation, the expression process occurs properly.

The open reading frames (**ORFs**) were considered carefully, taking into consideration that gene translation occurs according to the ORFs during transcription and translation. According to the Expasy programme from the SIB Swiss Institute of Bioinformatic. There are six ways of reading ORFs. The sphingomyelinase gene is in the **ORF1** form (in the sense 5‘ to 3’, from left to right). On the other hand, the *cat* gene, where the sphingomyelin will be inserted, is in the **ORF-3** form (antisense 5‘ to 3’, from right to left).

After the codon optimization, the correct ORF orientation, **ORF-3**, of the sphingomyelinase gene was performed to ensure the correct translation during the protein expression phase. According to this strategy, the antibiotic resistance gene (*cat*) was replaced by the sphingomyelinase cassette in the pSM657 vector, using instead tGFP as selection marker.

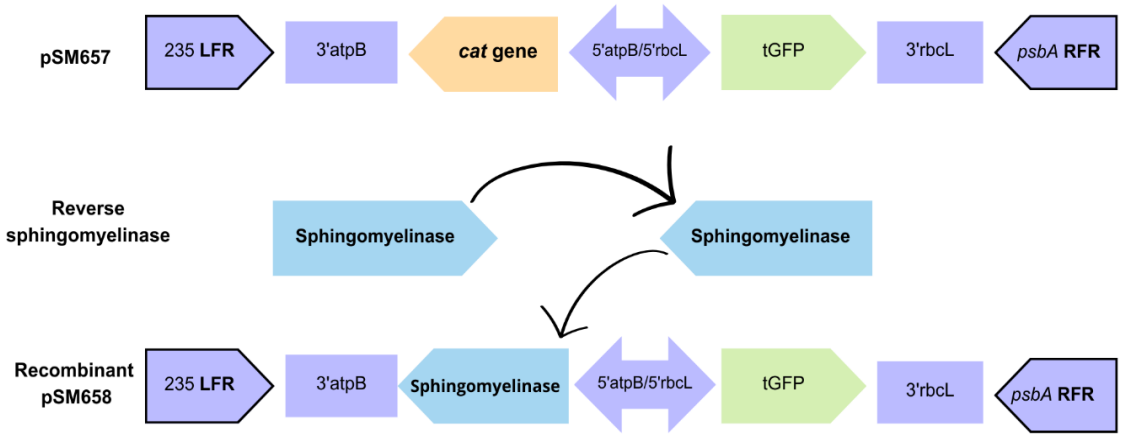

Figure 9. Diagrammatic representation of the construction of the recombinant pSM658 vector for heterologous expression of sphingomyelinase. This graphic representation, inspired by the work of Gutiérrez et al. (2020), was developed to explain the vector modification process, highlighting the replacement of the cat gene with the optimized sphingomyelinase sequence

In **Fig. 9** it's possible to see a detailed diagram illustrating the adaptation of the orientation (ORF) of the sphingomyelinase gene sequence during the design process, considering the specific orientation of the regulatory elements (divergent promoter) into the pSM657 vector.

In the diagram above, the original pSM657 vector can be observed, highlighting the fundamental elements, such as cloning flanks (LFR and RFR), promoter, and the *cat* gene, which is codified in the **ORF-3**. On the other hand, the tGFP gene and the sphingomyelinase sequence is codified in **ORF1**. Subsequently, the reversal of the orientation of the sphingomyelinase sequence is illustrated (**ORF-3**), which is essential to guarantee correct ORF during the transcription and translation processes. In the last section of the diagram, the sphingomyelinase sequence is ligated into the pSM657 vector, resulting in the recombinant vector pSM658. The arrows point to the specific region of the *cat* gene, indicating the insertion site. The lower part of the diagram shows the recombinant vector, now containing the sphingomyelinase gene sequence (codon optimized and in the correct ORF antisense orientation).

2.3. Synthesis of the recombinant vector

After the bioinformatic analyses for replacing the gene in the recombinant vector pSM658, the file (FASTA) with the sequence obtained in the Geneious Prime was sent to the GenScript company to be synthesized. This step was essential to ensure that the gene of interest was correctly inserted and expressed in the plasmid, allowing future works.

2.3.1. Transformation of *E coli* competent cells

After receiving the synthetized vector, it was necessary to ensure its multiplication, so that more vectors were available to work with. To do this, the process of bacterial transformation in *E. coli* (NEB 5-alpha) was carried out to amplify and obtain a larger quantity of plasmids. This *E.coli* strain has characteristics that allow it to be artificially induced into a competent state, which means that it is capable of absorbing and incorporating foreign DNA (Riva et al., 2020).

For this procedure, the high-efficiency NEB 5-alpha protocol from New England Biolabs (C2987H/C2987I) was used. After 10 min on ice, 5 µL of the recombined plasmid pSM658 was added to 50 µL of competent cells. To neutralize the membrane, the mixture was incubated for 30 min. A heat shock was performed by transferring the mixture from ice to a 42 ºC water bath for 40 sec. The cells were placed on ice for 2-3 min and then 250 µL of LB-Broth without antibiotic. The cells were left to recover for 1h at 37 ºC with constant stirring. Then, 40 µL of the LB with bacteria was pipetted into a LB-plate with antibiotic (AMP_{100}) and the remaining volume was pipetted into another plate with antibiotic. The plates were incubated overnight at 37 ºC (no more than 16 h). After this time, we collected 4 individual colonies from each plate and added 2 ml of LB medium with antibiotic (AMP_{100}) to each.

2.3.2 Isolation of the recombinant vector pSM658 from transformed cells

An aliquot of 1.5 ml of each bacterial culture was centrifuged at 16.000 x g for 30 sec to pellet the cells, and the supernatant was discarded. Then, 200 µl of plasmid resuspension buffer was added, followed by the addition of 200 µl of plasmid lysis buffer. The mixture was

gently inverted to avoid excessive lysis of the contamination with chromosomal DNA. It was left to incubate for 1 min at room temperature and 400 µl of plasmid neutralization buffer was added to stop the lysis. A centrifugation was carried out at 16.000 x g for 5 min. The supernatant was then transferred to a collection tube with a spin column, where it was centrifuged for 1 min to allow the plasmid DNA to bind to the column membrane. Then, 200 µl of plasmid wash buffer 1 was added to the column, centrifuged for 1 min and the flow-though removed. The membrane was washed again with 400 µl of plasmid wash buffer, centrifuged again for 1 min and discarded. After washing, the column was transferred to a new microcentrifuge tube. Then, 30 µl of nuclease-free water was added to the center of the column, incubated for 1 min and centrifuged for 1 min.

The isolated plasmid DNA was quantified by spectrophotometry, using a fluorometer spectrophotometer Thermo Fisher NanoDrop™ to determine the concentration and purity of the DNA obtained. Storage was carried out at -80 ºC. The concentration of plasmid DNA was determined from the absorbance at 260 nm using the specific extinction coefficient for double-stranded DNA (dsDNA). The recombinant plasmid sample with the highest concentration and best levels of purity and contamination was used for the next steps (383.055 ng/µL).

2.3.3 Confirmation of the correct assembly of the vector by restriction analysis

*Hind*III is a type II restriction enzyme that recognizes and cleaves the sequence at 5'-AAGCTT-3'. This sequence corresponds to the end of promoter *5'atpB* and *3'atpB* UTR region, including the whole sequence of the sphingomyelinase gene (~ 1500 bp in total). The recombinant vector was digested with the *Hind*III restriction enzyme to verify the correct insertion of the sphingomyelinase gene.

The New England Biolabs Time-saver protocol for digestion with the *Hind*III enzyme (NEB #R3104) was used for this step. The correct volumes of *Hind*III enzyme, reaction buffer, recombinant vector DNA, and nuclease-free water were calculated based on the proportions recommended in the protocol. The enzyme and reaction buffer were inactivated on ice according to the manufacturer's instructions. In a sterile microcentrifuge tube, water (40µl), *Hind*III reaction buffer (5 µl), recombinant plasmid pSM658 DNA (4 µl, 1 µg total) and finally NEB *Hind*III enzyme (1 µl, 10U/µl) were added. It was centrifuged briefly to ensure that the components were mixed correctly. It was incubated at 37 ºC for 1h. To stop the

enzymatic activity of *Hind*III, the reaction mixture was heat-activated at 80 ºC for 20 min. The result of the digestion was visualized by electrophoresis using a 1% agarose gel stained with GreenSafe Premium from NZYtech. This operation was carried out with TBE (1x) at 110 V for 60 min, using an NZYDNA Ladder I from NZYtech as a molecular weight marker. The resulting bands were visualized under UV light to confirm the presence of the fragments of the sphingomyelinase gene. In the same gel, it was also added the sample of the original and undigested recombinant vector.

As explained previously in this work, the agarose gel containing the individual bands was scanned under UV light and the band corresponding to the sphingomyelinase fragment was extracted with a scalpel. The band from the gel was then purified using a specialized gel extraction kit (NZYGelpure Kit; NZYtech), following the manufacturer's protocol. The concentration and purity of the amplified fragments were measured using a Thermo Fisher NanoDrop™ spectrophotometer. After purification, the extracted product was submitted for Sanger sequencing analysis by the i3s institute.

3. Results

3.1. Primer design for sphingomyelinase detection in *T. maritimum*

The parameters of the primers pair P1F/P1R and P2F/P2R, including the GC%, the existence of secondary structures including hairpins and self-dimers, as well as the melting temperature, are presented in the **Table 8**.

Table 8- Characteristics of the primers used in the study. The parameters included are the percentage of guanine and cytosine base pairs, the melting temperature (Tm), of the hairpin (if applicable), the melting temperature of the self-dimer (if applicable), the melting temperature of the primer, the melting temperature of the pair dimer (if applicable) and the approximate size of the amplified product.

Parameters	P1F (430 F)	P1R (940 R)	P2F (211 F)	P2R (724 R)
% GC	45.5%	52.4%	45,00%	45.45%
Hairpin Tm	None	44.1ºC	None	2ºC
Self-Dimer Tm	None	1,0ºC	4ºC	6ºC
Tm	59.6ºC	59.4ºC	58.40ºC	58.40ºC
Pair Dimer TM	None	None	None	None
Product Size (bp)	511	511	514	514
Sequence	**5'** AG-CAACTCTCCTTTCG-TAACCA **3'**	5' GGGGTT-GTTTTCCCTGCTTAC **3'**	**5'** AGAG-CAGAAGAC-CAAAAGAA **3'**	**5'** CGTTA-GAAGGAA-TTTTCCCCGA **3'**

These details are essential for the success of the PCR reactions, assuring the specific amplification of the target sequences.

Additionally, it was carried out an alignment analysis. This step is crucial to confirm the presence of the sphingomyelinase sequence in the genome of *T. maritimum,* predicted to be approximately 1350 base pairs (bp). The primer pairs, P1F/P1R and P2F/P2R, targeting different regions of the antigen gene, were aligned with the *T. maritimum* complete genome using the bioinformatics tools provided by Geneious Prime. The **Fig. 10** and **Fig. 11** show the result of the alignment of primers P1F/P1R (in purple) and P2F/P2R (in orange) with the genome of *T. maritimum*.

The alignment of the primer pair, 940R, 430F and the pair 724R, 211F, with the genome of *T. maritimum* revealed the presence of the sphingomyelinase sequence. The alignment also revealed a specific binding of the primers to the bacterium genome, which occurred around 1500 bp, as predicted. The specificity of the primers is highlighted by the absence of binding at unexpected sites. This result indicates that the primers were designed with high specificity for the desired region, minimizing the possibility of amplifying unintended regions.

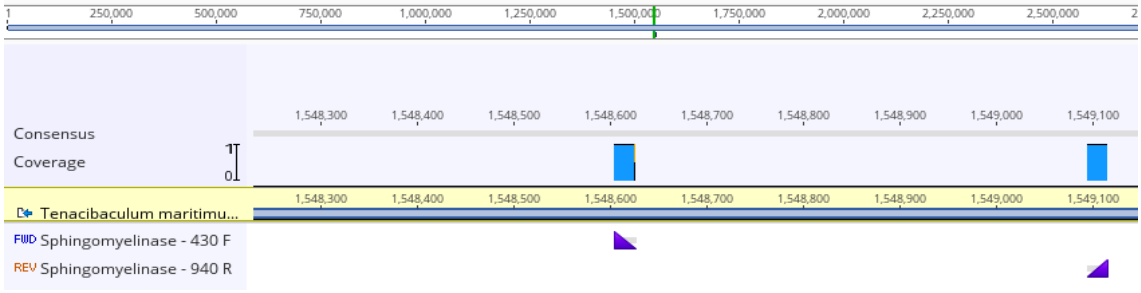

Figure 10. Alignment of the P2F/P2R primers with the *T. maritimum* genome. The horizontal bars indicate the distance scale in base pairs (bp) for reference of the location of the primers in the genome. The orange markers show the binding site of the P2F and P2R.

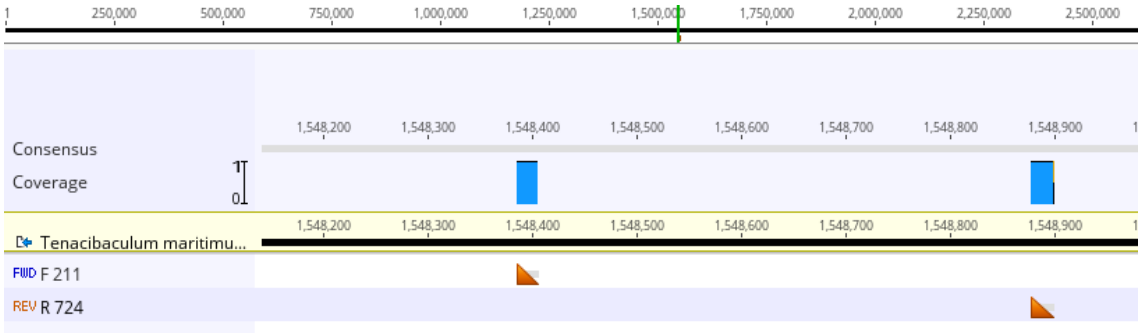

Figure 11. Alignment of the P1F/P1R primers with the *T. maritimum* genome. The horizontal bars indicate the distance scale in base pairs (bp) for reference of the location of the primers in the genome. The purple markers show the binding site of the P1F and P1R primers in the genome.

3.2. PCR amplification and isolation of fragments for sequencing analysis

PCR amplification revealed the presence of two bands, both located in the region around 500 bp, corresponding to the expected size of the final amplification products **(Fig. 12).** The first band was associated with primer pair P1 (430F/940R), and the second with primer pair P2 (211F/724R).

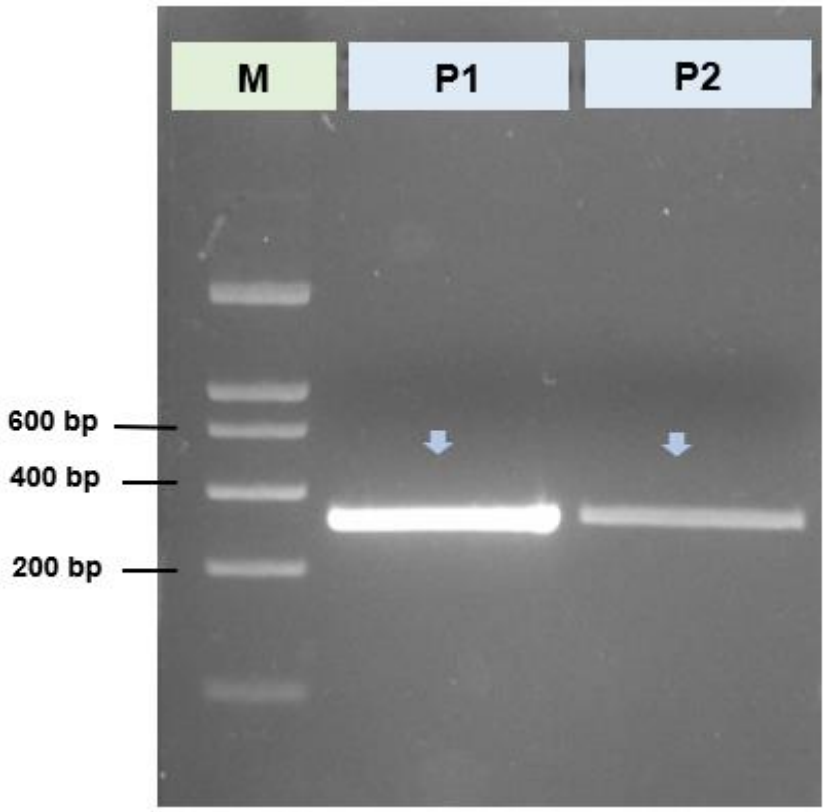

Figure 12. Gel electrophoresis of PCR products to verify the presence of the sphingomyelinase antigen in *T. maritimum*. The bands indicate the different amplified samples: NZYDNA Ladder I from NZYtech (M), cDNA (C), amplification product with P1 primers (430F and 940R), and amplification product with P2 primers (211F and 724R). The arrows highlight the specific bands of interest, confirmed by the expected size.

The presence of both amplicons confirmed the specificity of the primers used, confirming in fact the expression of the target gene sphingomyelinase in the strain of *T. maritimum* used in this study.

The concentration of the PCR product samples was evaluated using a Thermo Fisher NanoDrop™ spectrophotometer. The results revealed different concentrations for the products generated by primer pairs P1 and P2. The P1 primer pair showed a concentration of 26,577 ng/µl, demonstrating a considerable abundance of the amplified product. In contrast, the P2 primer pair showed a concentration of 7,053 ng/µl, revealing a lower relative quantity of the product compared to P1. This result is in accordance with the results of the gel electrophoresis, where the band corresponding to the product of primer P1 was visually more intense than the band of primer P2.

The combined analyses of the results of PCR amplification, gel electrophoresis band purification and sequencing analysis provides a comprehensive perspective for confirming the presence and expression of the sphingomyelinase protein in *T. maritimum*.

3.3. Substitution of *cat* gene for the optimized sphingomyelinase sequence

Bioinformatic analyses were crucial in the construction of the recombinant vector for the heterologous expression of sphingomyelinase in the chloroplast of *T. suecica*. The results obtained during these processes were fundamental to assure the validity and efficacy of the construction and for further proceedings.

3.3.1. Codon optimization

The identity analysis revealed that, after codon optimization, the original and optimized sphingomyelinase sequences showed 100% amino acid correspondence **(Fig. 13)**. This confirms the integrity of the optimized sequence and ensures that the changes to the codons have not affected the amino acid composition of sphingomyelinase during the protein translation process. The **Fig. 13** shows the alignment of the original and optimized sphingomyelinase sequences obtained, using the Geneious software. This alignment has a key role to perform in validating the codon optimization, permitting a detailed comparative analysis of the changes introduced and ensuring the integrity of the optimized sequence.

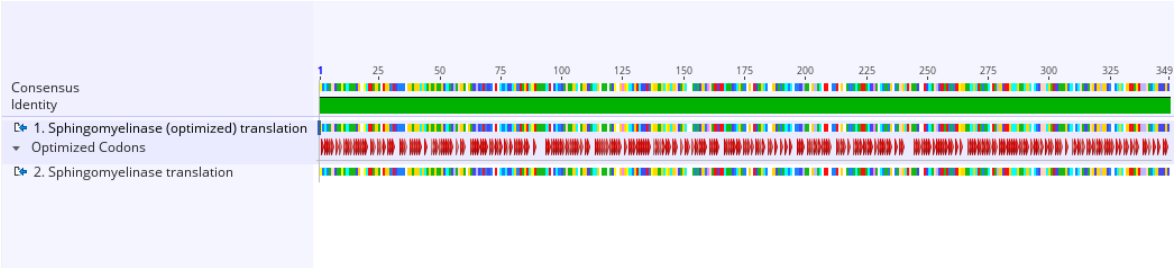

Figure 13. Alignment of the optimized sphingomyelinase (1) and original sphingomyelinase (2) sequences using the Geneious program. The green line represents Consensus Identity, which indicates areas of 100% identity between the two sequences.

3.3.2. Design of the optimized sphingomyelinase gene sequence to be inserted into the recombinant vector pSM658

The construction of the modified vector pSM658 for heterologous sphingomyelinase expression required, according to our strategy, the precise insertion of the optimized sphingomyelinase sequence (codon optimized and in the correct ORF) at the position of the *cat* gene, and under the control of the dual divergent promoter, specifically the *atpB* promoter.

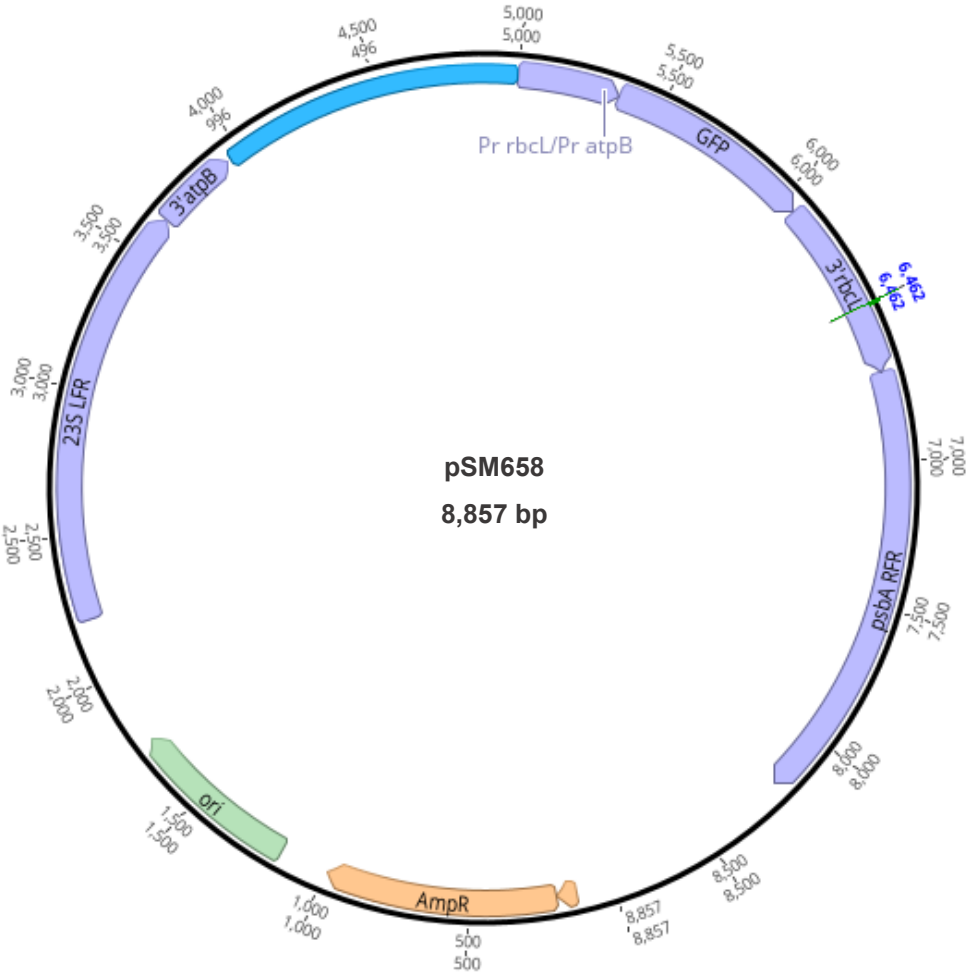

Figure 14. The modified vector pSM658 resulting from the insertion of sphingomyelinase gene at the position of cat gene, is represented in blue. The image was produced using Geneious Prime software and highlights the gene of interest, as well as important elements such as the green fluorescent protein (tGFP) gene, the left and right flanking regions (LFR and RLF), specific promoters and the origin of replication.

The **Fig. 14** illustrates the structure of the vector pSM658, the result of the insertion of sphingomyelinase sequence (codon optimized and under the right ORF), in the position originally occupied by the *cat* gene. Using the Geneious Prime software, the image shows the gene of interest (sphingomyelinase) in blue, providing visual evidence of the modification **(Fig. 14)**.

The vector pSM658 preserves essential elements, including the tGFP gene as reporter and selection gene, LFR (23S-5S) and RFR (*psb*A) to guarantee the integration of the vector in the chloroplast genome of *T. suecica*, specific promoters for expression regulation and the origin of replication to allow the vector to be reproduced. Following the insertion of sphingomyelinase, a detailed analysis was carried out to evaluate the structural integrity of the modified vector (whole-plasmid sequencing analysis). Using bioinformatics techniques and structural analysis tools, no significant changes were observed in the secondary structures, suggesting that the vector's general conformation was conserved. This result confirms that the insertion of sphingomyelinase has not compromised the structural characteristics of the rest of the vector, ensuring the overall stability and functionality of the construct.

3.4. Confirmation of the correct cassette integration into the recombinant vector pSM658

3.4.1 Purification and quantification of recombinant plasmid pSM658 DNA

Plasmid pSM658 DNA, obtained of 4 clones, was quantified and assessed for purity using a ByNovix spectrophotometer. All the purified DNA samples had an A260/A280 ratio close to 1.8, indicating high purity with little or no protein contamination. The A260/A230 ratio was higher than 2.0, suggesting the absence of significant organic contaminants. The different concentrations obtained were as follows: **1**: 240,702 ng/nL; **2**: 378.284 ng/nL; **3**: 383.055 ng/nL; **4**: 357.807 ng/nL.

3.4.2 Concentration and analysis by agarose gel electrophoresis of the sphingomyelinase cassette

Gel electrophoresis was used to analyze the results obtained from digesting the recombinant plasmid pSM658 with the *Hind*III restriction enzyme. The gel was run with samples of the digested recombinant pSM658 plasmid (PR), and NZYDNA Ladder I (200-1800 bp) (M) from NZYtech **(Fig. 15)**. In the well of the recombinant plasmid pSM658, there was a band higher up, corresponding to the cut recombinant plasmid. There was also a lower

band, around the 1500 bp region, which corresponds to the sphingomyelinase fragment. These results confirm that the digestion with the *Hind*III restriction enzyme went as expected and that the gene of interest was correctly inserted into the plasmid.

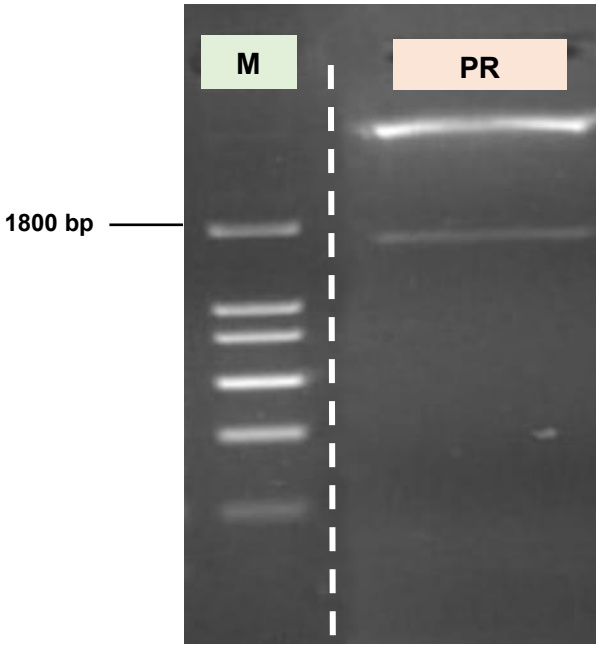

Figure 15. Gel electrophoresis of PCR products to verify the correct digestion and insertion of the sphingomyelinase gene into the recombinant pSM658 plasmid. The bands indicate the different samples amplified: NZYDNA Ladder I from NZYtech (M), and recombinant pSM658 cut with *Hind*III (PR). The arrow highlights the specific band of interest, corresponding to the sphingomyelinase gene. *

* **Note:** The gel image was cropped and adjusted to remove samples that were irrelevant to this study, which were between the samples of the intended results, in order to better observe the results. No data manipulation was performed.

After extraction, the purified sphingomyelinase fragment had an A260/A280 ratio close to 1.8, indicating high purity with little or no protein contamination. The A260/A230 ratio was higher than 2.0, suggesting the absence of significant organic contaminants. The concentration obtained was 4,711 ng/µl. Subsequently, those fragments were submitted to Sanger sequencing analysis to corroborate the results.

4. Discussion

In recent years there has been an increase in demand for organisms to produce recombinant proteins (Lomonossoff & D'Aoust, 2016), with microalgae emerging as potential hosts of interest (Taunt et al., 2018). Nevertheless, genetic engineering of chloroplasts is still very restricted to a few microalgae because the lack of information on the genome (Mauro, 2018).

The objective of this chapter was to develop a recombinant vector, named pSM658, for the expression of sphingomyelinase, an antigen presents in the *T. maritimum,* with the future intention of transform the plastome of the microalgae *T. suecica*, to use as a platform for oral vaccination in aquaculture. For this, the antibiotic resistance (*cat),* present in the original pSM657 vector, was replaced with the sphingomyelinase sequence.

The design of chloroplast transformation plasmids are consequently important in these processes because they need to have promotor and UTR regions that controls the expression of the foreign genes, and also LFR and RFR that rule the site directed integration into the plastome by homologous recombination (Bateman & Purton, 2000). The initial vector used was the pSM657 that has a divergent promoter region consisting of *5'rbcL* and *5'atpB* promoters (Gutiérrez et al., 2020). This feature is very important, as this region controls the expression of the gene of interest (in this case sphingomyelinase), and at the same time of the tGFP gene (used in this case as selection marker). Moreover, a series of optimization steps of sphingomyelin expression cassette were crucial

Codon optimization was indispensable to designing the expression cassette and ensuring that the sphingomyelinase sequence was adapted to maximize translation in microalgae (Fu et al., 2020) This procedure consisted of adapting the genetic sequence of our gene of interest to the microalgae (*T. suecica)* expression system, considering its codon preference (codon usage). After this, an alignment comparison between the original sphingomyelinase sequence (GenBank accession: WP_136438322.1) and the optimized sequence showed integrity and 100% amino acid correspondence. This whole process was carried out using bioinformatics tools and the programs mentioned previously. Once obtained the correct codon optimized sphingomyelinase sequence, the ORFs were taken in consideration, ensuring that translation occurs correctly. The original optimized sphingomyelinase sequence is represented in ORF1, and it was necessary to invert the sequence to insert in the orientation of the *cat* gene (ORF-3), which is controlled by the promoter *5'atpB.* This process ensures the correct ORF during the transcription and translation process, and the non-production of non-functionals proteins.

After the design of the correct sphingomyelinase cassette (codon optimized for *T. suecica* and in ORF-3), and its substitution for the *cat* gene, the vector pSM658 was synthesized by GenScript Biotech. To confirm the correct insertion of our gene of interest, it was performed a digestion with *Hind*III restriction enzyme, that cleaves the sequence corresponding between the end of promotor *5'atpB* and UTR *3'atpB* region of the sphingomyelinase gene (Gutiérrez et al., 2020). The results of the PCR showed a band, locating in the 1500 bp region, corresponding to the sphingomyelinase fragment. These results confirm that the digestion with *Hind*III restriction enzyme went as expected and the gene of interest was correctly inserted into the plasmid. Such result was also corroborated by Sanger sequencing analysis of the fragment isolated form the agarose gel.

It is important to notice that it was used the tGFP as a selection marker instead of an antibiotic resistance gene. This approach promotes a more accepted practice, avoiding concerns about human and environmental health (Costa et al., 2015). In addition, it is a solution to the problem of antibiotic resistance in bacteria (Daniell et al., 2001).

After the development of the recombinant vector for the expression of sphingomyelinase, the microalga *T. suecica* will be transformed by the insertion of the recombinant vector into the alga's chloroplast. The transformation process will be performed outside Portugal, in collaboration with the Laboratory of Molecular Engineering of Microalgae of the Pontificia Universidad Católica de Valparaiso (PUCV), Chile.

The transformation of the microalga *T. suecica* will be carried out using the biolistic method (Christou, 1995; Doron et al., 2016). This method involves the use of gold microparticles which are coated with DNA from the recombinant vector **(Fig. 16)**. These particles are then accelerated to high speed and fired into the target cells of the microalgae, using gas pressure at 1350 PSI (Christou, 1995). Once they reach the target cells, the microparticles break through the cell walls membrane (outer and chloroplast membranes, respectively) and release the recombinant vector DNA inside the cells. The DNA of the vector is then integrated by homologous recombination into the chloroplast genome of the microalgae (plastome), allowing the expression of the gene of interest, in this case, sphingomyelinase (Doron et al., 2016).

To achieve a correct integration, it's important to re-emphasize the importance of the LFR and RFR in the success of this process **(Fig. 17)**. The pSM657 plasmid contains two flanking regions derived from *T. suecica*: LFR (23S-5S) and RFR (*psb*A), respectively (Gutiérrez et al., 2020). These flanking regions are essential for the specific integration of the vector into the chloroplast genome by homologous recombination. These regions are used during the transformation process to guide the integration of the vector into the host

genome (Cooper & Adams, 2022). The only special feature of these regions is that they are homologous to the chosen target site and can be targeted without modifying the regions of the sequence (Gutiérrez et al. 2020). This is particularly important for minimizing the position effect on gene expression of an exogenous gene inserted into a host organism (*T. suecica* in this case). When using flanking regions that are homologous to the host genome, it is possible to direct the insertion of the gene of interest to specific locations in the genome which are known to carry consistent levels of gene expression. Thus, flanking regions are key factors for the integrity and stability of the cloning vector during the transformation process and during integration into the host genome (Kumar & Ling, 2021). Moreover, according to Gutiérrez et al. (2020), the chosen flanking regions (23S-S5 and *psb*A) are in the inverted repeat areas (IR), this also represents an advantage because of the integration in one IR is followed by a process of copy correction, which implies the duplication of the introduced transgene into the other IR as well (Beeramganti et al., 2012); therefore, leading to a higher level of expression of the recombinant protein.

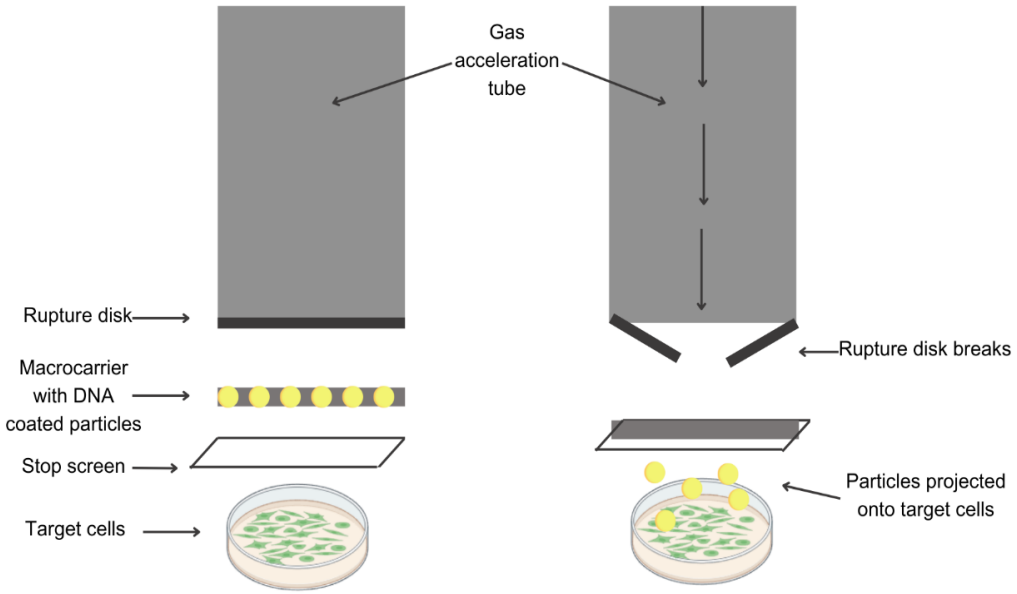

Figure 16. Diagram illustrating the process of chloroplast transformation by particle bombardment (biolistic).

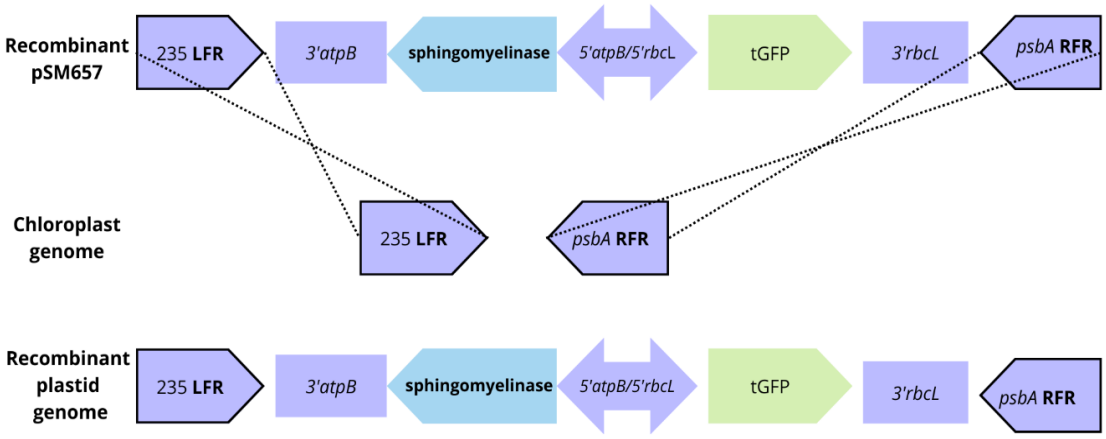

Figure 17. Diagrammatic representation of homologous recombination in the chloroplast. This graphic representation, inspired by the work of Gutiérrez et al. (2020), was developed to explain homologous recombination of the vector into the chloroplast genome, mediated by the presence of the LFR and RFR.

For detecting the successful transformed *T. suecica* cells, it would be used the screening method based on the tGFP fluorescence. This technique allows the detection and selection of clones transformed with the pSM658 vector, which contains the tGFP gene as a selection/reporter marker. After the biolistic process, the transformed clones would be selected by the detection of the fluorescent produced by the expression of the tGFP gene. The expression of this gene, which is regulated by the dual-promotor, indicates that the transformation has taken place correctly and that the cell has the desired mutation (Maor et al., 1998), in this case the gene sequence of the sphingomyelinase protein. Transformants that show fluorescence will be visualized using fluorescence microscopy. The intensity of the fluorescence can indicate a higher or lower level of expression of the gene of interest (Sproles et al., 2022). Another potential technique to detect transformed cells is flow cytometry and confocal scanning microscopy. Both techniques are based on the detection of the fluorescence intensity emitted by tGFP in the transformed microalgae cells, which can be discriminated from the natural chloroplast auto florescent produced by the presence of the chlorophyll and other accessory pigments (Gutiérrez et al., 2020). Therefore, it will be possible to simultaneous measure and distinguish between the auto-fluorescent and fluorescent produced by wild-type *T. suecica* and transformed clones, respectively. Finally, after

selection of *T. suecica* transformants cells, additional analyses, such as total protein extraction, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western Blot, and/or mass spectrometry (MS), would be used to confirm the correct expression of the sphingomyelinase protein.

5. Conclusion

The development of vectors capable of expressing genes of interest in heterologous platforms such as the microalgae *T. suecica*, represents an innovative and beneficial approach for aquaculture, creating possible new vaccination strategies.

The work described in the chapter of this dissertation successfully demonstrated the development of a recombinant pSM658 vector for the expression of the sphingomyelinase gene. The use of bioinformatics and molecular techniques, such as the optimization of codons and the correct orientation of ORFs, resulted in the correct insertion and translation of the protein of interest. The results obtained by electrophoresis after digestion of the recombinant vector proved and validated its correct assembly. The result obtained in this work provides a solid basis for the future integration of the recombinant vector into the chloroplast of the microalga *T. suecica*, through the process of biolistic. It is necessary to do further studies, using and exploring different algae and different genes, in order to develop sustainable and effective strategies to improve fish health.

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