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NUTRIMU and EPPO Internship: Larval rearing and fish health in Aquaculture

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NUTRIMU and EPPO Internship: Larval rearing and fish health in Aquaculture

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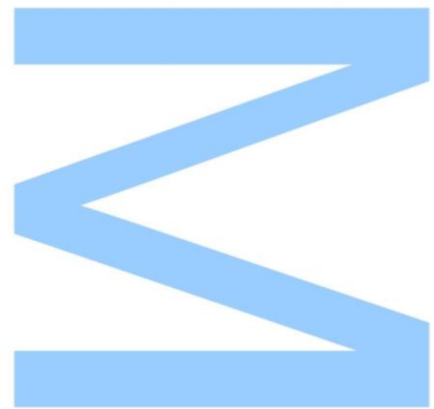

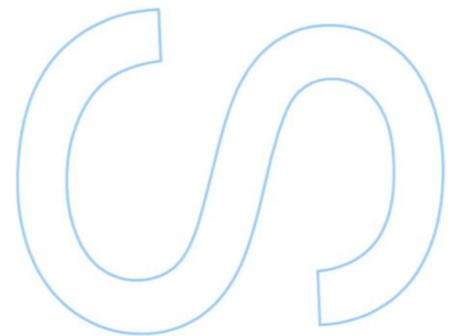

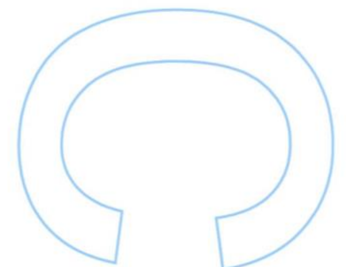

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pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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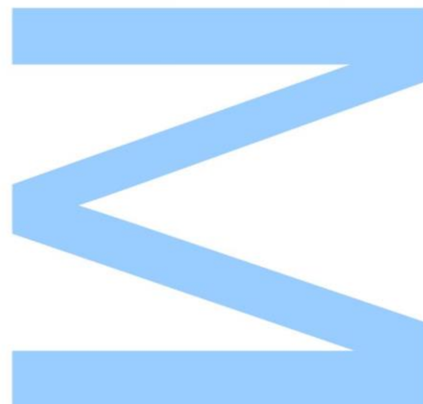

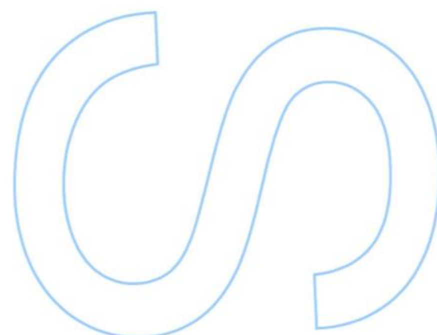

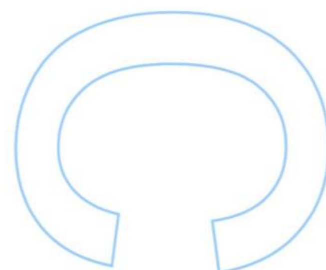

Vista aérea da Estação Piloto de Piscicultura de Olhão do IPMA onde foi realizado este trabalho.

Este trabalho foi parcialmente (2ª parte) financiado e realizado na Estação Piloto de Piscicultura de Olhão (EPPO) do IPMA utilizando corvinas nascidas nesta Estação e as infraestruturas e meios disponíveis para o cultivo, amostragens biológicas e análises laboratoriais. Ao IPMA reserva-se o direito de utilizar toda a informação nela contida para efeitos de publicações, relatórios da entidade financiadora e divulgação ao público em geral.

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Resumo

Atualmente, a humanidade enfrenta o enorme desafio de proporcionar nutrição segura e adequada para uma população mundial em constante crescimento. Neste contexto, a aquacultura desempenha um papel fundamental para responder à demanda contínua de pescado, uma vez que o setor pesqueiro estabilizou nas últimas décadas. Hoje em dia, a nível mundial, a aquacultura fornece mais da metade do pescado para consumo humano. Portugal, um país com uma forte ligação ao mar e com o maior consumo de pescado a nível da UE, com 61,5 kg per capita, possui uma produção aquícola baseada na cultura de peixes, moluscos e crustáceos em águas salobras e marinhas, que correspondem a 93,4% da aquacultura nacional total em 2019.

O presente relatório descreve o trabalho desenvolvido durante o meu estágio curricular, no ano letivo de 2019/2020, no âmbito do Mestrado em Recursos Biológicos Aquáticos, da Faculdade de Ciências da Universidade do Porto. O estágio foi dividido em duas partes, cada uma com objetivos e instituições diferentes.

A primeira parte deste estágio foi desenvolvida no grupo NUTRIMU, do CIIMAR, e foram analisadas amostras de robalo europeu (*Dicentrarchus labrax*) com uma patologia desconhecida, utilizando técnicas histológicas e de biologia molecular. As análises histológicas revelaram a existência de vários granulomas, que parecem estar ligados ao tecido adiposo que envolve o fígado e o trato digestivo e no interior do fígado. As análises moleculares revelaram a presença de bactérias dos géneros *Pseudonomas* e *Vibrio*. De forma a clarificar a etiologia da patologia que afetou os robalos, no futuro, seria relevante realizar uma análise molecular mais exaustiva de modo a descobrir quais as espécies específicas associadas aos géneros *Vibrio* e *Pseudomonas* e se estas são de facto espécies patogénicas de peixe responsáveis pela patologia que afetou os robalos.

A segunda parte foi desenvolvida na EPPO, pertencente ao IPMA, e foram realizadas tarefas relacionadas com a criação de larvas marinhas. Para além disso, foi realizado um ensaio alimentar com corvina (*Argyrosomus regius*), que teve como objetivo avaliar os efeitos da suplementação dietética de *Fucus vesiculosus* e *Nannochloropsis gaditana* no crescimento, sistema imunológico e respostas antioxidantes das corvinas. Para tal, também foi executado um desafio bacteriano, utilizando *Photobacterium damselae* subsp. *piscicida* (*Phdp*) como um stressor biótico para avaliar os efeitos da suplementação de algas no estado imunológico e capacidades antioxidantes das

corvinas. Em relação ao crescimento, não foram encontradas diferenças estatisticamente significativas na eficiência alimentar e nos parâmetros de crescimento analisados, entre os tratamentos dietéticos. Em relação ao desafio bacteriano com *Phdp*, apenas a mortalidade que ocorreu após o desafio foi analisada até o momento e as diferenças na mortalidade cumulativa entre os grupos dietéticos e o controlo negativo não foram estatisticamente significativas. Futuramente, nas amostras recolhidas do ensaio alimentar e do desafio bacteriano serão realizadas análises de expressão génica e de stress oxidativo. Estas análises irão trazer novos dados importantes para melhor compreender o presente estudo e o papel dos extratos de *F. vesiculosus* e *N. gaditana* na dieta de corvina na modulação do sistema imunológico de peixes contra *Phdp*.

Concluindo, este estágio abrangeu várias áreas da investigação científica em aquacultura e permitiu-me adquirir diversas competências bastante distintas, extremamente relevantes para a minha formação profissional e para a minha futura carreira na área da aquacultura.

Palavra-chave: aquacultura, robalo, patologias, histologia, criação de larvas marinhas, corvina, extratos de algas, desafio bacteriano

Abstract

Presently, humanity faces the massive challenge of delivering safe and adequate nutrition to a world population significantly rising. In this context, aquaculture plays a key role to respond to the continuous demand of fish, since the fisheries sector has stabilized over the past decades. Nowadays, aquaculture supplies more than half of the world's fish for human consumption. Portugal, a country with a profound connection to the sea and with the highest consumption of seafood at EU level, with 61.5 kg per capita, possess an aquaculture production based on the culture of fish, molluscs, and crustaceans in brackish and marine waters, that correspond to 93.4% of the total national aquaculture in 2019.

The present report describes the work carried out during my curricular internship, in the academic year of 2019/2020, as part of the Master's in Biological Aquatic Resources, in the Faculty of Sciences of the University of Porto. The internship was divided in two parts, each one with different aims and institutions.

The first part was developed in the NUTRIMU group, from CIIMAR, and samples from European Seabass (*Dicentrarchus labrax*) with an unknown pathology were analysed, using histological and molecular biology techniques. The histological analyses revealed the presence of several granulomas that appear to be connected to the adipose tissue that involves the liver and the digestive tract, and inside the liver. The molecular biology analyses revealed the presence of bacteria of the genus *Pseudonomas* and *Vibrio*. The aetiology behind the diseased European seabass remains unclear and, in the future, it would be worth conducting a more exhaustive molecular analysis with the goal of unveiling the bacterial species within the genus *Vibrio* and *Pseudomonas* and if they are indeed pathogenic species of fish responsible for the granulomas observed on seabass.

The second part was developed at EPPO, belonging to IPMA, and tasks related with marine larvae rearing were carried out. Additionally, a feeding trial with meagre (*Argyrosomus regius*) was performed, that aimed to assess the effects of *Fucus vesiculosus* and *Nannochloropsis gaditana* extracts supplementation to meagre diets on growth performance, disease resistance, immune and antioxidant responses. For that a bacterial challenge was executed, using *Photobacterium damselae* subsp. *piscicida (Phdp)* as a biotic stressor to assess the effects of algae supplementation in meagre disease resistance, immune status and antioxidant capacities. Regarding zootechnical performance, no statistically significant differences were found in the growth parameters

and in the feed efficiency, amongst the dietary treatments. Concerning the bacterial challenge with *Phdp*, only the mortality that occurred post-challenge was analysed so far and the differences in the cumulative mortality between the dietary groups and the negative control were not statistically significant. Furthermore, in the future, gene expression and oxidative stress analysis will be carried out in samples from the feeding trial and the bacterial challenge. This will bring new important data to better understand the present study and the role of *F. vesiculosus* and *N. gaditana* extracts in meagre´s diet in the modulation of the fish immune system against *Phdp.*

Overall, this internship covered various areas of aquaculture scientific investigation and allowed me to acquire several and very distinct skills, extremely relevant to my professional training and my future career in the aquaculture area.

Keywords: aquaculture, European seabass, pathologies, histology, marine larvae rearing, meagre, algae extracts, bacterial challenge

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Abbreviations

ANOVA - analysis of variance

CIIMAR - Interdisciplinary Centre of Marine and Environmental Research

DGGE - denaturing gradient gel electrophoresis

DGI- Daily Growth Index

DNA - deoxyribonucleic acid

EPA - eicosapentaenoic acid

EPPO - Olhão Aquaculture Research Station

EU – European Union

FAO - United Nations Food and Agriculture Organization

FBW - final body weight

FCR - feed conversion ratio

FE - feed efficiency

H&E - Hematoxylin Eosin

I- Ingestion

IBW - initial body weight

INE- National Statistics Institute

IPMA - Portuguese Institute for Sea and Atmosphere

NUTRIMU - Nutrition and Immunobiology Research Group

PCR - polymerase chain reaction

Phdp- *Photobacterium damselae* subsp. *piscicida*

PUFA - polyunsaturated fatty acid

RNA - ribonucleic acid

rRNA - ribosomal RNA

WG - weight gain

1. Introduction

1.1. The state of global Aquaculture

The global consumption of fish grew at an average annual rate of 3.1% between 1961 and 2017, an increase greater than the observed consumption of any other animal protein foods, which increased by 2.1% per year in the same period. Besides, the global fish consumption per capita increased from 9.0 kg in 1961 to 20.5 kg in 2018, regarding the live weight of fish (FAO, 2020). Moreover, the human population is rapidly increasing and is expected to reach 9.7 billion by 2050 (Guillen et al., 2019; Panagiotaki and Malandrakis, 2019). This translates into an escalation in the global food demand, particularly in seafood, also due to its recognized health benefits (Bohnes et al., 2020). Fish and other seafoods are an excellent source of protein and healthy fats like omega-3 fatty acids, as well as a source of various essential micronutrients like calcium, iron, zinc, vitamin B12 and D (Cantillo et al., 2021; Telli-Karakoç and Barlas, 2019). By 2030, it is expected that the world will require an extra 25 million tons of aquatic food to provide the current per capita fish consumption (FAO, 2020).

Thus, humanity encounters the considerable challenge of delivering safe and adequate nutrition to a world population significantly rising and, when it comes to seafood, aquaculture plays a key role. Fish and other seafood can come from both capture and aquaculture and, given that the fisheries sector has stabilized over the past decades, this increase in fish consumption is largely supported by aquaculture (Figure 1) (FAO, 2020)

Aquaculture consists in the monitored production of aquatic organisms in freshwater, brackish water, and saltwater, in both coastal and inland areas. In the concept of aquaculture, it is implied that some degree of human intervention exists, in at least one stage of the species' life cycle, to increase productivity, reduce financial losses, prevent damage to animal welfare, and to diminish impacts on public health (FAO, 2020).

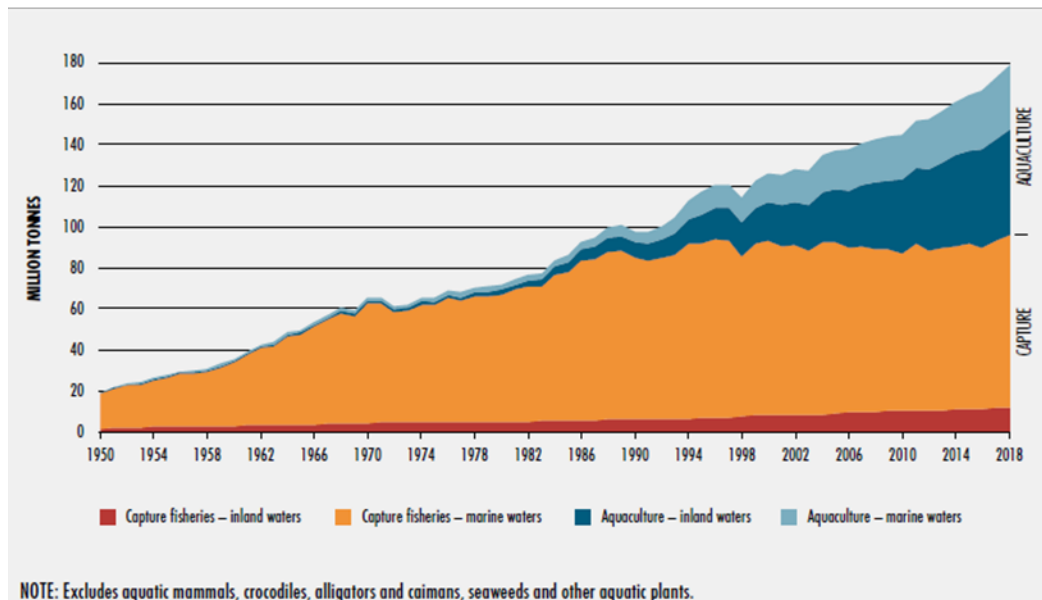

Figure 1- World production of aquatic animals: contribution of fisheries and aquaculture, in inland and marine waters. Retrieved from FAO (2020).

Aquaculture has a 4000-year long history, which began in China as a way to aggregate wild fish for a simpler harvest. This aquaculture was typically unfed, low-density production and may have begun as fishponds. Aquaculture was staged at this level for millennia and progressed slowly through the centuries, often relying on traditional knowledge, passed on from generation to generation, and advances gained through farmers' curiosity and needs. In the twentieth and twenty-first centuries, aquaculture has benefited from scientific progress about the biology of aquatic species and the aquatic environment combined with the introduction of formulated feeds along with increasing global trade. The result in terms of growth has been unparalleled, and nowadays aquaculture supplies more than half of the world's fish for human consumption (FAO, 2020; Tlusty, 2020). This increase of aquaculture production worldwide, by more than a four-fold growth since the 1990s, was primarily driven by Asian countries which produced 92% of the world aquaculture products, with China being the most important producer, producing 58% of the global aquaculture products (STECF, 2021).

According to the most recent statistics by FAO (2020), in 2018, world aquaculture production accomplished a new record high of 114.5 million tonnes in live weight, which correspond to 82.1 million tonnes of aquatic animals, 32.4 million tonnes of aquatic algae and 26 000 tonnes of ornamental seashells and pearls. Moreover, the prospects for aquaculture are quite positive, with the World Bank estimating that nearly two-thirds of the seafood consumption will be farm-raised by 2030 and after this date, it is expected

that aquaculture will dominate global fish supply (Gutiérrez et al., 2020; Panagiotaki and Malandrakis, 2019).

1.2. The state of European Union Aquaculture

In 2018, aquaculture production in the European Union (EU) Member States reached 1215 million tonnes and accounted for €4100 million (STECF, 2021). In the EU, the aquaculture sector can be divided into four main sectors: seaweed, shellfish, freshwater finfish, and marine finfish, with the last being the most valuable economically. The main species farmed in the EU countries are: mussels, oysters and clams regarding shellfish, trout and carp considering freshwater finfish, and seabream, seabass, turbot and salmon regarding marine finfish (Gutiérrez et al., 2020). Regarding the seaweed cultivation in the EU, it is not yet widely commercially viable and it is mainly taking place at the pilot scale, being expected that a full-scale production may be operational within the coming decades (Billing et al., 2020; Blaas and Kroeze, 2014).

In 2018, the EU per capita consumption of fishery and aquaculture products was 24.56 kg, higher than the global consumption rate, which was 20.50 kg (EUFOMA, 2020; FAO, 2020; STECF, 2021). However, even with the increasing demand of seafood in the EU and the increase in aquaculture production worldwide, the aquaculture sector in the EU is not expanding at the same rate as the rest of the world (Hofherr et al., 2015). According to the Economic report 2020 of STECF (2021), the aquaculture production in the EU has increased by 24% from 1990 although since 2007, the production has only increased by 6%. This translates into a low number of new farming licences issued in recent years, demonstrating a clear sign of the struggles for the sector to expand (Hofherr et al., 2015). These struggles had been explained by a set of factors such as the limited access to space and water, obstacles in accessing financial investment and the challenge of battling with third countries, that have less severe regulatory standards and where aquaculture production is cheaper due to lower labour costs and less advanced production technologies (DGRM, 2014; Gutiérrez et al., 2020).

Moreover, the European Union aquaculture production represented only 1.0% of the world aquaculture production in terms of weight and 1.5% in value. In 2018, the aquaculture sector provided around 20% of the fish and shellfish supply in EU and, in the same year, the EU was the largest fish importing market (34% in terms of value),

characterized by a fish trade deficit, depending enormously on external supplies (FAO, 2020; STECF, 2021).

Despite the reduced growth and low world contribution, the EU aquaculture has some characteristics that deserve to be highlighted such as, high technology standards optimized by qualified labour, educational and research institutions, the guarantee of food quality and safety, and an adequate climate for the cultivation of certain species (DGRM, 2014; Gutiérrez et al., 2020; Neculita, 2017).

Furthermore, in order to respond to these difficulties, the European Commission jointly with EU countries are trying to boost the EU aquaculture sector, with a planned investment of €1.2 billion for the period of 2014–2020 (Gutiérrez et al., 2020). This investment in the sector, among others, will result in a projected growth of 51.4% of EU aquaculture by 2030, with aquaculture being identified as one of the sectors with higher potential for sustainable jobs and expansion in the EU (Gomes Ferreira et al., 2020; Gutiérrez et al., 2020).

1.3. The state of Portugal´s Aquaculture

Portugal, an oceanic country with a deep connection to the sea, has a coastline of approximately 2500 km and one of the biggest exclusive economic zones of the world that stretches over 1.7 million km^2. Additionally, the Portuguese maritime triangle, which englobes the Portuguese Mainland, Madeira and Azores, constitutes approximately 50% of all marine waters that are under the jurisdiction of the EU, regarding areas adjacent to the European continent (DGRM, 2021). Moreover, in 2018, Portugal had one of the highest consumption of fishery and aquaculture products at EU level, with 60.92 kg per capita and year (EUFOMA, 2020).

The Portuguese aquaculture has a large market and a long tradition of producing molluscs and fish in fresh and marine water, which in recent years has used advanced and modern technology to increase production (DGRM, 2021). More explicitly, in 2019, the total aquaculture production in Portugal was 14 336 tons, a result that represents an increase of 2.5%, when compared to 2018. Furthermore, in the same year, sales revenue was 118.5 million euros, an increase of 22.4% when compared to 2018 (INE, 2021). Currently, the national aquaculture is characterized by the production in brackish and marine waters, which correspond to 93.4% of the total production in 2019 and, focusing in the marine sector, it increase 39% regarding the produce weight and 87% in value,

between 2008 and 2017 (INE, 2021; STECF, 2021). Most of the aquaculture products produced in Portugal are for national consumption, though the export sales are growing, with an increase of 6% to 33% from 2012 to 2018 in the total of sales (STECF, 2021).

Despite this, Portugal has a shortage of domestic suppliers that can meet the consumption needs of seafood, being forced to import the majority of the seafood, negatively impacting the trade balance of fishery products (STECF, 2021). Even so, Portugal has been investing in aquaculture production, in order to respond to market needs, taking into account the increasing request for fresh seafood for human consumption in the market at national level as well as at the European level (STECF, 2021). Therefore, the national aquaculture is an important alternative to traditional forms of seafood supply and it has gained more relevance since national fisheries production cannot respond to the actual demand of seafood and also due to the limitations in the capture of marine fish (DGRM, 2021). Nevertheless, in 2019, national fishing catches reached an approximate value of 93 thousand tons against 14 336 tons of aquaculture products (INE, 2021).

In 2019, the production in brackish and marine waters was the most significant segment of national aquaculture, and roughly half of the production consisted in the production of fish, mainly in intensive and semi-intensive regime, and the other half in the production of molluscs and crustaceans, predominantly in extensive regime. Regarding distinct species, in Portugal, the main marine fish species are turbot (*Scophthalmus maximus*), seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*), which correspond to 96% of the total production of fish in brackish and marine waters, according with the INE (2021) (Table 1). On the other hand, the production of molluscs and crustaceans decreased compared with the previous year, having represented only 46.6% of the total aquaculture production, compared to 67.2% in 2018, with clams remained the most relevant species, representing nearly 50% of the molluscs' production, followed by oysters and mussels (Table 1). Additionally, in 2019, the production in inland waters was practiced exclusively in the intensive regime, and accounted for 6.6% of the total aquaculture production in Portugal, consisting almost entirely of rainbow trout (*Oncorhynchus mykiss*) (INE, 2021).

Table 1- Aquaculture production in brackish and marine waters, by species. Adapted from INE (2021).

MAIN SPECIES OF BRACKISH AND MARINE WATERS	TONS	%
TOTAL	13 387	93,4% (of the total national production)
FISH	6 657	49.7% [1]
➢ TURBOT	3 580	53.8% [2]
➢ SEABREAM	1 953	29.3% [2]
➢ SEABASS	882	13.2% [2]
➢ SOLE	234	3.5% [2]
MOLLUSCS AND CRUSTACEANS	6 685	49.9% [1]
➢ CLAM	3 276	49.0% [3]
➢ OSTER	1 690	25.3% [3]
➢ MUSSEL	1 457	21.8% [3]
➢ COMMON COCKLE	250	3.7% [3]

[1] of the production in brackish and marine waters

[2] of fish produced in brackish and marine waters

[3] of molluscs and crustaceans produced in brackish and marine waters

1.4. Internship Aims

The internship was divided in two parts, each one with different aims and institutions. The first part was developed in the NUTRIMU (Nutrition and Immunobiology Research Group) from CIIMAR (Interdisciplinary Centre of Marine and Environmental Research), and the second part was performed at EPPO (Olhão Aquaculture Research Station), belonging to IPMA (Portuguese Institute for Sea and Atmosphere).

In the internship with NUTRIMU, the main goal was to obtain skills related with the diagnosis of an unknown pathology in the European seabass, *Dicentrarchus labrax*. It included acquiring expertise in histological analysis techniques and molecular biology techniques of diagnosis as well as bibliographic research related to the topic. Furthermore, this part also included assisting in surveillance campaigns of internal parasites for a commercial aquaculture. At EPPO, the main goal was to develop skills related with marine larvae rearing and to assist in the production of live prey since EPPO is a maternity for the main aquaculture fish species currently produced in Portugal. Furthermore, the internship with EPPO also involved performing an experimental feeding trial followed by a challenge trial that aimed to investigate the effect of dietary algal extracts on meagre growth, feed utilization and disease resistance.

2. Internship with NUTRIMU Group

2.1. Introduction

2.1.1. Fish Health in Aquaculture

Around the world, infectious diseases, caused by bacteria, viruses, fungi, and parasites, are threatening aquaculture´s sustainability and causing enormous economic losses. These diseases cause high fish mortalities, by negatively affecting fish health, growth rate and reproduction, being one of the biggest constraints for aquaculture expansion (FAO, 2005c; Flores-Kossack et al., 2020; Li et al., 2002). A disease is any biotic or abiotic phenomenon that causes abnormality or other disadvantages in a living organism, and include nutritional deficiencies, genetic problems, physical harm, pollution, and pathogens which are disease-causing organisms (Austin, 1989).

In fish, an infectious disease occurs when the host and pathogen are present, in an environment that allows the infection to occur. In aquaculture, due to the aquatic milieu, a large number of species are being produced, along with the diversity of aquatic pathogens, which increases the potential for disease occurrence and propagation (Tlusty, 2020). Furthermore, farming practices alter the natural equilibrium between host and pathogens found in the wild, favouring the emergence of diseases and posing a major problem for the aquaculture industry (Machado et al., 2015).

To maintain fish health in aquaculture systems, a robust approach is to prevent and diagnose (Tlusty, 2020). Prevention implies good handling practices, strict veterinary hygiene, and vaccination. The diagnosis of fish diseases is, if possible, an on-the-spot investigation, however, some fish diseases can only be properly diagnosed with the observation of the dissected fish body and tissue using specific laboratorial material, such as dissection material or microscope (Li et al., 2002). After the diagnosis, it is also required to apply a treatment by disinfection and use of antimicrobial or antiparasitic drugs (Flores-Kossack et al., 2020).

In the diagnosis, there are normally some steps involved. The first step is to observe the aquaculture environment and changes in fish behaviour, and collect information on: provenance, age, and fish species, number and average weight of fish per tank and behaviour of the affected fish. Specifically, some behaviours like not eating, self-isolation, gasping at the surface or jumping out of water, are typical warning signs. It is also essential to collect information on the physico-chemical parameters such as

temperature, pH, ammonia and dissolved oxygen levels (Li et al., 2002). The second step is to observe individual animals, by choosing moribund and/or specimens with abnormal behaviour, to detect clinical signs of a disease, such as fin erosion, cuts, lesions, swollen/damaged gills, and swellings (Li et al., 2002). The third step is to perform a post-mortem examination of the same individuals, with the purpose to find internal abnormalities in organs and tissues, as well as an appropriate collection of samples for further analysis, namely bacteriological, virological and histology examination (Austin, 1989).

2.1.2. Histology and molecular biology in diagnosis of fish diseases

Histology is an area of biology that consists in the microscopic examination of stained tissue sections, cut in the micra scale, with the goal to study their structure and function. Histology is often used for disease investigation in animals, since it can expose signs of disease and pathologies not easily recognized on gross examination, allowing the detection of a wide range of pathogens, being of great interest in the study of fish health (Aranguren and Figueras, 2016; Genten et al., 2009). Additionally, histological observations can reveal the presence of pathogens within infected material even when the animals are not yet clinically diseased (Austin, 1989; Kent et al., 2013). Hematoxylin and eosin (H&E) is a frequently used combination of stains, wherein hematoxylin stains the cell nucleus and other acidic structures in blue and eosin stains the cytoplasmic proteins and an array of extracellular structures from pink to red (Genten et al., 2009). This type of stain is the oversight method of first choice for histology and is commonly used in fish histological samples since it allows to discerning between nuclei and cytoplasm in tissues by staining them in different colours, enhancing contrast between cellular structures (Kuru, 2014; Mumford et al., 2007).

In response to injury caused by a pathology, a variety of changes in cell morphology and function can occur. Injuries often induce non-lethal and reversible changes in cellular structure, such as acute cellular swelling, hydropic change, and fatty change. However, cell injury can proceed to a point of no return, where the cell is incapable to adapt and homeostasis is no longer viable, originating an irreversible injury and cellular necrosis. There are distinct kinds of necrosis that can be recognized histologically such as coagulative, caseous and liquefactive necrosis (Mumford et al., 2007).

In general, inflammation is a nonspecific defence mechanism and it is the basic protective response to tissue damage, being defined as the series of changes that takes place following the injury (Roberts, 2012). Considering a pathology that leads to histological changes, an inflammatory response is initiated in order to resolve the infection, repairing the damage and re-establishing homeostasis, leading to an influx of phagocytic cells to the infection focus, like macrophages and neutrophils that efficiently attack pathogens (Machado et al., 2015). Inflammation can be divided into acute and chronic forms which differ in duration and in histological appearance. The acute inflammatory reaction is characterized by vascular events and exudation, ending when the injurious agent is eliminated, lasting approximately 3 to 10 days. The chronic inflammation, marked by cellular proliferation, can continue from weeks to the lifetime of the host, persisting as long as the injurious agent endures (Mumford et al., 2007). A form of chronic inflammation frequently observed in fish, is the granulomatous inflammation, which consists in a mixture of cells, such as plasma cells, fibroblasts, and leukocytes, oriented around the place of injury (Mumford et al., 2007). Granulomas or granulomata, which may also be observed in granulomatous inflammation, are defined nodular collections of macrophages and debris, surrounded by a layer of lymphocytes, in turn bordered by a layer of fibroblasts (Mumford et al., 2007). Granulomas are white to yellow lesions, which may have a cheesy or hard consistency or even be calcified (Roberts, 2012). Furthermore, granulomas constitute an immune system strategy to isolate microbes or material incapable to be eliminated (Martínez-Lara et al., 2021).

Incited by several mechanisms, including some types of infection, neoplasia is another pathologic event. It is a disturbance of cell growth characterized by the loss of responsiveness to normal cell control mechanisms, resulting in the formation of a tumour (Mumford et al., 2007).

Despite the described advantages of the histological techniques in the diagnosis of fish diseases, a method that allows the detection of a wide range of pathogens, it also has some disadvantages such as being a time consumption method that requires specialized personnel. Furthermore, this method relies on interpretation of results by the personnel, wherein lack of personal experience and familiarization with histological observations difficulties the interpretation (Aranguren and Figueras, 2016; Singla et al., 2017). Moreover, it can also be difficult to definitively diagnose diseases based exclusively on histological analyses (Aranguren and Figueras, 2016). Due to this, molecular biology methods, which are more sensitive, rapid and specific methods, have currently become an important option in the diagnosis of fish diseases, particularly nucleic acid

amplification technology (Aranguren and Figueras, 2016; Kent et al., 2013). The amplification of DNA fragments of a pathogen and subsequent sequence analysis can confirm infections in different hosts (Austin, 2019; Austin and Newaj-Fyzul, 2017). Nevertheless, the molecular methods also have some shortcomings. The detection of the pathogen's DNA with molecular methods reveals the presence of the infectious agent although it does not provide information concerning the viability of the agent to cause an real infection or in which tissue the pathogen is located (Aranguren and Figueras, 2016). Furthermore, false positive or false negative results can occur when using molecular methods (Aranguren and Figueras, 2016; Austin, 2019). Moreover, due to being an ultrasensitive method, molecular methods can detect very low quantities of the infectious agent which are not necessarily relevant for disease diagnosis (Austin, 2019). Therefore, a methodology combining traditional histologic analyses with innovative molecular biology assessments may be an appealing approach in the diagnosis of fish diseases, since this combination can overcome the disadvantages of the methods when used individually.

Polymerase Chain Reaction (PCR) is the nucleic acid amplification technology used most frequently in the diagnosis of fish diseases and is used to amplify certain regions of DNA, being a highly specific and sensitive method of detecting pathogens (Austin and Newaj-Fyzul, 2017; Cunningham, 2002). Within PCR methods, the PCR amplification of rRNA genes allows the detection and identification of pathogens species or strains in aquaculture (Austin and Newaj-Fyzul, 2017; Cunningham, 2002; Wang et al., 2005). This technique focuses on the ribosomal RNA (rRNA) genes, targeting the 16S rRNA or the 18S rRNA, a component of the small sub-unit of the procaryotic and eucaryotic ribosomes, respectively. These genes are ideal targets for diagnosis because they contain highly conserved regions which enable the use of universal primers that will amplify these genes from virtually any eucaryote or procaryote. These genes also contain enough variable regions, which vary between groups and species of pathogens, allowing to distinguish organisms at the appropriate taxonomic level and they occur in various locations of the chromosome, providing multiple targets for primer binding (Cunningham, 2002).

While in pure samples (i.e., samples containing DNA from a single organism), PCR amplicons of 16SrRNA or 18SrRNA can be sequenced directly, in complex environmental samples such as the ones analysed in this study, PCR amplicons must be first separated (e.g., cloning; DGGE) prior to identification through sequencing.

DGGE, which stands for denaturing gradient gel electrophoresis is a highly sensitive technique in separating DNA fragments that differ by as little as a single base substitution. This method allows to differentiate fragments of equal size but with different nucleotide sequences, which migrate to a different position in a gradient denaturing acrylamide gel based on the physical nature, melting behaviour and mobility of double stranded and partially denatured DNA (Ji et al., 2004; Wang et al., 2005). Partial denaturation during DGGE analysis is achieved by adding a GC clamp to the 5´end of one of the oligonucleotide primers used for PCR amplification.

The combination of PCR amplification of rRNA genes and DGGE allows to screen a number of species in a sample, along with the taxonomic identity of each species by sequencing individual DGGE bands, thus leading to a robust detection and identification of pathogens (Wang et al., 2005).

2.1.3. *Dicentrarchus labrax*

The European seabass (*Dicentrarchus labrax*, Linnaeus, 1758) (Figure 2), belonging to Moronidae family, is a coastal marine teleost fish, which lives in shallow waters, along the north-eastern Atlantic Ocean to the Mediterranean and the Black Sea. This fish is euryhaline (0–40 ppt salinity) and eurythermal (5-28 °C) (FAO, 2005c; Vandeputte et al., 2019).

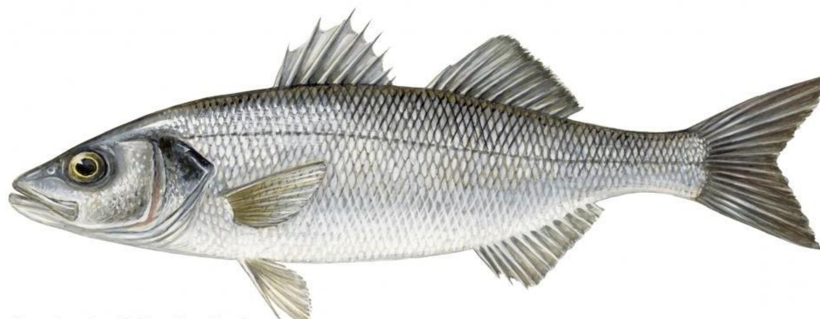

Figure 2- *Dicentrarchus labrax*, Linnaeus, 1758 (European seabass). Source: https://ec.europa.eu/oceans-and-fisheries/ocean/marine-biodiversity/seabass_en

The seabass was the first marine non salmonid species to be commercially cultured in Europe, being currently produced under intensive, semi-intensive or extensive systems (FAO, 2005c; Machado et al., 2015). Regarding the aquaculture status, this species is a widely distributed farmed fish with great importance for the Mediterranean aquaculture,

wherein seabass culture is essentially located, mainly in Turkey, Greece, Egypt and Spain, which accounts for 94% of the production (Stavrakidis-Zachou et al., 2019; Vandeputte et al., 2019). In 2019, the global production of this species reached 263 215 tons (Figure 3), with Mediterranean aquaculture as a major contributor (FishStat, 2021).

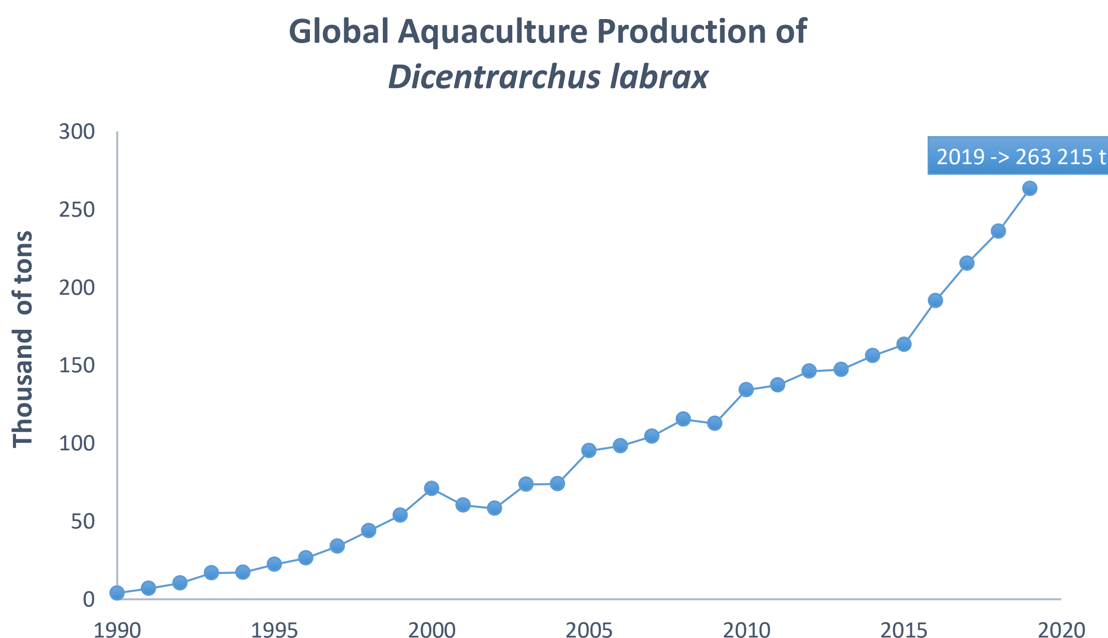

Figure 3- European seabass production throughout the years. Data retrieved from FAO **FishStat (2021)**.

Along with the gilthead seabream (*Sparus aurata*, Linnaeus, 1758), it is the main finfish cultured in Mediterranean areas. Furthermore, after Atlantic salmon, seabream and seabass are the most valuable species in the EU aquaculture sector (Llorente et al., 2020). Moreover, their combined production almost doubled during the 2008 – 2018 period from 245 thousand tonnes in 2008 to 464 thousand tonnes in 2018 and, in Portugal, its offshore aquaculture has started to emerge (STECF, 2021).

Even though a sturdy species under rearing conditions, the European seabass is subject to a variety of diseases, with stress being considered as an important co-responsible for disease outbreaks. In aquaculture, the most common diseases in seabass are related with viruses (e.g. the nervous necrosis virus (NNV) which is the causative agent of viral nervous necrosis); bacteria (e.g. *Vibrio anguillarum; Vibrio ordali; Photobacterium damselae* subsp. *piscicida*; *Tenacibaculum maritimum*; *Mycobacterium marinum)* and parasites (e.g. *Amyloodinium occelatum; Cryptocaryon irritans; Philasterides dicentrarchi; Shaerospora dicentrarchi; S. testicularis; Ceratomyxa labraci; Diplectanum aequans; Anisakis* spp.*; Ceratothoa oestroides; Nerocilla orbiguyi; Anilocra physoides*) (FAO, 2005c; Zrncic, 2020). Additionally, members of the genus *Mycobacterium*

alongside *Aeromonas veronii* bv *sobria*, which is an opportunistic pathogen of fish both in freshwater and in the marine environment, are earning notoriety in the Mediterranean aquaculture industry (Zrncic, 2020).

2.2. Internship Description

At the internship with the NUTRIMU group, which started in September 2020, European Seabass specimens with an unknown pathology were analysed. The fish, belonging to the same batch, came from the "Estação de Zoologia Marítima Dr. Augusto Nobre". This batch was suspected to have a pathology of unknown aetiology, which was later confirmed by the necropsy of fish found dead in the tanks. To diagnose the pathology four fish from the aforementioned batch were sacrificed in June 2019. Next, they were weighed and necropsied, being grossly observed internally and externally. Gross examination of the abdominal cavity revealed several granules of different sizes and textures connected to peri-visceral fat and adjacent tissues. After the necropsy, organ samples (liver, spleen, digestive tract, skin, adipose tissue) and several granules were collected and fixated in buffered formalin (10%, pH 7.4) for 24h and further preserved in ethanol 70% until histology analysis. Similar samples were collected and preserved in RNAlater, for future molecular biology analysis. Summarizing, the internship with NUTRIMU consisted in performing the histological and molecular biology analysis of the four fish who were sacrificed as well as the bibliographic research related to the theme.

Furthermore, I also participated in surveillance campaigns of internal parasites for a commercial aquaculture. I performed the standard histotechniques in sampled organs of the digestive tract of *Sparus aurata.* It included processing the samples as well cutting and staining with H&E. Additionally, I also contributed to the microscope observation of the obtained slides.

2.3. Material and Methods

2.3.1. Histotechniques and microscopical observation

Samples were previously collected from fish with signs of disease, properly fixed in phosphate buffered formalin (10%, pH 7.4) for 24h and stored in ethanol 70% until analysis. The organs were cut in appropriately sized pieces and placed gently in histology cassettes, correctly labelled. After that, all samples were processed using standard techniques in an automatic tissue processor Citadel 2000 (ThermoScientifc), by dehydration in a graded ethanol series up to 100% alcohol followed by clearing in xylol (which is miscible with both 100% alcohol and paraffin) and embedded in paraffin wax. Next, the samples were transferred to an embedding mould of melted paraffin, properly placed and oriented, and hardened at room temperature.

Following, samples were cut into 4 µm thickness sections with a Leica microtome (Leica Systems) and the obtained sections were placed in labelled glass microscope slides and dried in an oven at 40ºC. Subsequently tissue sections were stained with hematoxylin and eosin (H&E) in an automatic slide stainer (Varistain, ThermoScientifc), mounted with glass coverslip using a mounting medium (ThermoScientific), and left to dry for 24 hours.

Protocols regarding the tissue processing and the H&E staining are present in the Annexes (Table 5 and 6 respectively)

The slides were observed under an optical microscope and photographed using the ZEN lite v.3.2 blue software. Histological changes as well as pathological signals and lesions were registered. An extensive literature review was done to verify if the observations correspond to previously reported cases. Since these observations were not sufficient to identify the pathological agent, a molecular biology approach was performed to reach a more accurate identification.

2.3.2. Molecular biology analysis

Regarding the molecular biology analysis, organs previously collected and preserved in RNAlater were analysed. Samples were subjected to different DNA extraction methods, including a manual protocol (Table 7 in the Annexes, adapted from Pitcher et al. (1989)) and the ZymoBIOMICS™ DNA Miniprep (Zymo Research Corp., Irvine, CA, USA), to maximize the success of extracting DNA from the target samples.

Bacterial 16S rRNA and eucaryotic 18S rRNA gene fragments were amplified from the extracted DNAs, by a touchdown PCR on a T100™ Thermal Cycler (Bio-Rad), using primers pairs described in Table 2. PCR mixtures (50 µL) contained 24.75 µL of water (Sigma), 10 µL of GoTaq Buffer 5X (PROMEGA), 5 µL of each dNTPs (2 mM, PROMEGA), 2.5 µL of each primer (10 µM Forward and Reverse), 0.25 µL of GoTaq polymerase (PROMEGA), and 5 µL of DNA template. A 94ºC incubation for 5 min was followed by 10 cycles of 64ºC, 1 min, 65ºC, 1 min and 72ºC, 3 min. The annealing temperature was decreased at every cycle 1ºC, until reach 55ºC. Thus, final 20 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 3 min. Final extension was at 72ºC, 10 min. The PCR products (5 µL of each amplified produc) and a 1 kb Plus DNA Ladder (Thermo Scientific) were resolved by electrophoresis in TAE at 100 V for 30 minutes in a 1% agarose gel stained with 4% of GreenSafe Premium (Nzytech). The gel was visualized in a Gel Doc™ EZ Imager (Bio-Rad, EU) using the Image Lab™ Software v5.2 (Bio-Rad, EU) to analyze PCR product size and purity.

A DGGE approach was then employed to rule out the presence of several organisms and the DGGE methodology was performed according to the method described by Serra et al. (2018). For that, primers containing a GC tail, which allows DNA bands partial denaturation or separation using a denaturing gradient were used. The primers are present in Table 2 and two types of primers were applied, one type for targeting procaryotic DNA and the other type for targeting eucaryotic DNA. Bacterial 16S rRNA and eucaryotic 18S rRNA gene fragments were amplified from the extracted DNAs, by a touchdown PCR performed in a T100™ Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal). PCR mixtures (50 µL) contained 24.75 µL of water (Sigma), 10 µL of GoTaq Buffer 5X (PROMEGA), 5 µL of each dNTPs (2 mM, PROMEGA), 2.5 µL of each primer (10 µM Forward and Reverse), 0.25 µL of GoTaq polymerase (PROMEGA), and 5 µL of DNA template. A 94ºC incubation for 5 min was followed by 10 cycles of 64ºC, 1 min, 65ºC, 1 min and 72ºC, 3 min. The annealing temperature was decreased at every cycle 1ºC, until reach 55ºC. Thus, final 20 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 3 min. Final extension was at 72ºC, 10 min. The PCR products (5 µL of each amplified product) and a 1 kb Plus DNA Ladder (Thermo Scientific) were resolved by electrophoresis in TAE at 100 V for 30 minutes in a 1% agarose gel stained with 4% of GreenSafe Premium (Nzytech). The gel was visualized in a Gel Doc™ EZ Imager (Bio-Rad, EU) using the Image Lab™ Software v5.2 (Bio-Rad, EU) to analyse PCR product size and purity. Then, 10 µL of each PCR product were resolved by DGGE electrophoresis on an 8% polyacrylamide gel composed by a denaturing gradient of 40–80% 7 M urea/40% formamide. DGGE was performed using a DCode universal mutation

detection system (Bio-Rad Laboratories Lda.) during approximately 16 h, at 60 °C, 65 V in 1 × TAE buffer. After that, the gel was stained for 1 h with agitation, with SYBR-Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) and imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal). Finally, distinctive selected bands were excised from the gel and eluted in 20 µL ultrapure water preceding to DNA re-amplification using equal oligonucleotide primers as above, but without the GC clamp. Each PCR product was again resolved by electrophoresis on a 1% agarose gel to confirm successful amplification and integrity of the DNA, before sending to sequencing along with the respective primers (StabVida, LDA, Caparica, Portugal).

Ultimately, the sequenced bands were explored with SnapGene® software and phylogenetically analysed, to identify the closest known species, by comparison with sequences in the GenBank non-redundant nucleotide database with BLAST (http://www.ncbi.nlm.nih.gov).

Table 2- List of primers used for DGGE analysis. Direction: Forward (F) and Reverse (R). The primers 16S-355F and 16S-517R were used to targeted procaryotic DNA. The primers EuK1A and Euk516R were used to targeted eucaryotic DNA.

Primer Name	Sequence (5' – 3')	Reference
16S-355F [1]	GGCACGGGGGGCCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
16S-517R	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
EuK1A	CTGGTTGATCCTGCCAG	(Díez et al., 2001)
Euk516R [1]	ACCAGACTTGCCCTCC	(Díez et al., 2001)

[1] contains a GC clamp at the 5' end

2.4. Results

2.4.1. Histologic analyses

All the organs preserved in ethanol 70% were observed and evident histological changes were detected in the following organs and tissues: liver, pyloric ceca, stomach, spleen, and adipose tissue.

Regarding the spleen, it appeared to have various melanomacrophage centers (Figure 4).

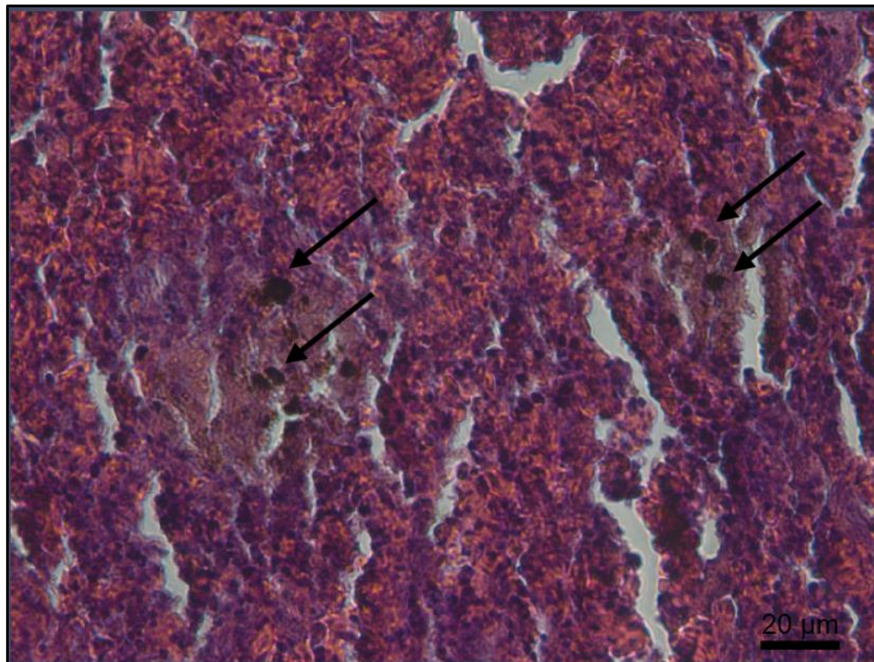

Figure 4- Histological changes in the spleen, melanomacrophage centers (arrows) in the spleen.

Regarding the liver, it appeared to have granulomas surrounding the organ (Figure 5A) as well as in the parenchyma (Figure 5B) and it appears to be vacuolated, with high hepatocytes vacuolization (Figure 5C). A normal liver of *Dicentrarchus labrax*, also stained with H&E, as a comparison term with Figure 5C, is present in Figure 5D.

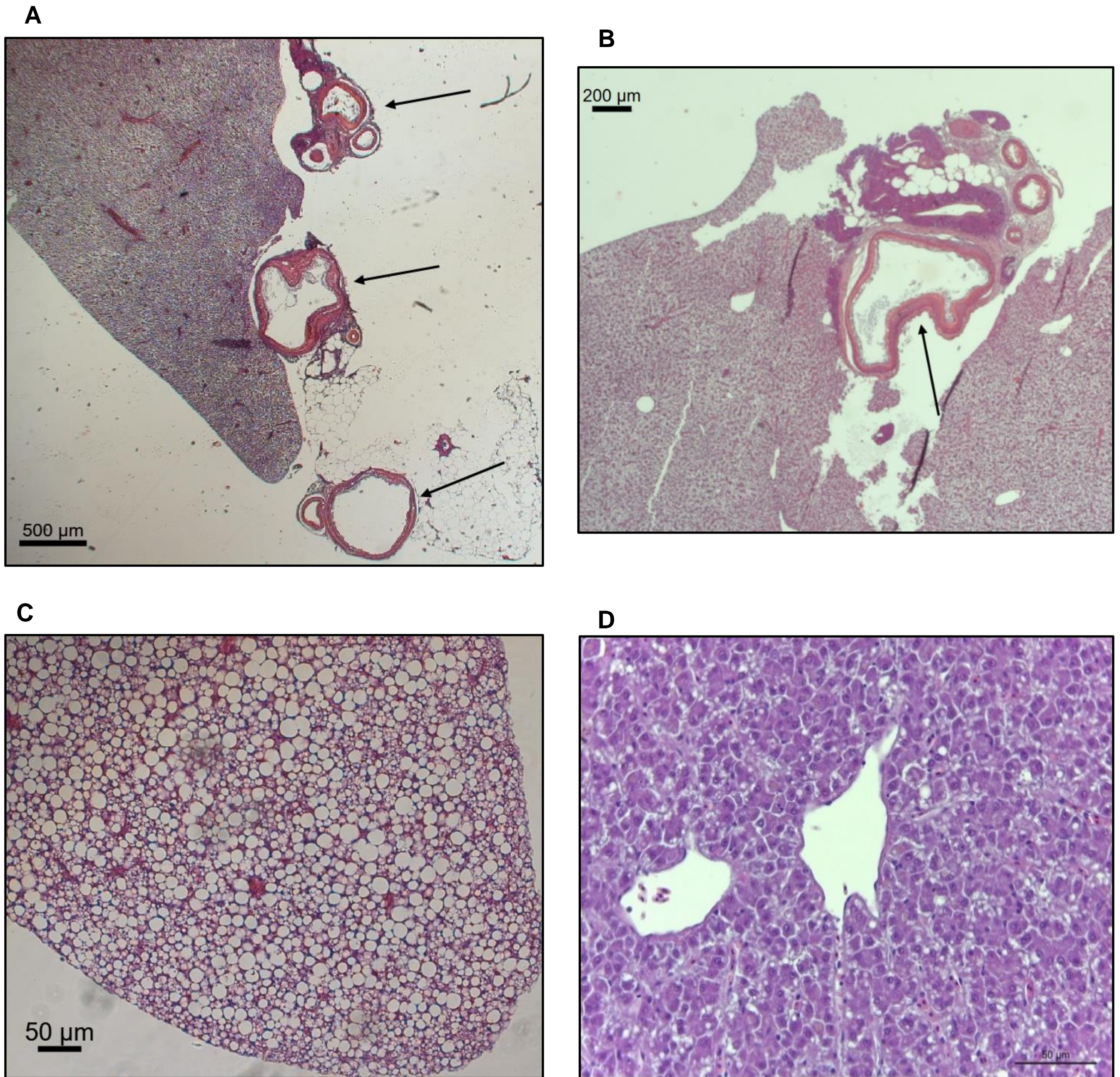

Figure 5- Histological changes in the liver. **A.** granulomas (arrow) in the surrounding of the liver. **B.** granulomas (arrow) in the liver parenchyma. **C.** liver with high hepatocytes vacuolization. **D.** Normal liver of *Dicentrarchus labrax* retrieved from Saraiva et al. (2015).

Regarding the digestive tract, the pyloric caeca and stomach appeared to have granulomas in the adipose tissue surrounding the organs (Figure 6A and 6B, respectively).

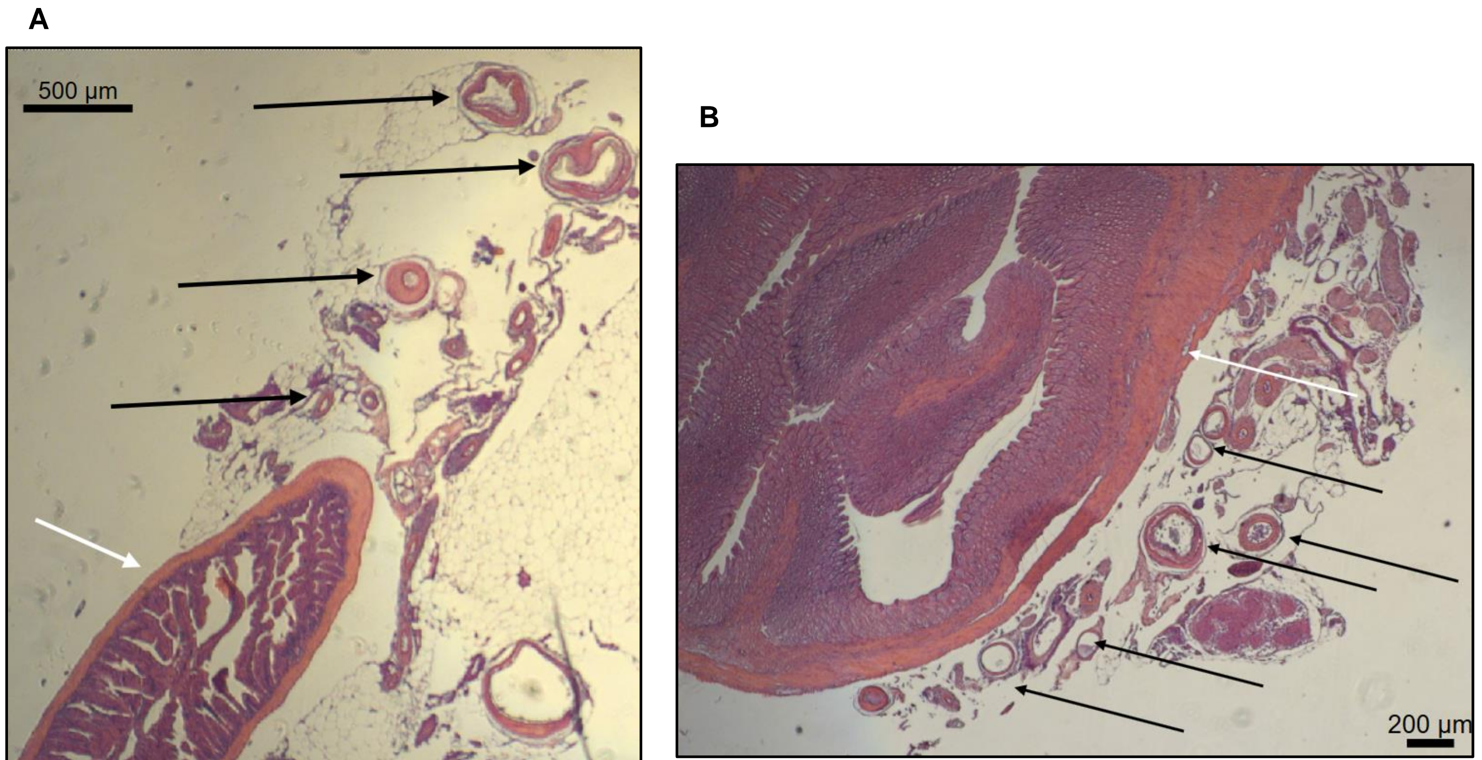

Figure 6- Histological changes in the digestive tract. **A.** pyloric caeca (white arrow) surrounded by a dense granuloma formation (black arrows) in the adipose tissue. **B.** stomach (white arrow) surrounded by dense granuloma formation (black arrows) in the adipose tissue.

Regarding the granulomas (in more detail in Figure 7), they appear to be inside the liver (Figure 7A-B) and connected to the adipose tissue surrounding the liver and the digestive tract (Figure 7C-D), as reported above. In detail (Figure 7D), an onion ring granuloma, with epithelioid macrophages surrounding a necrotic centre, appears connected to the adipose tissue (Talaat et al., 1998).

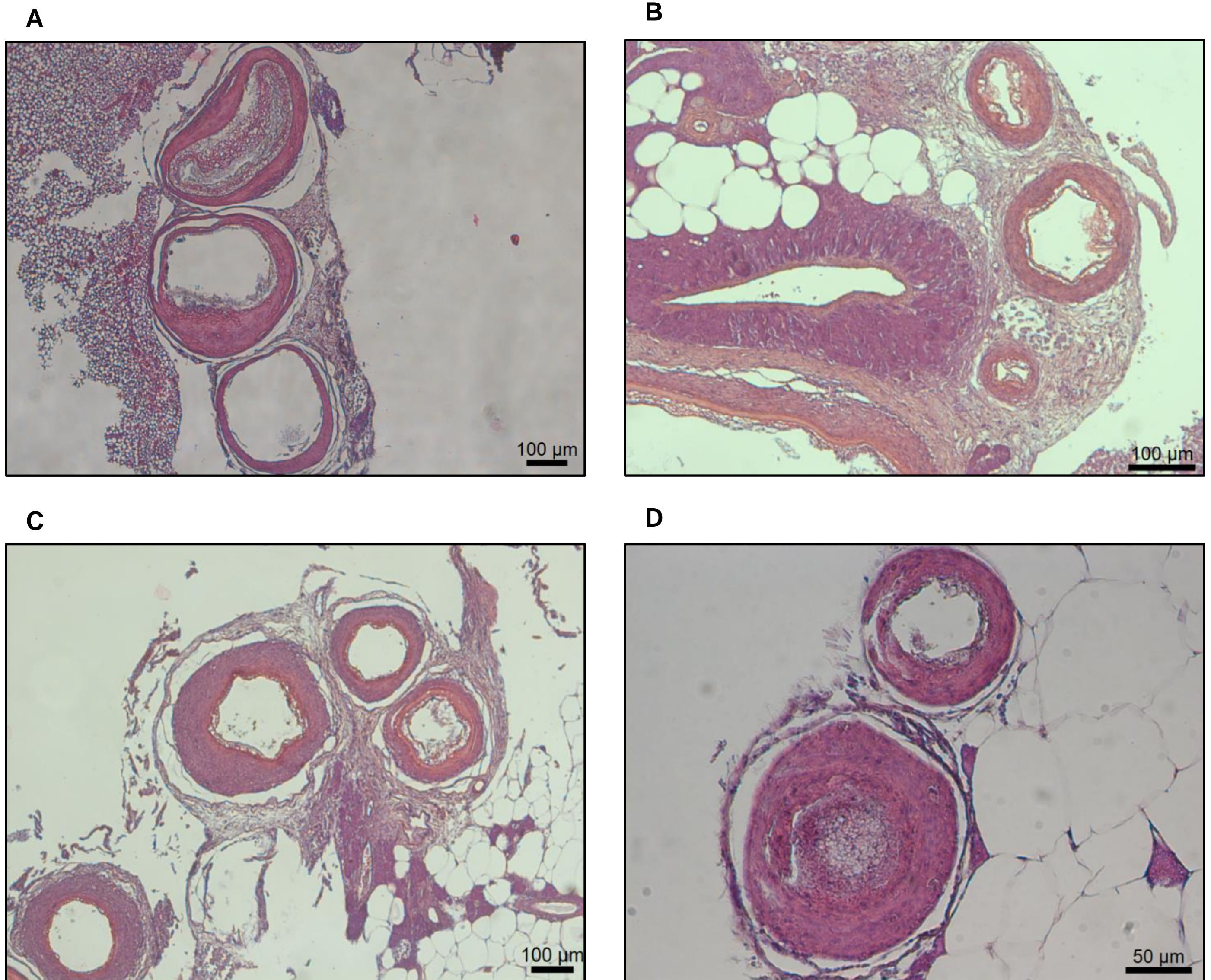

Figure 7- Detailed view of the granulomas. **A-B.** granulomas in the liver. **C-D.** granulomas in the adipose tissue that surrounding the liver and the digestive tract. **D**. onion ring granuloma, with epithelioid macrophages surrounding a necrotic centre.

2.4.2. Molecular biology analyses

To obtain the most diversified data set, bands with different sizes were selected from the samples loaded into the DGGE (Figure 8). Besides differential sizes, bands were chosen according to intensity (which correlates with concentration) and novelty.

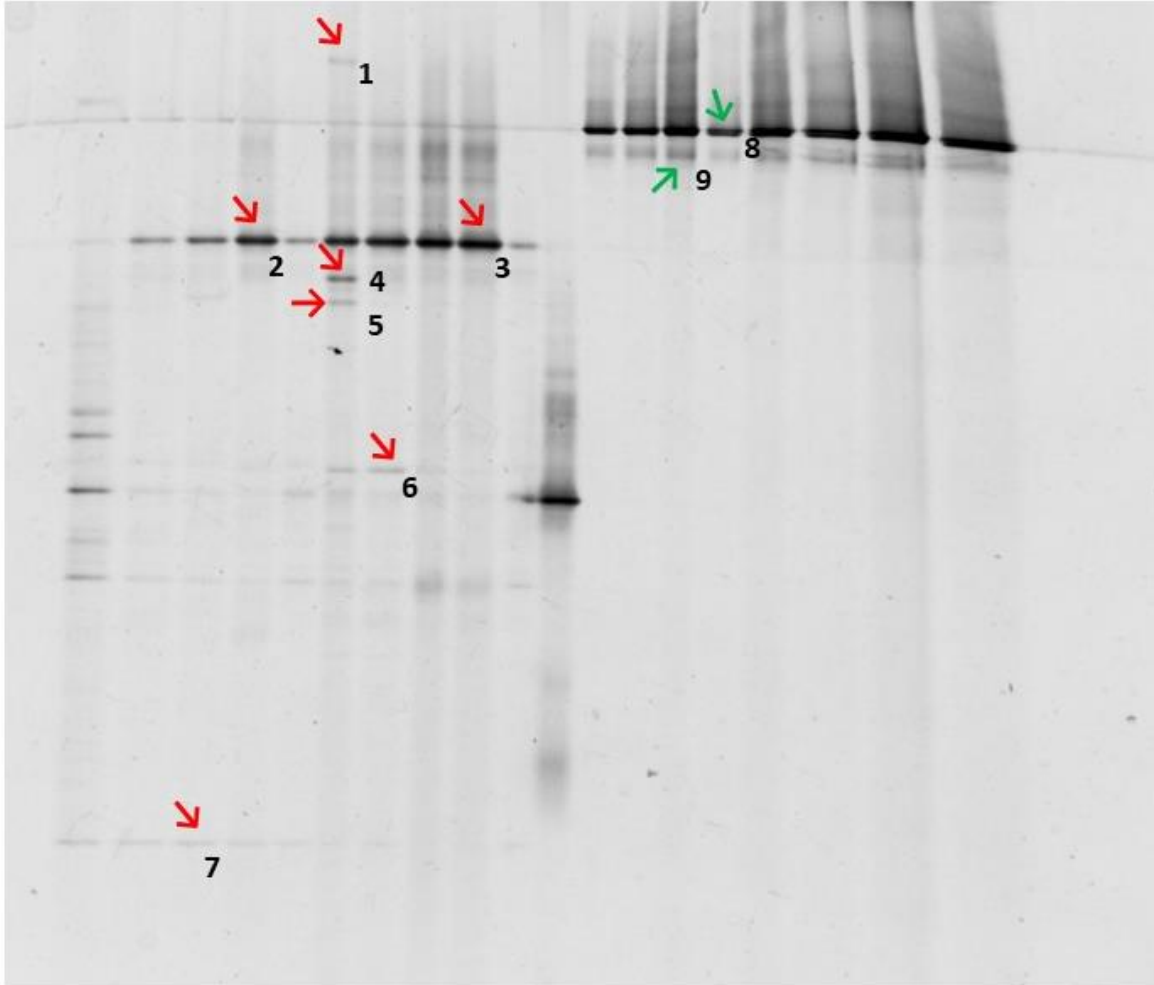

Figure 8- DGGE selected bands. Selected bands (band 1 to 7) amplified with procaryotic primers (red arrows), and selected bands (band 8 and 9) amplified with eucaryotic primers (green arrows). Column correspondence: **1º** - Plus DNA Ladder; **2º to 5º** - DNA extracted with **manual protocol** and amplified with **procaryotic primers**; **6º to 9º** - DNA extracted with **DNA Miniprep** and amplified with **procaryotic primers**; **10º** - negative control; **11º** - positive control; **12º to 15º** - DNA extracted with **manual protocol** and amplified with **eucaryotic primers**; **16º to 19º** - DNA extracted with **DNA Miniprep** and amplified with **eucaryotic primers**

Identification of the selected DGGE bands from the collected organs of the European seabass is shown in Table 3. The bands 1 to 7 were amplified with primers for procaryotic organisms and band 8 and 9 were amplified with primers for eucaryotic organisms.

Table 3- Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the DNA extracted from the collected organs of European seabass.

Band	Nearest neighbour	Similarity to nearest neighbour (%)	Accession number of nearest neighbour
1[1]	Uncultured *Pseudomonas* sp.	87	MF034595.1
2[1]	*Xiphias gladius*	99	MH331278.1
3[1]	*Xiphias gladius*	99	MH331278.1
4[1]	*Pseudomonas azotoformans*	92	MN733445.1
5[1]	*Pseudomonas* sp.	87	MH236690.1
6[1]	*Vibrio* sp.	98	DQ659046.1
7[1]	Uncultured bacterium clone	96	KF064537.1
8[2]	*Morone saxatilis*	100	XR_004797324.1
9[2]	*Morone saxatilis*	100	XR_004797324.1

[1] bands amplified with procaryotic primers

[2] bands amplified with eucaryotic primers

Sequence analysis showed that the bands amplified with primers for procaryotic organisms corresponded to uncultured bacteria or to bacteria of the genera *Pseudomonas* and *Vibrio* in addition to *Xiphias gladius,* which is a fish species known by the common name of swordfish. Moreover, the bands amplified with primers for eucaryotic organisms correspond to *Morone saxatilis,* which is the striped seabass.

2.5. Discussion

Considering the histological observations, the most significant finding is the presence of granulomas, that appear to be connected to the adipose tissue that involves the liver and the digestive tract and inside the liver. A result of chronic inflammation, granulomas can be associated with biotic agents as well as abiotic ones which the macrophagic lysosomes cannot digest (Colorni, 1992). Other findings with the histological observations were high hepatocyte vacuolization and the presence of various melanomacrophage centers in the spleen.

There are several agents know to be behind granulomatous lesions. The most frequent organisms associated with fish granulomatous infections are bacteria belonging to the genera *Mycobacteria* spp., *Nocardia* spp. and *Francisella* spp. (Martínez-Lara et al., 2021). The granulomas are considered to be vital host-protective structures against virulent pathogens, by constraining the growth of infection as an attempt to enclose the bacteria (Elgendy MY et al., 2015).

Particularly, the most common disease associated with granulomas is the fish mycobacteriosis, also known as piscine tuberculosis and it can affect the European seabass (Baumgartner and Hawke, 2011; FAO, 2005c; Zrncic, 2020). This is normally a chronic progressive disease caused by various species of the genus *Mycobacterium,* where *Mycobacterium marinum* is the most common etiological agent of this pathology, representing a significant threat to seabass culture in the Mediterranean (Davidovich et al., 2020; Zrncic, 2020). Regarding the clinical signs of this disease, since typically it is a chronic progressive disease, it could take some time to develop clinical signs, where external clinical signs are usually non-specific. Furthermore, internally, the infected fish may exhibit spleen, kidney and liver enlargement as well as granulomas in these internal organs (Zrncic, 2020), which was not the case in the sampled fish, except in the liver, that exhibited granulomas.

The clinical signs of disease from nocardiae are similar to mycobacteriosis (Baumgartner and Hawke, 2011).

Another disease that affects seabass and is associated with granulomas is the pasteurellosis, where, in a more chronic form of the disease, typical pseudotuberculi, that resemble tubercle of tuberculosis but due to other causes, develops primarily in the spleen and/or kidney parenchyma, which are creamy-white granulomatous nodules, which was also not the case in the sampled fish. The causative agent of this disease is the *Photobacterium damselae* subsp. *piscicida* (FAO, 2005c; Zrncic, 2020).

However, a metabolic disorder also can cause granulomas, similar to systemic granulomas observed in other cultured fish species, characterized by multiple granulomas in all soft tissues (Tsertou et al., 2018). For example, systemic granulomatosis in meagre has been linked with vitamin deficiencies in the diet, principally vitamins from the C and B complexes (Soares et al., 2018).

Regarding the high hepatocyte vacuolization observed in this study, it may not be a pathological situation, since the livers of farmed fish have a tendency to be moderately to heavily vacuolated, as a result of storage of carbohydrate or lipid reserves, which suggests an excess of energy intake (Wolf and Wheeler, 2018). Therefore, the fat content of the liver is quite variable in fish (Mumford et al., 2007). Despite this, since the histological results showed granulomas in the liver, the high hepatocyte vacuolization can also be related with the granulomas.

Concerning the presence of various melanomacrophage centers in the spleen, they are a characteristic immune cell type of teleosts prevalent in this organ and consist in aggregates of pigmented cells, wherein pigments such as melanin act as a scavenger for free radicals (Mumford et al., 2007; Noga, 2010). Furthermore, these centers also act as metabolic dumps since macrophages, replete with particulate matter of microbial origin as well as metabolic waste products as a result of phagocytosis, migrate selectively to these centers (Roberts, 2012). The role of melanomacrophage centers in the fish immune system is not clear, however unhealthy fish tend to have more and larger melanomacrophage centers as well as chronically stressed fish (Genten et al., 2009; Noga, 2010; Sales et al., 2017). Nevertheless, it is necessary to know the normal prevalence of melanomacrophage centers in a particular fish species and age to extrapolate their abnormal aspect and quantity (Noga, 2010). A study by Elgendy MY et al. (2015), where moribund European seabass were examined, histopathological alterations such as different granulomas in the liver, kidney and spleen, with hyperactivity of melanomacrophage centers in spleen were detected, wherein the majority of the diseased fish were infected with *P. damselae* subsp. *piscicida*.

Although not detecting the most common bacterial pathogens associated with granulomatosis (e.g., *Mycobacteria* spp., *Nocardia* spp. and *Francisella* spp), the molecular analyses of seabass lesions revealed the presence of bacteria of the *Vibrio* (band 6) and *Pseudomonas* (band 1, 4, 5) genera.

The genus *Vibrio* comprises more than 130 species of bacteria, which are halophilic, facultative aerobic, gram-negative, motile, ranging in size and morphology from coccobacilli to definite rod-shape cells, being the most common shape the curved-rod

(comma -like) (Abdelaziz et al., 2017; Colwell and Grimes, 1984; Möller et al., 2021). This genus is found in brackish and marine water, being the most dominant heterotrophic bacteria in marine environments, also found on the surface and internal organs of higher organisms, such as fish, being waterborne bacterial pathogens (Arab et al., 2020; Jun and Woo, 2003). Moreover, these bacteria vary from symbiotic to pathogenic, wherein stress can compromise the animal host, resulting in a commensal species shifting to pathogenicity (Arab et al., 2020; Colwell and Grimes, 1984; Möller et al., 2021).

Vibriosis, caused by bacteria of the genus *Vibrio*, is a devastating epizootic disease affecting most wild and farmed fish species worldwide and has become a severe limiting factor for the development of intensive mariculture industry, causing high mortalities with severe economic losses worldwide (Abdelaziz et al., 2017; Jun and Woo, 2003). This disease is associated with deterioration of culture conditions and factors like high salinity (30–35 ppt), organically polluted water, high temperature, parasitic infestation, and mechanical injuries, which increase fish susceptibility to vibriosis (Abdelaziz et al., 2017; Jun and Woo, 2003; Noga, 2010). Among the various fish species susceptible to vibriosis, the European seabass is included (Arab et al., 2020; FAO, 2005c; Korun and Timur, 2008; Zrncic, 2020). *Vibrio anguillarum* is the most common fish - pathogen vibrio (Noga, 2010). Regarding the clinical signs of vibriosis, most of the infected fish exhibit loss of appetite, abnormal swimming behaviour with head floating near the water's surface, darkened body coloration and pale gills (Jun and Woo, 2003; Korun and Timur, 2008). Additionally, clinical signs such as haemorrhagic areas near the mouth and at the base of fins, skin ulceration, exophthalmia, corneal opacity, rotted fins and tails, abdominal distension are usually present in severely infected fish. Internally, moribund fish show severe anaemia wherein the liver is pale and with petechiae, and the kidneys and spleen are congested (Colwell and Grimes, 1984; Jun and Woo, 2003; Korun and Timur, 2008). Histologically, diseased fish may exhibit haemorrhage in the affected organs such as skin, gills, kidney and liver, reduced areas of hemopoietic tissue and deposition of hemosiderin in the melanomacrophage centres in the spleen and necrosis in the interstitial tissues of both kidney and liver. Spleen, kidney and liver are the main target organs of vibrio infection (Jun and Woo, 2003; Korun and Timur, 2008). Furthermore, granulomas, which can be caused by a very wide range of pathogens, can also be associated with *V. anguillarum*, that may cause bacterial granulomas in a chronic phase, wherein skin lesions, that can extend deep into the muscle, may organise and become granulomatous lesions in chronically infected fish (Noga, 2010; Roberts, 2012).

Nevertheless, the genus *Vibrio* is also composed of probiotic species wherein some species produce hydrolytic enzymes assisting in the breakdown of dietary components, acting as symbionts (Egerton et al., 2018).

Regarding the presence of bacteria of the genus *Pseudomonas* in the examined samples (band 1, 4, 5), the species *Pseudomonas azotoformans* (band 4) is a bacterium that infects cereal grains, especially rice, and is included in the *Pseudomonas fluorescens* phylogenomic group, based in phylogenetic studies (Circella et al., 2020; Fang et al., 2016; Garrido-Sanz et al., 2017).

The genus *Pseudomonas* comprises more than 144 species, which are gram-negative, oxidase positive, aerobic bacteria (Circella et al., 2020; Gomila et al., 2015; Roberts, 2012) . Regarding their habit, they are commonly found in decaying organic material like rotting leaves and soil, being ubiquitous inhabitants for oxygenated environments, with high adaptation capability, which allows them to survive in a large range of environmental conditions (Circella et al., 2020; Urku, 2021).

Additionally, various *Pseudomonas* species are pathogenic for humans, animals and aquatic organisms, being one of the most common bacterial infectious agents of cultured fish, triggering stress-related diseases particularly under farming conditions (Duman et al., 2021). Usually, *Pseudomonas* spp. is described as opportunistic pathogens of fish, wherein under stressful conditions such as malnutrition and overcrowding, the bacteria become pathogenic, causing serious illness. Even so, some species of the genus also have been identified as the primary pathogen of several diseases in farmed fish, and fish pathogenic pseudomonas have been reported increasingly in the past decade (Duman et al., 2021; Urku, 2021).

Pseudomonas fluorescens is one of the pathogenic species of this genus, usually associated with environmental stress, mainly high temperatures or overcrowding, being recognised as one of the causal agents of bacterial haemorrhagic septicaemia in fish (Roberts, 2012). Another pathogenic species is the *Pseudomonas aeruginosa*, an opportunistic pathogen, associated with septicaemia in aquatic animals, as well as associated with stress (Lamari et al., 2017). One more species reported to be pathogenic in fishes is the *Pseudomonas putida*, also considered an opportunistic human pathogen (Oh et al., 2019). Fish suffering from *Pseudomonas* spp. infections may exhibit external signs such as discoloration of the skin, tail/fin rot and skin lesions and, in more severe cases of illness, haemorrhagic septicaemia, gill necrosis, abdominal distension, splenomegaly, friable liver, and congested kidney (Algammal et al., 2020; Lamari et al., 2017).

Among the various fish species susceptible to *Pseudomonas spp*., infection is the European sea bass (Duman et al., 2021; Urku, 2021). A study by Urku (2021) described a *Pseudomonas* infection caused by *P. putida* in European sea bass, where the diseased fish presented white nodules in the liver and kidney and histologically, the liver and kidney presented granulomatous lesions and gram-negative bacilli shaped bacteria in these lesions. Furthermore, of the 10 specimens analysed in the study, only 3 exhibit haemorrhagic skin lesions, wherein the rest of the specimens didn't appear to have external clinical signs.

Despite this, the *Pseudomonas* genus contains potential probiotics species for the aquaculture industry, being abundant in aquatic environments and in fish gut. Moreover, according to Serra et al. (2021), it even can be part of the European seabass core microbiota. Furthermore, a survey by Nikouli et al. (2018), revealed the existence of a core gut microbiota consisting of *Pseudomonas* in the European seabass.

Regarding the correspondence of amplified bands with procaryote primers to *Xiphias gladius* (band 2, 3), a fish commonly known as swordfish, which is eucaryote, is justified by the non-specific ligation of the procaryote primers to sequences of eukaryotic origin due to the unrestricted conditions of the performed PCR, affecting the amplification of 16S rRNA gene sequences (Vieira, 2017).

Moreover, the amplified bands with eucaryote primers matched to *Morone saxatilis* (band 8,9), which is the striped seabass, a fish that belongs to the same family (Moronidae) as the European seabass (El Mohajer et al., 2022). This is justified by the resemblance of the 18S rRNA gene portion in closely related species and by the fact that the amplified sequence, part of the 18S rRNA gene, had approximately 500bp which is relatively small to discriminate to the species level. Furthermore, since the eucaryote primers only amplified fish DNA, this indicates that no other eucaryote species was presented in the examined samples, thus eliminating the possibility of existing parasitic species in the samples.

2.6. Concluding remarks

The exact aetiology behind the diseased European seabass remains to be clarified. The main bacterial pathogens traditionally associated with fish granulomatous infections (i.e., *Mycobacteria* spp., *Nocardia* spp. and *Francisella* spp.) have not been detected in this study. At the lesions level, the molecular analyses point out to bacteria belonging to the genus *Vibrio* spp. or/and *Pseudomonas* spp., which can be supported by the histological findings of the granulomas, according with Roberts (2012) and Urku (2021), respectively. Our results might thus provide an additional indication that species of these genera can indeed be responsible for the development of granulomas in seabass.

Regardless, to gather more definitive assumptions, it would be worth conducting in the future a more thorough molecular analysis with the main goal of unveiling the particular specie(s) within the genera *Vibrio* and *Pseudomonas* and if they are indeed pathogenic fish species or not. Additional molecular analysis such as multiplex PCR assay, could be used as tools for discriminating similar species within both genera found, as exemplified by Kim et al. (2019) to the genus *Vibrio*. Additionally, a high-throughput approach such as Next Generation Sequencing (NGS), due to its much higher sequencing depth, enabling higher sensitivity, might be a powerful tool to discover rare, still unidentified species associated with granulomatosis (Subasinghe et al., 2019)

Nevertheless, this unknown aetiology may even not be related with the genus *Vibrio* or *Pseudomonas,* since it can be related with an abiotic phenomenon, such as the metabolic disorder observed in other cultured fish species, characterized by multiple granulomas in soft tissues (Tsertou et al., 2018), being the presence of *Vibrio* or *Pseudomonas* at the lesion site, an opportunistic phenomenon.

3. Internship at Olhão Aquaculture Research Station (EPPO)

3.1. Introducing EPPO

The Olhão Aquaculture Research Station (EPPO), located in the Ria Formosa Natural Park, Olhão, Algarve, belongs to the Portuguese Institute for Sea and Atmosphere (IPMA). It is an infrastructure of notable features, designed to perform and conduct scientific research as well as development and experimental demonstration at pilot scale of innovative solutions relevant to the national aquaculture.

Regarding the EPPO facilities, it occupies an area of about 7 ha, containing a hatchery area, a pre-fattening area in fiberglass tanks and an area for fish growing in earth ponds (Figure 9) (IPMA, 2021). With this, EPPO has the capability to complete the entire reproductive cycle of various marine species adapted to captivity, and to perform trials at different stages of the fish production cycle, including studies of welfare, pathology, physiology, and nutrition. Grouper (*Epinephelus marginatus*), meagre (*Argyrosumus regius*), sole (*Solea senegalensis*), sardine (*Sardina pilchardus*), seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) are some of the species currently produced at the station. Additionally, different types of marine algae, such as microalgae and macroalgae, and echinoderms, such as sea urchin, are also cultured at the station (IPMA, 2021; Teixeira, 2016).

In parallel with the marine aquaculture there is also the production of phytoplankton and zooplankton constitutes what is known as support culture, important to produce live prey to feed the numerous marine species produced at EPPO. Concerning the live prey, the zooplankton organism's rotifers (*Brachionus* sp.) and *Artemia* sp. are produced at EPPO. Regarding the phytoplankton, microalgae are used to feed the rotifers and to enrich both the rotifers and artemia.

The activities carried out at EPPO including scientific research and various trials are supported by national and international funding projects, also relying on the cooperation of other research institutes, universities, and private companies. Furthermore, this station plays an important role in both professional and academic training, frequently accepting members of the aquaculture sector as well as students from national and international educational organizations, supporting numerous internships and dissertations.

Figure 9- Outside view of EPPO, highlighting the earth tanks. Retrieved from Teixeira (2016).

EPPO also has a remarkable humanitarian aid feature, donating products produced in the station to charities, especially since the beginning of the pandemic of coronavirus disease, wherein the EPPO already offered at least 4.2 tons of fish (Sul Informação, 2020).

3.2. Internship Description

The internship at EPPO started in April 2021 and lasted 3 months, finishing in July 2021. This part of the internship was divided in two phases. In the first phase, I performed a feeding trial with meagre, using algae extracts supplemented in fish diets, followed by a bacterial challenge with *Photobacterium damselae* subsp. *piscicida*. The other phase consisted in following the daily routines carried out at EPPO, wherein tasks associated with marine larval cultivation, assessment of larval mortality and assisting in the production of live prey for the larvae were carried out and are described in this report. The marine larvae species that I was involved with during the work period were sardine, seabass, seabream, and meagre, which were produced according to the availability of eggs and necessity at the time, either for larval trials or to produce adult fish.

Moreover, I also participated in various occasional activities occurring at EPPO. It included assisting in fisheries in the exterior earth tanks (Figure 10), with the goal of monitoring the occurrence of fish parasites. I also aided in several fish samplings of different experimental trials as well as transportation of fish between various tanks.

Figure 10- Fishing in the exterior earth tanks, using a fishing rod with a hook attached to a mussel, used as bait. Picture of me catching a seabream, in April 2021.

3.3. Zebralgrae Trial

3.3.1. Introduction

3.3.1.1. *Argyrosomus regius*

Meagre (*Argyrosomus regius*, Asso 1801) (Figure 11) is a marine fish belonging to the Sciaenidae family, being widespread all over the Mediterranean Sea and in the Black Sea, also found along the Atlantic coasts of Europe and Africa (Duncan et al., 2013; Papadakis et al., 2018). Furthermore, it is a carnivorous species that, in the wild, feeds on small crustaceans and fish and inhabits coastal ecosystems near the continental shelf, frequently near lagoons or river deltas. (Mansour et al., 2017; Papadakis et al., 2018). In its natural habitat, this species can grow up to two meters and reach more than fifty kilograms (FAO, 2005b).

Figure 11- *Argyrosomus regius*, Asso, 1801 (Meagre). Source: https://www.diversifyfish.eu/meagre-workshop.html

Regarding meagre aquaculture status, is an emerging and promising species in European aquaculture due to some interesting biological features. These features include fast growth rate in captivity (around 1kg per year), thus reaching large commercial sizes quite rapidly and low feed conversion ratios (Duncan et al., 2013; FAO, 2005b; Soares et al., 2018; Tsertou et al., 2018). Additionally, this species appears to tolerate temperature and other environmental fluctuations, having the capacity to endure diverse environmental conditions (Soares et al., 2018; Stavrakidis-Zachou et al., 2021). Moreover, from a consumer perspective, meagre has low fat content and appreciated flesh quality and flavour, with its specific epithet *regius* standing for royal quality of the fish flesh (Costa et al., 2017; Pérez et al., 2014; Tsertou et al., 2018). Since meagre is a relatively new species in the aquaculture industry, one of the requirements for its

successful introduction is to know the main pathological problems that may arise (Tsertou et al., 2018).

Concerning meagre production, it has been cultured in increasing amounts throughout the Mediterranean region (Papadakis et al., 2018). Moreover, according to Mansour et al. (2017) the total aquaculture production of this species has risen 40-fold in the Mediterranean region, between 2007 and 2017. The global production of meagre has expanded (Figure 11) and, in the space of two decades, rose from 30 tons to 37 thousand tons, reaching 37 526 tons in 2019 (FishStat, 2021). Furthermore, meagre has the potential to become a mass market species, shifting from the present position of a niche species with a limited production (Monfort, 2010).

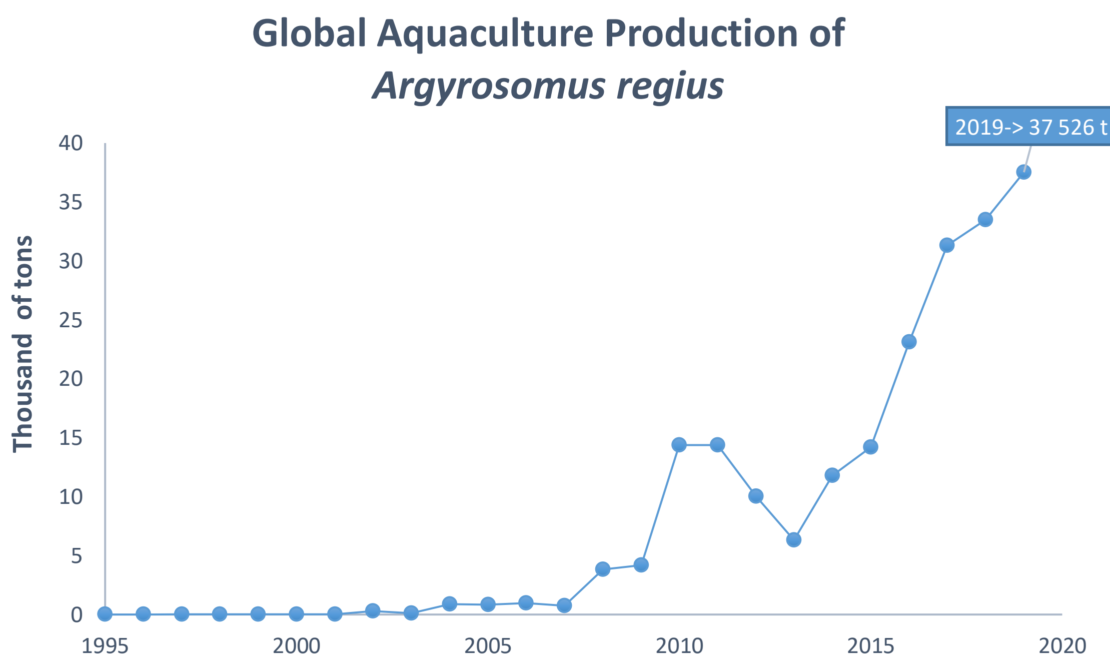

Figure 12- Meagre production throughout the years. Data retrieved from FAO FishStat (2021).

3.3.1.2. *Photobacterium damselae* subsp. *piscicida*

Photobacterium damselae subsp. *piscicida* (*Phdp*), belongs to genus *Photobacterium* and family Vibrionaceae, is a halophilic Gram-negative, non-motile, obligate halophilic bacterium (Zappulli et al., 2005; Zrncic, 2020) .This bacterium and *Photobacterium damselae* subsp. *damsela* are genotypically homogeneous subspecies of *Photobacterium damselae* (Zrncic, 2020). Both subspecies are considered pathogenic for marine fish though only the subspecies *piscicida* causes acute infections with high

mortality rates, with the subspecies *damselae* associated with skin ulcers (Egerton et al., 2018; Zappulli et al., 2005).

This bacterium is the etiological agent of the photobacteriosis disease also named fish pasteurellosis (Machado et al., 2015; Soares et al., 2018). The former name of this bacterium is *Pasteurella piscicida,* therefore the term "pasteurellosis" is still used to refer to this disease (Noga, 2010; Zrncic, 2020). Is one of the most serious bacterial diseases in warm and temperate marine aquaculture causing massive mortalities and massive economic losses in several marine fish species worldwide, especially in the larval and juvenile phases (Aslam et al., 2018; Elgendy MY et al., 2015; López et al., 2012; Noga, 2010). This disease provokes major losses among cultured fish species in Europe, with meagre amongst the affected species, with mortalities reaching 50% (Costa et al., 2017; Zrncic, 2020). Moreover, this disease is associated with high temperatures, wherein outbreaks occur when the water temperature rises above 18ºC (Noga, 2010; Zrncic, 2020).

Regarding the clinical signs, pasteurellosis is associated with the presence of white nodules and haemorrhagic septicaemia in the internal viscera (López et al., 2012; Peixoto et al., 2017). Furthermore, this disease can evolve to an acute or chronic form. In the acute form, a limited number of clinical signs appear, wherein may occur small haemorrhages around the gill or fins, abnormal skin pigmentation and enlarged spleen and kidney. In contrast, in the chronic form, small lesions, composed of bacteria that incite a chronic inflammatory response, may appear in the kidney and spleen, reminiscent of the granulomas caused by fish mycobacteriosis (Noga, 2010).

3.3.1.3. Algae as immunostimulants in functional feeds

In aquaculture, feeds are formulated to supply the fish basic nutritional requirements to perform its normal physiological functions (Encarnação, 2016). Nowadays, functional feeds are emerging, and consist in diets that aim to improve fish health and welfare, through the inclusion of dietary ingredients that promote growth, health, environmental and economic advantages ahead of traditional feeds (Encarnação, 2016; Olmos Soto et al., 2015).

Immunostimulation, which is the enhancement of the immune system of an organism, appears as a potential approach in improving host immunity and preventing diseases in aquaculture fish (Baleta and Bolaños, 2019). Within the existing immunostimulants,

algae are receiving increased attention due to their diverse taxonomy, their bioactive molecules with antioxidant properties and the complex polysaccharides they contain that can stimulate non-specific host immunity and inhibit bacterial activity function as well as act as prebiotics (Peixoto et al., 2019; Ringø et al., 2014; Thépot et al., 2021).

The use of algae in diets for fish has been explored in several species produced in aquaculture and it has been showed that algae do have overall positive effects on fish immune responses without compromising fish growth (Baleta and Bolaños, 2019; Martínez-Antequera et al., 2021; Peixoto et al., 2019; Peixoto et al., 2017; Thépot et al., 2021). There are various approaches to include algae in fish diets, wherein the most frequent consists of the use of the whole-algae and algae extracts. Comparing these approaches, extracts may be more beneficial to use then whole-algae, since algae have rigid cell walls, high fibre content and various antinutritional substances, that may interfere with food utilization, lowering the digestibility and nutrient bioavailability when included in fish feed, especially for carnivorous species (Agboola et al., 2019; Gong et al., 2020; Monteiro et al., 2021a). Moreover, an algae extraction approach has the advantage to use and extract only the compound of interest and to avoid the inclusion in fish feed of harmful compounds, that might be present in the whole-algae (Barkia et al., 2019). Another advantage of using algae extracts is the possibility of standardizing the final product, which is a requirement for marketed products (Michalak and Chojnacka, 2014).

3.3.1.4. *Fucus vesiculosus*

Fucus vesiculosus is a brown macroalgae from the class Phaeophyceae and is also the most well-known species from the genus *Fucus* (Catarino et al., 2018; Circuncisão et al., 2018). This macroalgae is widely distributed in the intertidal areas of various cold and warm temperate regions in the Northern Hemisphere, usually dominating shallow macroalgae communities (Catarino et al., 2019). Moreover, this species can grow on the mid-tide zone of high salinity waters in depths ranging 0.5 to 4 m (Catarino et al., 2017; 2018; Ferreira et al., 2019)

Concerning the nutritional value, *F. vesiculosus* is a balanced source of bioactive nutrients and phytochemicals, such as fucoidans and phlorotannins, in addition to being capable to accumulate high amounts of essential minerals, specifically iodine (Ferreira et al., 2019). Due to their high content in iodine, *F. vesiculosus* supplements are used in

the treatment of goitre, which is generally caused by iodine deficiency, and other thyroid-related complications (Catarino et al., 2018). Fucoidans are sulphated polysaccharides exclusively produced by brown algae, being characterized by their antioxidant, antimicrobial and anti-inflammatory activities (Catarino et al., 2017; Ferreira et al., 2019; Tian et al., 2019). Concerning phlorotannins, it represents the major phenolic compounds in *F. vesiculosus*, associated with promising health benefits including antioxidant, anti-inflammatory and antibacterial properties (Catarino et al., 2019; Ferreira et al., 2019; Wang et al., 2012). In fact, according to Wang et al. (2012), brown macroalgae have been found to possess remarkably high antioxidant activity in vitro, which is well correlated with their total phlorotannin content.

On the topic of fish diet supplementation with this macroalgae, a study by Pereira et al. (2019) with gilthead seabream fed a macroalgae-enriched diet, that included *F. vesiculosus*, revealed that algae-enriched feed exhibited anti-genotoxic properties that reduced oxidative DNA damage, which enhanced fish welfare. Moreover, a study by Peixoto et al. (2016) that analysed the supplementation of selected macroalgae, including *Fucus sp.*, in diets for European seabass, observed improvements on immune and antioxidant responses in fish fed with these diets. Furthermore, a study performed by Monteiro et al. (2020), using the same *F. vesiculosus* extracts as the present study, showed that these extracts displayed higher antioxidant capacity, when compared to other macro and microalgae extracts. Additionally, other study carry out by Monteiro et al. (2021b), in this same extracts, showed that the inclusion of a *F. vesiculosus* extract as a functional ingredient stimulated anti-inflammatory activity in zebrafish larvae and that these extracts also exhibited in vitro antibacterial action against a broad range of pathogens, including *Phdp*.

3.3.1.5. *Nannochloropsis gaditana*

Nannochloropsis gaditana is a microalgae belonging to the class Eustigmatophyceae and the genus *Nannochloropsis* (Pousão- Ferreira, 2009). This genus is characterized by unicellular microalgal species, with a spheric form and diameter ranging from 1 to 4 µm, with a polysaccharide cell wall structure that only contains one chloroplast (Abdelghany et al., 2020; Gbadamosi and Lupatsch, 2018; Pousão- Ferreira, 2009).

In Mediterranean hatcheries, *N. gaditana* is used both for rotifer production and to improve the water quality in the larval tanks (Dhont et al., 2013; FAO, 2005a; Ma et al.,

2020). Furthermore, this microalga is also being used in the industry of human nutrition supplements (FAO, 2020).

Concerning the nutritional value, this species has a high protein content and is also an important source of PUFAs (polyunsaturated fatty acids), especially EPA (eicosapentaenoic acid) (20:5v3), which are nutritionally required by marine fish (Castro et al., 2020; Pousão- Ferreira, 2009; Sørensen et al., 2017; Zanella and Vianello, 2020). Furthermore, under culture conditions, this species has a favourable growth rate and high cell density, being especially appealing to use in aquaculture (Zanella and Vianello, 2020). Additionally, this species also has a high content of antioxidants along with unique bioactive compounds that include carotenoid pigments, vitamins and sterols, that possess several biological functions, including immunomodulatory, antioxidant, and antibacterial activities (Castro et al., 2020; Cerezuela et al., 2012; Valente et al., 2019; Zanella and Vianello, 2020).

There are several studies highlighting the beneficial usage of microalgae of the genus *Nannochloropsis* in the diets of finfish species (Abdelghany et al., 2020; Castro et al., 2020; Gbadamosi and Lupatsch, 2018; Zanella and Vianello, 2020). Focusing on the modulation of the immune system, a study by Cerezuela et al. (2012), that examined the supplementation with *N. gaditana* in gilthead seabream's diets, demonstrated that this microalgae has an enhancing effect on fish immune system. Another study by Carballo et al. (2020) also demonstrated the enhancement of the *Senegalese sole* larvae immune response when fed *N. gaditana* extracts.

3.3.2. Trial Aims and contextualization

The present trial aimed to investigate the effects of *Fucus vesiculosus* and *Nannochloropsis gaditana* supplementation on meagre growth performance as well as to evaluate if meagre immune and antioxidant responses are modulated by algae supplementation and if it provides higher protection against bacterial infections. For that, the two algae extracts, with putative immune stimulant properties, were incorporated in balanced diets for meagre. Furthermore, a bacterial infection was done, using *Photobacterium damselae* subsp. *piscicida* as biotic stressor.

As part of my internship, I performed the trial, which included conducting the trial throughout its entire duration, by everyday monitoring the fish, and to execute the final sampling, with the support of a multitask team from EPPO. I also did the bacterial challenge.

The dietary extracts were made by PhD student Marta Monteiro, member of the NUTRIMU group. The experimental design was performed by PhD student Marta Monteiro, Dra. Ana Couto (from CIIMAR) and Dra. Florbela Soares (from EPPO).

This trial is included in the ZEBRALGRE project from CIIMAR (https://www2.ciimar.up.pt/projects.php?id=20). Part of the project, regarding the use of macro and microalgae as functional ingredients in diets for meagre are described in Monteiro et al. (2018).

3.3.3. Material and Methods

3.3.3.1. Experimental diets

The algae extracts were made by PhD student Marta Monteiro. Briefly, algal extracts were prepared from freeze-dried biomass from *F. vesiculosus* obtained from Alga+ (Ílhavo, Portugal) in methanol/water 50:50 v/v and *N. gaditana* obtained from Buggy Power S.L. (San Pedro del Pinatar, Murcia, Spain) in ethanol/water 80:20 v/v (Monteiro et al., 2020).

The diets were produced by SPAROS, Lda. (Olhão, Portugal). Regarding the diets, algae extracts were incorporated in balanced diets for meagre and three diets were made: a diet with *F. vesiculosus* supplementation at 1% (FUCUS), a diet *with N. gaditana* supplementation at 1% (NANNO) and a control diet without supplementation (CTR). These diets were microdiets, adapted to post-larvae phase. For each diet, three types of pellets with different diameter ranges (600-800 µm, 1000 µm and 1200 µm) were used in order to accompany the meagre growth.

3.3.3.2. Trial and sampling procedure

The trial was conducted at EPPO, Olhão, Portugal, in 9 flat-bottom tanks of 200 L each (Figure 12), supplied with a continuous flow of filtered seawater that comes from an external reservoir. The trial lasted 28 days and a 14h:10h light: dark photoperiod was adopted. Regarding temperature, it varied according to the water coming from the outside, with an average of 20.4 ± 1.4 ºC during the trial. Concerning oxygen, it had an average of 6.5 ± 0.6 ppm and 87.0 ± 8.2 % saturation through the trial. 125 Specimens of *Argyrosomus regius*, obtained by spawning from a broodstock at EPPO, with 34 days and an average weight of 0.110 ± 0.026 g were randomly distributed to each tank.

Diets (CTR, FUCUS, NANNO) were randomly assigned to triplicate tanks and fish were fed with an automatic feeder and by hand, with 10 feeding moments per day, during the 28 days. The fish were fed *ad libitum*, with the automatic feeder discharging fed in intervals of 90 min between 9:00h and 16:30h and discharging one time between 21:00h and 22:00h and another time between 7:00h and 9:00h. The feeding by hand was done in the morning around 9:00h and in the end of the workday, around 17:00h, if the fish demonstrated hungry. The automatic feeder shots were adjusted according with the fish

development. Meagre must be fed several times a day in order to prevent cannibalism between individuals (Duncan et al., 2013).

As a day-to-day routine, the tanks were cleaned with a siphon, purged twice a day and the water flow checked and adjusted according to the water conditions and fish development. Feed consumption was recorded daily as well as mortality. Temperature, dissolved oxygen, and oxygen saturation was measured twice a day.

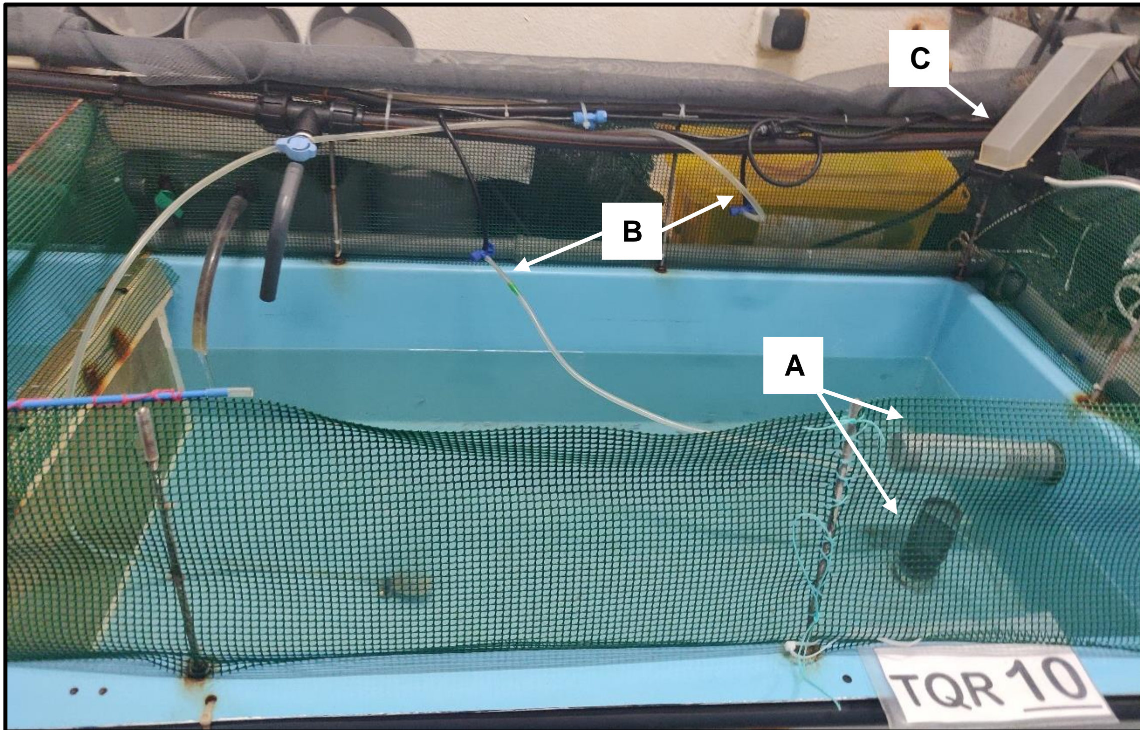

Figure 13- Blue flat-bottom fiberglass tank, equipped with outlet filters (A) (surface and depth filter, 1mm mesh), two airstones (B), and an automatic feeder (C) with an air blower. Picture taken by me in May 2021.

By the end of the trial, 21 fish were randomly selected from each tank, and were measured and weighed. Of these, 12 fish were euthanized with ice bath, and 6 whole fish were snap frozen in liquid nitrogen and stored at -80ºC until oxidative stress analysis. Another 6 whole fish were placed in RNAlater at 4ºC for 24 h and subsequently stored at -80ºC until gene expression analysis. The remaining fish returned to their respective tanks.

3.3.3.3. Fish performance parameters

To evaluate fish growth performance, weight gain (WG), ingestion (I), feed efficiency (FE), daily growth index (DGI) and mortality (%) were determined through the following equations, where IBW stands for initial body weight, FBW for final body weight and days stands for number of days that the trial occurred.

$$Weight\ gain\ (g) = FBW\ (g) - IBW(g)$$

$$Ingestion\ (g) = \frac{Feed\ given\ per\ tank}{Fish\ per\ tank}$$

$$Feed\ Efficiency = \frac{Weight\ gain\ (g)}{Ingestion\ (g)}$$

$$Daily\ growth\ index = \frac{FBW^{1/3} - IBW^{1/3}}{days} \times 100$$

$$Mortality\ (\%) = \frac{n^{\underline{o}}\ dead\ fish}{n^{\underline{o}}\ initial\ fish} \times 100$$

3.3.3.4. Bacterial Challenge and sampling

After the feeding trial, a bacterial challenge was carried out. Ten aquariums with 20L (Figure 13) were used, with salt water at a temperature of 20º C, with continuous aeration and without recirculation. The water was renewed manually, with 1/4 of the aquarium water being renewed twice a day.

From each tank used in the feeding trial, 50 individuals with an average body weight of 1.67 ± 0.68 g and total length of 5.2 ± 0.8 cm, were transferred to a corresponding

aquarium, thus maintaining the triplicates of origin, and left to acclimate to the new conditions for 24h.

Figure 14- Aquariums used in the bacterial challenge, equipped with airstones and covered with a net. Picture taken by me in May 2021.

Concerning the bacterial preparation, the bacterium *Photobacterium damselae* subsp. *piscicida*, strain C2, with reference CECT 5895 (https://www.cect.org/vstrn.php?lan=es&cect=5895) was used for the bacterial challenge. The bacterium was prepared by experienced staff from EPPO, using an internal protocol from EPPO (unpublish).

After the 24h acclimation period, fish were infected in a 30-minute bacterial immersion bath with a suspension of *Photobacterium damselae* subsp. *piscicida*. The bacterial immersion bath consisted of a 4.5L of salt water from fish aquariums, with continuous aeration, to which 0.5L of the previously prepared bacterial suspension was added. The dose of LD50 (median lethal dose) determined by Couso et al. (2003), 10^7 cells/ml, was used in the bath infection. The control group did not undergo this bath.

Sampling took place at 4h, 24h and 48h after the bacterial immersion bath, in which 8 fish were sampled per aquarium, totalling n=24 per dietary treatment at each sampling moment. Fish were euthanized with ice bath, 12 whole fish were placed in RNAlater at 4ºC for 24 h and subsequently stored at -80ºC until gene expression analysis, and the

other 12 whole fish were snap frozen in liquid nitrogen and stored at -80ºC until oxidative stress analysis. After the samplings, the remaining fish were monitored.

Fish mortality was monitored for a period of 12 days after the challenge, and dead fish were removed daily. When a mortality peak occurred, that corresponded to 3 or more dead individuals per day in a given aquarium, the fish were autopsied to sample the liver and spleen, that were placed in ethanol 70% and stored at -20°C until molecular analysis, to confirm the presence of the pathogen. Fish mortality was calculated as previously described. Fish were not fed during the experimental period.

3.3.3.5. Statistical analysis

Results were analysed using the IBM SPSS Statistics v27.0 software package for Windows. Data was tested for normality and homogeneity of variances using Shapiro-Wilk and Levene tests, respectively. Results were then analysed using a one-way analysis of variance (ANOVA) with factor “Diet”. Challenge test mortality was analysed using Kaplan-Meier Survival test and compared statistically using the log-rank (Mantel–Cox) test (Keup et al., 2020; Peto et al., 1977). Differences were considered statistically significant at $P<0.05$.

3.3.4. Results

3.3.4.1. Fish performance

Fish growth performance and feed utilisation parameters are presented in Table 4. No differences were found in any growth performance parameters or in feed utilisation, amongst dietary treatments (p value > 0.05).

Fish mortality was lower than 1% in all dietary treatments. Furthermore, all dietary treatments exhibited a 15-fold increase in their initial body weights (0.110 ± 0.026 g) after 28 days of feeding trial. No significant differences were observed on growth (Weight Gain range of 1.52-1.60; Daily Growth Index range of 2.78-2.86), ingestion (1.48-1.53) and Feed Efficiency (1.02-1.08), among dietary treatments.

Table 4- Growth performance and feed efficiency of meagre fed the experimental diets (CTR, FUCUS, NANNO) for 28 days; an initial body weight (IBW) of 0.100 g.

	Diet			**One-way ANOVA**
Growth Performance	**CTR**	**FUCUS**	**NANNO**	**p-value**
WG (g)	1.56 ± 0.26	1.52 ± 0.11	1.60 ± 0.02	*0.878*
Ing (g)	1.53 ± 0.01	1.48 ± 0,05	1.49 ± 0.02	*0.336*
FE	1.02 ± 0.16	1.03 ± 0.08	1.08 ± 0.02	*0.867*
DGI	2.81 ± 0.25	2.79 ± 0.11	2.87 ± 0.02	*0.873*
Mortality (%)	0.80 ± 0.653	0.80 ± 0.653	0.53 ± 0.38	*0.870*

WG - weight gain; Ing- ingestion; FE - feed efficiency; DGI- daily growth *index*
Mean initial body weight (IBW) of 0.100 g.
Data are represented as the mean ± SD. (n = 3 tanks/diet)

3.3.4.2. Bacterial challenge mortality

The mortality of the bacterial challenge is represented in Figure 14, with the three diets of the feeding trial (CTR, FUCUS, NANNO) and with CTR-, that represents the negative control group, that didn't endure the bacterial challenge.

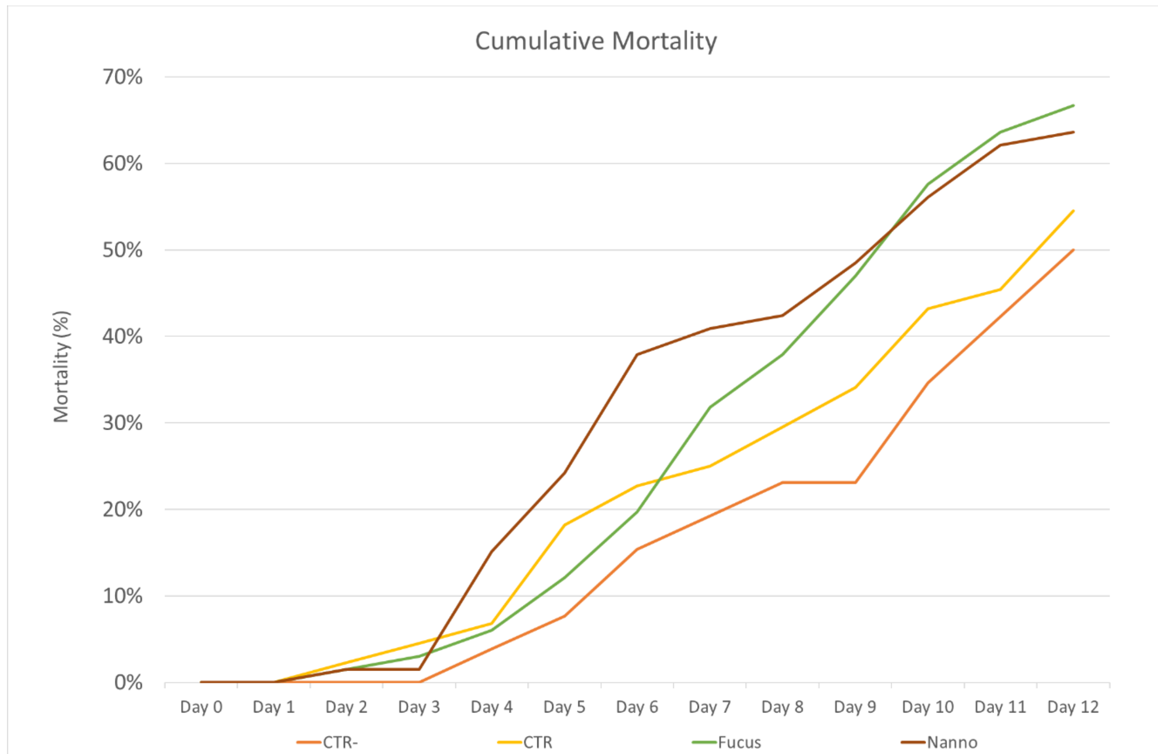

Figure 15- Cumulative mortality of meagre fed the experimental diets (CTR, FUCUS, NANNO) after the bacterial challenge (n = 3 aquarium/diet); negative control without bacterial challenge (CTR-)

By the end of the 12-days post challenge, the cumulative mortality reached 50% in the negative control group, 55% in the CTR diet group, 64% in the FUCUS diet group and 67% in the NANNO diet group. According to Mantel-Cox test obtained with the Kaplan-Meier Survival test (p = 0.346), comparing the mortality of the three dietary treatment´s group and the negative control group, the differences between treatments are not statistically significant and one cannot conclude that the mortality is significantly different between the treatment's groups (p value > 0.05).

3.3.5. Discussion

Functional feeds are an emerging innovative way to develop diets in aquaculture, with the aim to improve fish health and welfare, through the inclusion of specific dietary components. Among these components, algae have been recognized as a notable source of various bioactive compounds which act as immunostimulants in fish, making them of major interest for inclusion in new functional feeds.

In the present study, the findings that no significant differences were observed in terms of growth performance and feed utilization of fish fed different dietary treatments indicate that meagre growth was not compromised or influenced by dietary supplementation. This is consistent with results observed by Peixoto et al. (2017), wherein dietary seaweed supplementation, with *Gracilaria sp.* and *Alaria sp.* did not affect meagre growth performance. Additionally, it is also concurrent with results observed by Lobo et al. (2018), in which a diet with seaweed supplementation mix, containing *Fucus* sp., *Gracilaria* sp. and *Ulva* sp., did not significantly influenced fish growth performance of juveniles European seabass. Furthermore, the results in the present study are also in agreement with those of Peixoto et al. (2016), wherein dietary supplementation of selected algae such as *Fucus* sp. or *Gracilaria* sp. or *Ulva* sp. or a mix of the three, in diets for European seabass, did not have a significant effect on fish growth performance. Moreover, regarding the DGI in the present study, it is consistent with findings by Valente et al. (2019), in which European seabass, feed with various inclusion levels of *Nannochloropsis* sp., presented DGI levels not significantly different from the control diet (algae-free).

Nevertheless, the results in the present study are not in concordance with some studies. For example, a study from Abdelghany et al. (2020), that examine the effects in Nile tilapia (*Oreochromis niloticus*) fed on diets supplemented with different levels of *Nannochloropsis* sp., observed that fish fed with this microalgae exhibited significant superior weight gain, better feed conversion ratio (the inverse of the feed efficiency of the present study) and superior feed intake (the equivalent of ingestion in the present study). Moreover, Sørensen et al. (2017) study with Atlantic salmon, wherein fish were fed diets with two different inclusion levels of *Nannochloropsis* sp. (10 and 20% inclusion) and a control diet (algae-free), observed that the weight gain was significantly lower in fish groups feed with the microalgae and the higher inclusion level exhibited significantly worse feed conversation rates. Furthermore, it is also important to take into account that dietary seaweed supplementation in aquafeeds is species and dose dependent, according with Peixoto et al. (2017).

Thus, the contradictory results found in literature may be due to differences in fish species used, algae species used and amount of inclusion, as well as type of inclusion (whole algae biomass/extract/other). Given our experimental design, with balanced diets with 1% of algae extract supplementation or without supplementation (control diet), it was not expected that growth performance and feed utilization parameters were influenced by these diets. This level of algae extract supplementation may not be enough to cause differences in terms of growth performance and feed utilization, which may explain the absence of statistical differences between dietary treatments. Moreover, being balanced diets for meagre, our experimental diets fulfilled the requirement for meagre normal growth which could also explains the lack of differences in terms of fish performance. Algae extracts may present an advantage only when fish are under sub-optimal conditions and therefore, under optimum rearing conditions meagre performs equally with or without extract supplementation.

Similarly, the lack of differences in the feeding trial mortality between dietary groups indicates algae supplementation did not had any negative effects on fish and also that under optimum rearing conditions the extracts do not represent an advantage. This was also previously observed in Abdelghany et al. (2020) study with Nile tilapia (*Oreochromis niloticus*), fed on diets supplemented with different levels of *Nannochloropsis* sp., wherein no significant differences were observed in the survival rate among all dietary groups. Furthermore, it is also in agreement with Gbadamosi and Lupatsch (2018), in which diet with *Nannochloropsis* sp. (the protein and lipid source was exclusively from the microalgae, with the inclusion of 820 g per kg of feed) compared to diets with fish meal and soybean meal, did not cause significant differences in the survival rate of *Oreochromis niloticus.*

Regarding the bacterial challenge with *Photobacterium damselae* subsp*. piscicida* (*Phdp*), only the mortality that occurred post-challenge was analysed so far thus, immune response and oxidative status will be presented elsewhere. The cumulative mortality was higher in the groups fed with *N. gaditana* (67%) and *F. vesiculosus* (64%) followed by the mortality in the control diet group (55%). However, the differences in the cumulative mortality between the dietary groups and the negative control were not statistically significant. The cumulative mortality (50%) observed in the control group, that did not undergo the bacterial challenge, could be associated with the lack of feeding post-challenge and the abiotic conditions of the challenge itself, since the challenge's aquariums did not have a recirculating water system, wherein the water renewed was done manually twice a day. Thus, it will be relevant to perform a new challenge in

aquariums with a recirculating water system, wherein the fish are fed, to reduce the biased mortality in the negative control group, that seemed to be a result of the rearing conditions, and by that, account for mortality only induced by the bacterial challenge itself.

Similarly to the results of the feeding trial, the differences in the cumulative mortality between the dietary groups and the negative control were not statistically significant. This could mean that the algae extracts do not protect fish in a challenge situation. On the other hand, our bacterial challenge was accompanied by a stress challenge, unintentionally induced by the abiotic conditions of the experimental like the lack of feeding and absence of a recirculation water system which, nevertheless, caused additional stress to the fish. Thus, the fish experimented a combined challenge, with a bacterial plus a stress challenge, wherein the algae extracts may not be strong enough to provide protection. Moreover, 1% algae extract dietary supplementation may be a low dose to have a beneficial effect in fish under the above-mentioned conditions. Thus, further experiments should be carried out to better understand the potential of the algae extracts as functional ingredients in diets for meagre.

3.3.6. Future Perspectives

The gene expression and oxidative stress analysis will be carried out in the future, in samples from the feeding trial as well as the 4h and 24h after the bacterial challenge. This will bring new important data, to better understand the present study and the role of *Nannochloropsis gaditana* and *Fucus vesiculosus* extracts in meagre´s diet in the modulation of the fish immune system against *Phdp.* Furthermore, it would also be important to repeat the bacterial challenge, under more controlled conditions, with aquariums with a recirculating water system and wherein the fish are fed, to reduce the biased mortality. Moreover, it would also be relevant to test different algae extracts levels in fish diets.

Furthermore, the liver and spleen sampled from the bacterial challenge dead fish, will be analysed to detect *Phdp* with molecular biology analysis, using internal protocol from EPPO, with specific primers for this bacterium.

3.4. Internship Routines at EPPO

3.4.1. Rearing of marine larvae

In aquaculture, broodstock is the commonly applied term for a group of specific fish that are used for producing the new stock of larvae, going through natural or induced spawns to produce eggs (De Silva et al., 2008). At EPPO, the majority of marine fish eggs are obtained through spawns of broodstock kept in captivity at the station.

At EPPO, there are 12 indoor (R´s tanks) and 5 outdoor tanks wherein broodstock specimens of different species are stocked, at low density in the ideal male/female ratio for the reproductive success of each species (Teixeira, 2016). The species produced at EPPO produce pelagic eggs, with a density lower than salt water, which causes them to float when they are in good condition and, on the contrary, to sink when they are not viable. Each broodstock tank has egg collectors that retain the eggs in a filter mesh. At the time of the collection, the viable eggs and the non-viable eggs are separated. The viable eggs are weighed and evaluated with a binocular magnifying glass to further identify egg viability and developmental phase (Figure 15). Non-viable eggs are also weighed to obtain a daily report of posture quality.

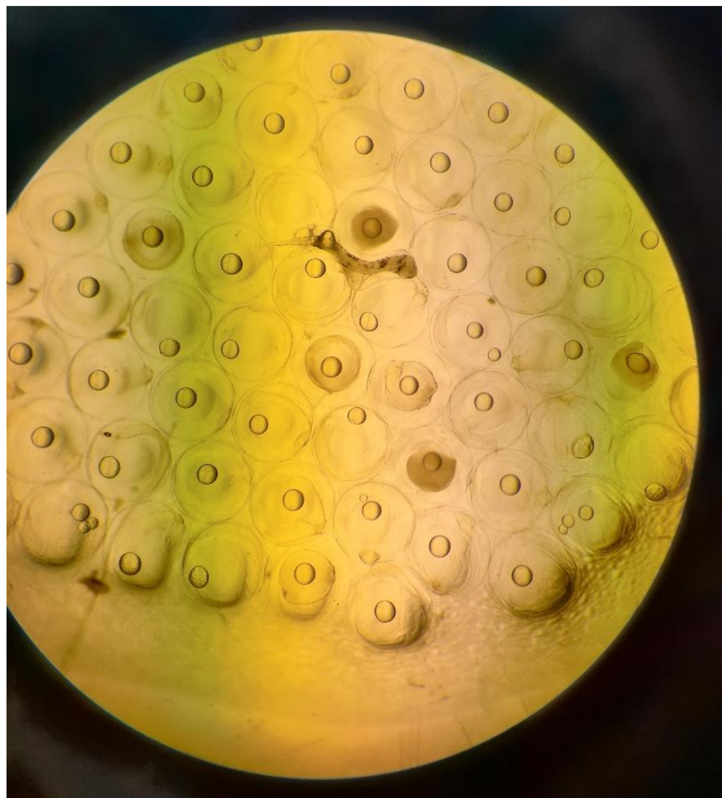

Figure 16- *Sparus aurata* eggs (neurula phase) evaluated with a binocular magnifying glass. Picture taken by me in April 2021

After egg collection, the eggs are sent to the trial room/maternity (Figure 16). There, the eggs are placed in tanks called “incubators” (I’s tanks with 200L), which are conical shaped and have sufficient water flow to prevent the eggs from sinking (De Silva et al., 2008).Conditions such as water temperature, oxygenation and light are adjusted for each species requirements. Eggs begin to hatch approximately 48 hours after incubation, at a water temperature around 19ºC (Teixeira, 2016).

This room possesses other types of tanks for larval rearing that vary in size and volume (A’s tanks with 300L, B’s tanks with 200L) as well as a special filtration system, with biological filters, UV light and mechanical filters. Following, larvae are placed in the A’s or B’s tanks and, when deemed convenient and according to larval development, larvae will be transferred to larger 1500L larval tanks, called the L´s tanks, outside the maternity room. The larvae can be used for various trials or to continue their growth to obtain juveniles / adult fish.

Figure 17- Trial room/maternity of EPPO. I’s tanks on the left (white arrow) and A’s tanks on the right (black arrow). Picture taken by me in July 2021.

3.4.1.1. Larvae rearing: Daily routines

Daily, the maternity routine is carried out which includes, purging and cleaning the tanks, feeding and observing the larvae, with more rigor than for other fish tanks, due to the sensitivity that characterizes this stage.

Regarding the purging, it consists of opening for a few moments the drain at the base of the tank, to eliminate a large part of the residues, including dead larvae, that accumulate at the bottom of the tank due to its conical shape. The purge is usually performed early in the morning and in the beginning of the afternoon.

Concerning the cleaning of larval tanks, it is performed at least twice a day, usually after purging the tanks, being a procedure that requires great care and should be done calmly not to damage the physical integrity of the larvae. The cleaning is done through the siphon method, wherein a tube with a brush at the tip is used to vacuum the dirt of the tank. The hose through which the dirt is sucked ends up in a collector with a filtering mesh, inside a bucket, to retain possible live larvae that were accidentally sucked, as well as dead larvae. After that, the content of the filtering mesh is placed in a smaller container, usually a plastic beaker, and left to decant during a few minutes, allowing the dead larvae and other organic material to sink down and the live larvae to stay at the surface. The live larvae return to their respective tank and the dead larvae are discarded, after being counted to assess the daily mortality of each tank. Furthermore, the tank filters are also washed with fresh water twice a day to avoid clogging.

In relation to the feeding, the larvae are normally fed four times a day and the quantities given per tank are registered. Depending on their stage of development, they may need rotifers, artemia or inert feed, or a mixture of these. When inert feed is provided with an automatic feeder, this is also cleaned daily with a brush to prevent clogging, since the water humidity causes aggregation of the feed grains.

Larvae observation, it is a task performed throughout the day, to ensure the normal larvae rearing. In the beginning and at the end of each day, the water flow is adjusted to values suitable for each species and stage of development. Furthermore, in each tank, the temperature (Cº), dissolved oxygen (parts per million), and oxygen saturation (in percentage) is typically measured two times a day, one time in the morning and the other in the afternoon, using an oximeter, to assess rearing conditions are adequate for the species and adjust as needed.

3.4.2. Production of live prey

Marine fish larvae are characterized by their small size, fragility, incomplete development of the digestive system, low mobility and predatory efficacy. Thus, in aquaculture, fish larvae consume live prey in proper quantities and quality as their first exogenous food. The live prey must have movements compatible with the mechanisms of capture and predation of the larvae, as well as appropriate dimensions to the size of their mouths, allowing an easy capture and ingestion (Pousão- Ferreira, 2009; Shields, 2001; Vadstein et al., 2018). At EPPO, the live prey provided to marine larvae are rotifers (*Brachionus* sp.) and artemia.

Live prey is offered to the larvae a few days after hatching, when most individuals in culture open their mouths and are able to ingest food, which varies among fish species. This phase is extremely critical and marked by great mortalities, since it requires adaptation and learning to catch prey that must be available in quality and quantity (Pousão- Ferreira, 2009).

At EPPO, there are two rooms to produce live prey, one for rotifers and one for artemia, each one with several tanks to produce each organism. Rotifers belong to the genus *Brachionus,* have a life cycle fully controlled in the laboratory and are continuously produced to provide the daily quantity of food required for the larvae. With the progressive development and the gradual growth of the larvae mouth, artemia, which is larger than rotifers, is given to the larvae. Concerning artemia, the cists are not produced at EPPO and come from commercial manufacturers. Usually, these cysts, which are a mechanism of artemia adaptation to adverse environmental conditions, are purchased in a dehydrated form. At EPPO, the decapsulation, incubation and growth of the artemia is performed to respond to the required daily production to feed the larvae.

When rotifers and artemia reach its ideal stage to be used as live feed, they are placed and kept in a cold container with continuous aeration to be read to use throughout the day.

3.4.2.1. Live prey: Daily routine

To monitor the growth of rotifers and artemia, regular counts of these organisms are carried out throughout the day with the aim of knowing the real density present in the production tanks as well as to plan the next production step. To conduct the counts, a

sample is taken from the desired tank. After that, using a 1ml graduated glass pipette, 1ml is taken from the sample and the organisms inside the pipette are counted using a magnifying glass. At least three counts of the same sample are performed, and then the obtained average is used to estimate the quantity of organisms in the total volume of the production tank. During the counts, if the quantity of organisms is too high that it becomes impossible to conclude the task, dilutions can be made with salt water and the dilution value is taken into consideration in the calculations. These counts are also executed with the rotifers and artemia in the cold container, to know the exact density of the organisms and therefore calculate the volume of culture necessary to give to the marine larvae.

4. Final Remarks

This internship covered various areas related with aquaculture scientific investigation, from diagnosis of fish pathologies with histological and molecular techniques to marine larvae rearing and to performing a feeding trial followed by a bacterial challenge. This allowed me to acquire several and very distinct skills, extremely relevant to my professional training and future career in the aquaculture area.

I had the privilege of working with various excellent professionals that taught me a lot, both with the NUTRIMU group and at EPPO. The whole experience was very enriching and enabled me to confront the reality of work in aquaculture scientific investigation. It was also a very important experience for my personal development, since it allowed me to grow as a more resilient and autonomous person and to surpass various obstacles that appear on my path, and to make some new friends along the way.

Therefore, this internship provided me with a set of enriching experiences that were extremely important for my personal and professional development and an added value in my education training and in finishing my Master´s in Biological Aquatic Resources.

5. Annexes

Table 5- Histological protocol regarding standard techniques in the automatic tissue processor Citadel 2000 (ThermoScientifc). Internal document.

Step	Reagent	Time (h)	Program Overnight
1	Formol	Pass	-
2	Formol	Pass	-
3	EtOH 70%	3	16h00
4	EtOH 90%	1	19h00
5	EtOH 100%	1	20h00
6	EtOH 100%	2	21h00
7	EtOH 100%	2	23h00
8	Xylol	Pass	-
9	Xylol	1,5	1h00
10	Xylol	2	2h30
11	Paraffin	2	4h30
12	Paraffin	2	6h30
	TOTAL	16,5	8h30

Table 6- Histological protocol regarding Hematoxylin and Eosin (H&E) staining in the automatic slide stainer (Varistain, ThermoScientifc). Internal document

Position	Reagent	time
1	Xylol	2'
2	Xylol	2'
3	Xylol: EtOH abs	2'
4	EtOH abs	2'
5	EtOH 95%	2'
6	EtOH 85%	2'
7	EtOH 75%	2'
8	H2Od	5'
9	Haematoxylin	3'
10	running H20	5'
11	Differentiator (EtOH 70% + 1%HCl)	PASS
12	H2O tap	PASS
13	Mordent	30''
14	running H2O	5''
15	Eosin	3'
16	H2O	PASS
17	EtOH 85%	1'
18	EtOH 95%	2'
19	EtOH Abs	3'
20	Xylol: EtOH abs	3'
21	Xylol	3'
22	END	

Table 7- Protocol regarding Bacterial Genomic DNA Isolation from fish samples. Version from Claudia R. Serra, 2014, adapted from Pitcher et al. (1989).

Step	Description
1'	Prepare 2 ml Precellys-homogenizer compatible tubes: 500 µl STE buffer + 0.4 g of glass beads (Sigma G8772).
2'	Weight up to 250 mg of fish tissue to the 2 ml tube previously prepared.
3'	Homogenize 3x 1 min in the Precellys @ 4500rpm (1min intervals on ice).
4'	Incubate @ 75ºC, 15 min, with gentle agitation every 5 min.
5'	Centrifuge 1 min @ 16000g or let stand for 10 min.
6'	Transfer 500 µl of supernatant to new sterile 2ml Eppendorf tubes.
7'	Add 100 µl of lysozyme (from a 10mg/ml solution freshly prepared) and incubate at 37ºC for 30 min.
8'	Add 5 µl of RNAse (from a 10mg/ml solution) and incubate at 37ºC for 30 min. (you can do steps 7 and 8 at the same time, incubating 1 h at 37ºC)
9'	Add 50 µl of 10%SDS and 3 µl proteinase K (from a 20mg/ml solution) and incubate at 55ºC for 30 min.
10'	Allow samples to cool on ice for 2-3 minutes
11'	Add 500 µl GES solution and mix by gently inverting tubes (DO NOT VORTEX).
12'	Add 250 µl ammonium acetate 7.5 M and mix by gently inverting tubes (DO NOT VORTEX).
13'	Incubate on ice for 10 min or more.
14'	Add 500 µl phenol:chloroform:isoamyl-alcohol (25:24:1) and mix well by inverting tubes.
15'	Centrifuge 10 min @ 13000 rpm, rt.
16'	Transfer aqueous (upper) phase to new 2 ml Eppendorf tubes.
17'	Add 500 µl chloroform:isoamyl-alcohol (24:1) and mix by gently inverting tubes.
18'	Centrifuge 10 min @ 13000 rpm, rt.
19'	Transfer aqueous (upper) phase to new 2 ml Eppendorf tubes properly labeled.
20'	Add 0.6 vol of Isopropanol and mix gently by inverting tubes (DO NOT VORTEX).
21'	Incubate on ice for 15 min.

22'	Centrifuge 15 min @ 13000 rpm @ 4ºC
23'	Carefully discard supernatant. You might see a pellet (most of times translucid).
24'	Add 500 µl 770% Ethanol (cold in freezer) to wash DNA pellet. Do this by flicking the tube with your fingers (DO NOT VORTEX).
25'	Centrifuge 10 min @ 13000 rpm @ 4ºC.
26'	Repeat the DNA wash with 500 µl 70% Ethanol (cold in freezer).
27'	Carefully discard supernatant. You should see better the pellet (most of times becomes white).
28'	Centrifuge 1 minute @ 13000 rpm, rt. Carefully remove as much remaining ethanol as possible without disrupting DNA pellet.
29'	Incubate tube with pellet (lid open) at 37ºC for ~10 min (to allow evaporation of remaining ethanol).
30'	Add 50-100 µl ddH2O to DNA pellet
31'	Incubate tubes (lid closed) at 37ºC for ~30 min (to add in DNA resuspension).
32'	Store DNA at 4ºC o/n to allow remaining DNA resuspension and then freeze at -20ºC.

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