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Pedro Emanuel
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O potencial da incorporação de halófitas em rações para peixes

Avaliação das propriedades antioxidantes e genoprotetoras

The potential of halophytes incorporation in fish aquafeeds

Evaluation of antioxidant and genoprotective properties

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, e da Doutora Sofia Isabel Antunes Gomes Guilherme, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) e do Departamento de Biologia da Universidade de Aveiro.

o júri

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"Precisas de aceitar o fato de não seres o melhor, e
ter o desejo de superar qualquer coisa que enfrentes"
Eiichiro Oda

palavras-chave Salicornia, Alimento funcional; integridade do ADN; aquacultura sustentável; *Dicentrachus labrax*

Resumo

Vários fatores, como as mudanças climáticas, o crescimento da população humana, o aumento da demanda por alimentos e bioprodutos, bem como o aumento da pressão das atividades industriais sobre a pesca e o meio ambiente, levantaram uma infinidade de questões que se tornaram o foco da Agenda 2030 das Nações Unidas para o Desenvolvimento Sustentável. Anualmente, parte dos produtos de pesca é convertida em farinha e óleo de peixe, embora nos últimos anos as taxas de inclusão em rações tenham diminuído (sendo utilizadas em estágios seletivos e em espécies produzidas), uma parte significativa é ainda utilizada para esse fim. Assim, é necessário encontrar ingredientes que sejam ambientalmente e economicamente sustentáveis, aliados ao desenvolvimento de rações funcionais. Espécies do género *Salicornia* mostraram conter importantes lipídios (1,5 a 5%) com valor nutricional e uma rica produção de metabolitos bioativos. Neste contexto, foi abordada a avaliação das potenciais propriedades genoprotetoras resultantes da incorporação da halófita *Salicornia ramosissima* na formulação de ração para robalo (*Dicentrarchus labrax*). Para tal, foram considerados três objetivos: (i) assegurar que a incorporação de *S. ramosissima* nas rações não apresenta toxicidade para o robalo, traduzida em alterações da saúde passíveis de afetar o crescimento e bem-estar dos peixes; (ii) avaliar a proteção promovida por *S. ramosissima* incorporada na dieta do robalo, especificamente na melhoria da integridade do ADN e cromossómica e reforço do sistema antioxidante; (iii) avaliar de que forma a estratégia testada pode contribuir para melhorar as práticas de aquacultura, e, ao mesmo tempo, cumprir com os objetivos de desenvolvimento sustentável da ONU. Os resultados demostraram que a condição fisiológica geral dos animais não foi afetada negativamente, nomeadamente em termos de integridade do DNA/cromossómica e ausência de peroxidação lipídica. Mais importante, as dietas com suplementação exibiram propriedades genoprotetoras nas células sanguíneas do robalo. Estes benefícios foram observados através do ensaio do cometa, especificamente no segundo mês da suplementação com Salicórnia, onde foi percetível uma melhoria na

integridade do ADN. Essa melhoria foi detetada em todas as percentagens de suplementação, mas com maior ênfase no nível mais elevado (10%). Além disso, uma aparente melhoria das defesas antioxidante foi detetada, nomeadamente envolvendo a regulação da glutationa e de enzimas dependentes da glutationa. Globalmente, estes resultados aparentam ser promissores no sentido da incorporação de halófitas como a *S. ramosíssima*, apontando uma potencial via para redução de custos, e, ao mesmo tempo, fortalecer a condição dos peixes, contribuindo, assim, para uma melhoria das práticas de aquacultura em linha com a Agenda 2030 para o Desenvolvimento Sustentável.

keywords Salicornia, Functional food; DNA integrity; Sustainable aquaculture; *Dicentrachus labrax*

Abstract

Several factors such as climate change, human population growth, increased demand for food and bioproducts, as well as the increased pressure of industrial activities on fisheries and the environment, have raised a plethora of issues that became the focus of United Nations 2030 Agenda for Sustainable Development. Annually, part of the finfish products is converted into fishmeal and fish oil, although in the last years the inclusion rates in aquafeeds have decreased (being utilized in selective stages and species produced), a significant part is still used for that purpose. So, it is necessary to find a more environmental and economical sustainable ingredients allied to a development of functional feeds. Species of the genus *Salicornia* showed to contain important lipids (1.5 to 5%) with good nutritional values, representing also a source of bioactive metabolites. In this context, the evaluation of potential protective properties resulting from the incorporation of the halophyte *Salicornia ramosissima* in the feed formulation for the European seabass (*Dicentrarchus labrax*) was addressed. For that, three different objectives were considered: (i) To ensure that the incorporation of *S. ramosissima* does not present toxicity for the European seabass, translated in health impairments passible to affect fish growth and welfare; (ii) to evaluate the protection promoted by *S. ramosissima*, specifically towards the improvement of DNA and chromosomal integrity and the strengthening of antioxidant system; (iii) to assess how the tested strategy can contribute to improve the aquaculture practices and, at the same time, meet the UN Sustainable Development Goals. The results demonstrated that the general physiological condition of the animals was not negatively affected, namely as DNA/chromosomal integrity and lack of lipid peroxidation. Most importantly, the supplemented diets exhibited genoprotective properties in the European seabass blood cells. This beneficial action was observed through the comet assay and specifically on the second month of Salicornia supplementation, where an improvement of DNA integrity was discernible. This positive impact was detected in all the supplementation levels, but with greater acuity at the highest level (10%). In addition, an enhancement of the antioxidant

defences was apparent, namely involving the regulation of glutathione and glutathione-dependent enzymes. Overall, these results seem to be promising towards the incorporation in aquafeeds of halophytes like *S. ramosissima*, offering a potential path to reduce cost of aquaculture while strengthening fish fitness, and thus, contributing to improve aquaculture practices in line with the 2030 Agenda for Sustainable Development.

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Chapter 1

Introduction

1. Introduction

Our planet is composed with 71% of water (approximately 96,5% of the water belongs to the oceans) and 29% of land (Shiklomanov, 2000). Land is extremely overexploited and overpopulated, and, for that reason, we switched the attention and efforts to the ocean, since it is an undeniable huge source of renewable and non-renewable resources with a significant contribute to the world's economy. Due to several factors such as climate change, growing population, increased demand for food and bioproducts, as well as the increased pressure of industrial activities on fisheries and the environment, a plethora of problems emerged as a focus of United Nations 2030 Agenda for Sustainable Development (Custódio et al., 2021; Satterthwaite et al., 2010).

The present work was developed in the context of the European Union's Horizon 2020 research project AQUACOMBINE (Integrated on-farm aquaponics systems for co-production of fish, halophyte vegetables, bioactive compounds, and bioenergy), using the principles of circular economy and the concept of marine biotechnology. The concept of biotechnology is defined by the Organization for Economic Co-operation and Development (OECD, 2005) as "the application of science and technology to living organism, as well as parts products, and models, to alter living or non-living materials to produce knowledge, goods, and services". The physical, biological, chemical, and genetic diversity of the marine species is the key toward a revolution in biotechnology (Blunt et al., 2009), and emphasizes the sustainable utilization of the ocean and its species to preserve for the next generations and attend to the needs of the present, contemplating the concept of sustainable development elaborated on the World Commission on Environment and Development in 1987 (WCED, 1987).

1.1. State of aquaculture and fisheries

Data from FAO (2022) indicate that the global consumption of finfish is increasing faster than the human population growth (3.1% *vs*. 1.6%), resulting in an estimated value of 178 million tonnes of finfish (fresh weight) produced and captured,

a slight decrease from the record of 179 million tonnes in 2018. Wild fisheries contributed with 90 million tonnes (a decrease from 96.4 million tonnes in 2018) and aquaculture 88 million tonnes (an increase from the 84.4 million tonnes in 2018) with a first sale value of USD 406 billion.

The total aquaculture production in 2020 contributed 49% of the global finfish production, showing it as a growing sector, essential for sustainable development. The main groups of animals produced in aquaculture are described in Table 1, with finfish representing approximately 65.7% of the total.

Table 1 - Aquaculture production for the main species groups in 2020 (millions of tonnes live weight). Adapted from FAO (2022)

Finfish	57.461
Crustacea	11.237
Molluscs	17.740
Other aquatic animals	1.062
Total	87.500

Inland compared to coastal aquaculture and mariculture produces more farmed aquatic animals, as shown in Table 2, with a total value of USD 109.8 billion.

Table 2-Inland *vs*. Marine and coastal animal aquaculture in 2018 (millions of tones, live weight). Adapted from FAO (2022)

Inland Aquaculture	54.384
Mariculture and coastal Aquaculture	33.116
Total world aquaculture	87.500

Aquaculture´s contribution to the global supply of aquatic food will likely intensify during the next decade, as most commercial fish stocks remain on the threshold of sustainably fished and overfished (FAO, 2018). It is expected in the future

the services and seafood provision be adhering to the sustainable development goals from the 2030 agenda.

There are several fish rearing systems and the choice of system, size, and unit type depends on several factors such as financial (costs more associated with the production expenses) and biological (intrinsic to the cultivated species (Tidwell, 2012)). Keeping in view the environmental sustainability, new approaches and the improvement of existing techniques are required, and much attention has been devoted to the development of Integrated multitrophic aquaculture (IMTA) in Recirculation aquaculture system (RAS).

IMTA systems translate a concept where the co-cultivation of species from different trophic levels occurs in the same production, and the wastes from the main produced organism are removed *in situ* by other organisms (Buck et al., 2018), making this approach a great candidate for long-term sustainable and profitable aquaculture (Biswas et al., 2020). Invertebrates are used for the removal of organic compounds, while aquatic plants, macroalgae, and microalgae are implemented for the bioremediation of inorganic compounds (Lara et al., 2021). In marine aquaculture, the integration of salt-tolerant extractive species is mandatory (Buhmann et al., 2015).

RAS are based on the reutilization and treatment of water through the application of mechanical and biological processes (Lara et al., 2021), where the water is recirculated through the rearing tanks reducing the overall water consumption in the systems (Qiu et al., 2022). The strict control over the water flow and quality in RAS ensures the health of the cultivated organisms and prevents the excessive release of nutrients into the environment.

Effective incorporation of IMTA into RAS can increase the benefits of aquaculture. Halophytes have been demonstrated to be effective in bioremediation of saline aquaculture wastes and their implementation in aquaculture allows the production of economically, nutritionally and environmentally valuable by-products (Buhmann et al., 2015; Oliveira et al., 2020; Ventura et al., 2011).

About 89% (approximately 157 million tonnes) of the 178 million tonnes of finfish product were utilized for direct human consumption, while the remaining 11% (about 22 million tonnes) were utilized for non-food purposes. Approximately 81% of the non-food purposes (16 million tonnes) were converted in fishmeal and fish oil, the remaining 19% (4 million tonnes) were utilized for ornamental fish and other purposes. Fish utilization and processing methods differ significantly across continents, regions, countries, and even within countries (FAO, 2020).

As indicated above, a percentage of world fisheries production is processed into fishmeal and fish oil, which can be produced from whole fish, fish trimmings, or other fish processing by-products, as the most nutritious and digestible ingredients for farmed fish. Although in the last years the inclusion rates of fishmeal and fish oil products in aquafeeds have decreased, being utilized in selective stages and species produced, about 75% of annual fish-oil production are still used for that purpose (Auchterlonie,2018).

1.2. European seabass (*Dicentrarchus labrax*) in aquaculture

1.2.1. Production and trade

European seabass is cultivated in marine water 86.67%, while the use of brackish and freshwater (with addition of salt) represents 13.33%. The main systems utilized are:

- Extensive lagoons
- Semi-intensive lagoons
- Floating cages
- Tanks and ponds

In 2019, the total production of seabass was approximately 236.000 tonnes, with Turkey being the biggest producer, accounting for 52.21% (Figure 1) of world production (FAO, 2021).

In the European Union (EU), in 2019, production came mainly from six countries: Greece (48.84%), Spain (29.9%), Croatia (7.22%), Italy (6.77%), Cyprus (3.36%), and France (2.91%). Malta, Portugal, and Slovenia had less than 1% of the EU production (FAO, 2021).

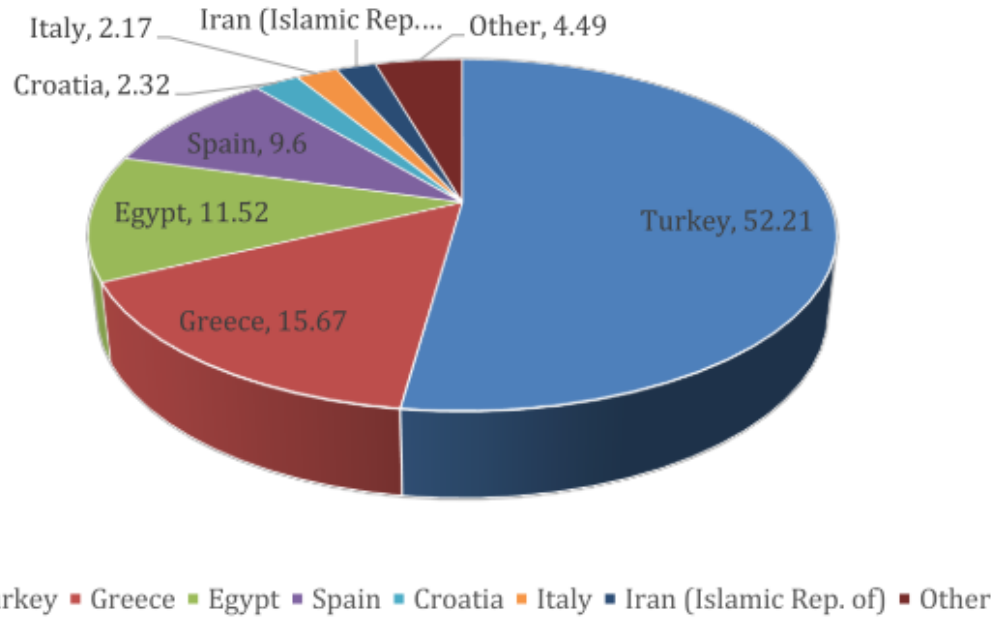

Figure 1-Global European production of European seabass (*Dicentrarchus labrax*) in 2019 (%). "Other" includes countries among 10 top countries with less than 2% share. From Compassion in world farming, 2021

In 2018, the largest exporters by volume in Europe and worldwide were Turkey (34.69%) followed by Greece (33.13%), and the Netherlands (8.08%). On the other hand, the largest importer was Italy (26.61%), followed by Spain (9.32%) and the Netherlands (7.23%).

Financially exports worldwide account for a value of USD 713 million, with the leadership of Greece (USD 239 million), followed by Turkey (USD 194 million) and the Netherlands (USD 73 million). In terms of imports, the total value was USD 739 million, and the country that spends the most is Italy, spending 26.85%, followed by the USA 10.44%, and finally Spain 8.06% (FAO, 2021).

Data for apparent consumption of European seabass was estimated by Llorent et al. (2020) due to lack of direct data, showing the Europe as the main consumer in

2018, followed by Asia, Africa, the Americas, and, lastly, Oceania. In Europe, Italy is the main consumer, followed by Spain, Greece, France, and Portugal.

1.2.2. Price *vs*. cost of production

It is difficult to find reliable and updated sources for production price and cost structure for European seabass, so the data are presented according to a few published papers, that are not always update or accurate, mainly due to the inclusion of different kind of costs (Bayramoglu, 2018; Bozoglu & Ceyhan 2009). In the biggest producers worldwide, the cost varies between 1.77 €/kg (in the Aegean Sea) and 3.62 €/kg in the Black Sea. In Italy, 2014, the cost of production varies between 4.3 €/kg and 4.29€/Kg (inshore cages and offshores cages, respectively (Di Trapani et al., 2014)).

The production cost for France, Italy, and Spain in 2016, for conventional production, was 6.41 €/kg, while for organic production was 8.61 €/kg (Prins et al., 2016).

Feeds represent the main cost of production, with an average of 39.97%, though it has been rising continuously over the years due to the increase in the price of raw materials. The second highest cost concerns juveniles/fingerlings, 20.71%, followed by labor, 17.76%, and then others, 15.08% (includes marketing, packing, medicine, and other concepts). The maintenance and fuels represent 4.32% and 2,15%, respectively (see Table 3).

Table 3 - Production cost structure (% of the total cost) for European seabass from different locations, systems, and years (Bayramoglu, 2018; Bozoglu & Ceyhan 2009; Di trapani et al., 2014).

	Italy 2014		France, Italy & Spain (2016)		Turkey Black Sea		Average
	Inshore	Offshore	No organic	Organic	2009	2019	
Feed	38.90	36.91	32.40	36.15	47.73	47.3	39.97
Juveniles	31.20	31.20	18.79	22.64	10.43	10.00	20.71
Labor	14.10	16.50	16.42	13.96	22.59	23.00	17.76
Repair & Maintenance	4.10	3.90	7.40	7.58	1.43	1.50	4.32
Fuels & Energy	1.90	3.30	2.81	2.09	1.33	1.50	2.15
Others	9.80	8.19	22.19	17.58	16.47	16.27	15.08

The selling price for the European seabass depends on the size of the fish: small (300-400 g) varying between 4-6 €/kg, medium (400-600 g) varying between 6-8 €/kg, and big (>600 g) varying between 7-11 €/kg (EUMOFA, 2019).

1.3. Functional feeds in fish farming

The successful replacement of animal protein sources in fish feed, partial or complete, with vegetable protein and complex carbohydrates sources, is very difficult to achieve (Soto et al., 2015) without affecting the growth rates and feed efficiency (Pueyo et al., 2016), but is mandatory as a consequence of increasing of economic value of fishmeal and fish oil, and environmental constrains (Bechard et al., 1998; Bomba et al., 2002; Boyd & Tucker, 1998; Chen et al., 1990). In this context, the development of functional feeds (FF), characterized as having physiologic effects beyond that of nutrient effects, is necessary (Guerreiro et al., 2019). FF are reported to improve growth, feed efficiency, stress tolerance, disease resistance and health performance in fish (Guerreiro et al., 2019). A properly formulated FF could be supplemented with high levels of vegetable protein, complex carbohydrates, or specifically selected innocuous probiotic bacteria (Olmos et al., 2011).

Several dietary ingredients have been tested to FF formulations, with emphasis on the soybean, due to lower cost (Pueyo et al., 2016). However, carnivorous marine fishes require or tolerate carbohydrate concentrations no greater than 10 % and need at least 50% protein concentration for a good development (Lupatsch et al., 2001). So, besides genetic engineering modified soy, several other ingredients have been tested, like insect meal, macroalgae and medicinal plants. Insect meals in conjunction with antimicrobial peptides have demonstrated immune-stimulating and antioxidant properties for the cultured species (Mousavi et al., 2020). The marine macroalga *Gracilaria vermiculophylla* has shown an improvement of immunological response in *Oncorhynchus mykiss* (Araújo et al., 2016; Marques, 2019) and land-farmed plants, like medicinal plant *Portulaca olearacea* incorporated in the feed of *O. niloticus* (improving growth, antioxidant and immunological responses) and *Aloe vera* in the

feed of *Cyprinus carpio* (improving blood parameters and antioxidant capacity) (Tadese et al., 2022). Theoretically, those ingredients could successfully replace fishmeal and/or fish oil while improving fish physiology, but they still need improvement or overcome several obstacles (Hua et al., 2019).

1.3.1. Functional feeds for the European sea bass

The European sea bass, of the Moronidae family, being a predatory fish, makes the replacement of traditional feed ingredients (fishmeal/or fish oil based) with vegetable meal more difficult, due to its dietary requirements already established according to life stage, weight, water temperature and, in some cases, oxygen levels (Kousoulaki et al., 2015).

In the last decades, different alternatives and/or a combination of ingredients have been tested in *D. labrax* and has been performed an evaluation for different aspects of fish physiology. Introduction of yeast as a complementary ingredient with no negative effects in terms of growth and an improved in feed efficiency (Oliva-Teles & Gonçalves, 2001). Medicinal plants such as *Yucca schidigera* improved hematological parameters and immunological responses (Tadese et al., 2022). Introduction of prebiotics on the feeds also acted as immunostimulants through the increase of phagocytic activity (Carbone & Faggio, 2016).

1.3.2. Halophytes as candidates for a novel functional ingredient

Halophytes are plants highly resistant to fluctuations in salinity, high temperature, and light intensities, as adaptations to the environment where they are found, namely saltmarshes and coastal areas worldwide (Lokhande & Suprasanna, 2012). This type of plants has developed several adaptive mechanisms, especially salt tolerance, including increased leaf and/or stem succulence, salt exclusion, osmolyte production, as well as the improvement of antioxidant defences (Lima et al., 2020).

In the European Union, Portugal is the most affected country in terms of desertification (followed by Turkey and Italy), with a third of its territory affected and a perspective of increasing in the next two decades (Branco et al., 2012), affecting the fauna and flora. In some regions, only a few species can survive and thrive in those adverse conditions, and halophytes are among those species with mechanisms developed for those environments.

A wide diversity of halophyte species exists in wild, so those that have the most economic potential and capacity to adapt under harsh conditions can be reliable as crops, contributing to sustainable agriculture in marginal lands (Panta et al., 2014; Ventura & Sagi, 2013).

Halophytes already demonstrated a great potential as a biofilter in marine and brackish water aquaculture (Buhmann et al., 2015; De Lange & Paulissen, 2016), namely within an IMTA framework (Custódio et al., 2017; Maciel et al., 2020), with the already demonstrated ability of successful bioremediation in aquaponics while adding value to the crops (Marques et al., 2017). Halophytes have been present in human food in the form of salads, soups, replacement of usual ingredients, and other dishes. In terms of nutritional value, they are considered a good source of proteins, fibers, minerals, and vitamins, having a high content of bioactive compounds (primary and secondary metabolites) with the potential for antioxidant (*S. ramosissima*, *S. perennis* subsp. *alpini* and *S. perennis* subsp. *perennis*, according to Barreira et al., 2017), antimicrobial, anti-inflammatory and anti-tumoral activities (*Cynara cardunculus*, *Artemisiae scopariae*) (Ksouri et al., 2012). About 2000 plants are known worldwide to possess some salinity tolerance, but only few species have been investigated for their antioxidant capacities, antimicrobial, anti-inflammatory and antitumoral activities, thus adding a greater scientific interest (Ksouri et al., 2012).

Species of the genus *Salicornia* showed to contain important lipids with good nutritional values and a rich production of bioactive metabolites mainly associated with antioxidant systems and DNA repair mechanisms (Barreira et al., 2017; Custódio et al., 2021; Kim et al., 2021). *Salicornia ramosissima* is an annual green tip plant

that can be found in saltmarshes from Artic to Mediterranean, characterized by not producing leaves, with a pleasant texture and a juicy and salty taste (Ventura et al., 2015). Thus, it is commonly used as a salt substitute for salads and considered to be a valuable nutritional and healthy food for humans, rich in minerals, fibers, and proteins and poor in fat (Barreira et al., 2017).

Nutrition may be simultaneously a source of antigenotoxic and genotoxic compounds (Marques, 2019). Those compounds able to reduce the impact of physical and chemical mutagens are known as antimutagens; however, considering that all mutagens are genotoxic, but not all genotoxic substances are mutagenic, those substances that can improve DNA integrity are called antigenotoxic agents. Antimutagens have been classified as desmutagens, if act before the mutagen attacks the DNA through partial or full inactivation (by enzymatic or chemical interactions), and bio-antimutagens, if the mutation suppression process occurs after genes are damaged, improving the repair and replication processes of the mutagen damaged DNA (Bhattacharya, 2011; Izquierdo-Vega et al., 2017).

Halophytes may be a source of novel compound along with providing a new source for known biologically active compounds, namely antioxidants. Those compounds with antioxidant properties are also candidates to be classifies, in the light of the previously presented concepts, as desmutagens.

1.4. Evaluation of functional feeds suitability

Functional feed assessment can follow a variety of strategies, and those choices can influence the outcome. According to Glencross et al. (2007), a series of five steps is essential to develop comprehensive data: 1. characterization; 2. palatability; 3. digestibility; 4. utilization; 5. functionality (processability). Sometimes, the traditional assessments (growth, feed intake/ efficiency) don't show any significant differences, so, according to Glencross et al. (2020), more steps need to be taken into count. Hence, one between the previously called steeps 4 and 5 (5. immunological and health allied assessments) tests other nutritional, immunological, and health parameters allied to

growth, and one last step must be to test product quality, where the sensory qualities like colour taste and smell of the product must be verified (7. product quality influences). In this new step 5, a variety of strategies can be adopted, such as immunological, *in vitro* assessments, near infrared and nuclear magnetic resonance spectroscopy (allows the assessment of the nutritional value of raw materials on a near-real-time basis), nutrigenomic and allied assessments, transcriptomic, proteomic, DNA and chromosomal integrity, haematological analyses and metabolomic analyses.

Despite the few studies in terms of the immunological and health of the organisms fed with new potential FF, in this work the focus will be in genome protection and haematological dynamics. The use of a battery of parameters is recommended since the assessment of just one biological response isn't enough to reflect the alterations to the physiology of living organisms and can lead to misinterpretations (Pacheco & Santos, 2002).

The quantification of the activity of enzymes that play a key role in common physiological processes, like detoxification, respiration, or antioxidant responses, is an approach developed to assess the biological stat of the animal and environment (Antunes et al., 2016; Nunes et al., 2020; Rebelo et al., 2020).

Antioxidant system

The antioxidant defences are essential to maintain cell homeostasis and when they fail or are overcome, oxidative stress products can induce DNA damage, enzymatic inactivation, and peroxidation of cell constituents (Guilherme, 2007). Defence systems that inhibit or limit the formation of reactive oxygen species (ROS) are only a part of antioxidant protection, which also include antioxidant enzymes. ROS are known to be biologically important in several physiological systems, including adaptation to hypoxia, immunity, differentiation, cognitive function, regulation of fertility and others (Bardaweel et al., 2018; Sena & Chandel, 2012). Organisms can adapt to ROS overgeneration by increasing antioxidant defences; however, the

amount of ROS that can be altered is limited, which may led to the disturbance of the redox state, also known as oxidative stress that may cause DNA damage (Barata et al., 2005; Kovacic, 1986; Lushchak, 2011).

To prevent damage, fishes can increase the levels of protective antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), and phase II metabolic enzymes such as glutathione S-transferases (GSTs), as well as non-enzymatic free radical scavengers like reduced glutathione (GSH). Assessing the antioxidant system of the species feed with FF helps to understand how compounds can, directly or indirectly, affect the organism's health (Marques, 2019).

DNA and chromosomal integrity

DNA integrity and stability are important to assure the health and survival of organism, especially in a time when exogenous and endogenous sources of genotoxic stress are getting bigger (Pereira et al., 2019). Aerobic organisms are more susceptible to DNA damage due to the presence of ROS formed as part of physiological processes (Oliveira et al., 2010), at the same time a higher quantity and variety of environmental agents with the potential to damage the DNA molecule (Geacintov & Broyde, 2010). One single alteration in the DNA molecule of an organism could originate serious biological consequences including cell death.

One of the most used tools for DNA integrity assessment is the comet assay which corresponds to the electrophoresis of individual cells on an agarose gel. This technique is based on the migration of fragments of DNA generated by the breaking of the strands, in an alkaline medium, due to the action of genotoxic agents that generate alkaline-sensitive sites. The greater the amount of DNA damage, the greater the migration of fragments from the degraded nucleus towards the positive electrode during electrophoresis, giving the appearance of a comet, which is an indication of the susceptibility of DNA damage (Marques et al., 2016).

Erythrocytic nuclear abnormalities (ENA) assay has been utilized in the past decades to assess chromosomal integrity in fish (Castro et al., 2018). The deformation of the erythrocyte nuclei is associated with chromosomes breakage or total loss of chromosome (clastogenicity), as well as mitotic spindle apparatus dysfunction (aneugenicity) (Fenech, 2000). Despite the undeniable utility of the assay to point nuclear lesions, it is unable to detect alterations in cell cycle progression and subsequent implications on the turnover of circulating erythrocytes.

FF may simultaneously be a source of antigenotoxic compounds (Izquierdo-Vega et al., 2017; Marques, 2019). So, the combination of both diagnosis tools above described represents a suitable and useful strategy to appraise potential protective properties (ensuring the lack of a genotoxic hazard as well) of FF through the measurement basal DNA integrity.

Hematological dynamics

To better comprehend the erythrocyte population dynamics and complement the information obtained with the genetic damage evaluation, the use of the erythrocyte mature index (EMI) has been adopted in the last decades (Marques et al., 2020). This method has been applied in fish erythrocytes, as a nucleo-cytoplasmatic ratio (Castro et al., 2018; Maceda-veiga et al., 2015; Maceda-Veiga et al., 2010), and can also provide an indication of the overall health status of the animals involved in dietary trials testing FF.

1.5. Objectives

In the present dissertation, the focus is the evaluation of potential protective properties resulting from the incorporation of the halophyte *Salicornia ramosissima* in the feed formulation for the *Dicentrarchus labrax*. For that, three different objectives must be considered:

(i) To ensure that the incorporation of *S. ramosissima* in the feeds does not present toxicity for the European seabass, translated in health impairments passible to affect fish growth and welfare;

(ii) To evaluate the protection promoted by *S. ramosissima* incorporated on the European seabass diet, specifically towards the improvement of DNA and chromosomal integrity and antioxidant system strengthening;

(iii) To assess how the tested strategy can contribute to improve the aquaculture practices and, at the same time, meet the UN sustainable development goals (1. No poverty; 2. Zero hunger; 3. Good health and well-being; 6. Clean water and Sanitation; 8. Decent work and economic growth; 9. Industry, innovation and infrastructure; 12. Responsible consumption and production; 14. Life below water).

Chapter 2

Materials and methods

2. Materials and methods

2.1. Experimental diets

Salicornia ramosissima (21.5 kg) was collected in Praia da Areia Branca, (Portugal), between the 3rd and 15th of June of 2020. The whole plant, except the green tips and the roots, was dried in Riasearch Lda. facilities (Murtosa, Portugal). The resulting 6 kg were sent to Sparos Lda. (Olhão, Portugal) for feeds formulation and manufacture (after proper grinding). A control diet (C) was formulated to include an indispensable amino acid (AA) profile, meeting the ideal pattern estimated for European seabass (Kaushik, 1998). Three test-diets were produced, incorporating *S. ramosissima* (as a whole plant), at the expense of wheat meal, at 2.5, 5, and 10% of feed weight (S2.5, S5, and S10, respectively). The formulation of experimental diets is presented in Table 4.

Table 4-Ingredients and chemical composition of the experimental diets

Ingredients (%)	**C**	**S2.5**	**S5**	**S10**
Fishmeal LT70[1]	35.00	35.00	35.00	35.00
Krill meal[2]	5.00	5.00	5.00	5.00
Soy protein concentrate[3]	13.00	13.0	13.00	13.00
Wheat gluten[4]	10.00	10.10	10.10	10.30
Corn gluten meal[5]	8.00	8.00	8.00	8.00
Wheat meal[6]	16.30	13.70	11.20	6.00
Vitamin and mineral pre-mix[7]	1.00	1.00	1.00	1.00
Monocalcium phosphate8	0.78	0.78	0.78	0.78
Yttrium oxide[9]	0.02	0.02	0.02	0.02
Fish oil (Sopropeche)[10]	5.20	5.20	5.20	5.20
Rapeseed oil[11]	5.70	5.70	5.70	5.70
Salicornia -Whole	-	2.50	5.00	10.00
Total	100.00	100.00	100.00	100.00
As fed basis (% dry matter)				
Dry matter	94.1	96.5	95.5	94.5
Crude protein	52.4	51.4	53.7	52.1
Crude lipids	16.8	16.8	17.3	17.3
Ash	8.7	10.3	9.7	12.0
Energy (KJ g-1 DM)	23.4	23.1	23.3	22.7

1: LT70 steam dried. 70.7% crude protein (CP). 8.1% crude fat (CF). Pesquera Diamante. Peru.
2: Krill meal: 52% CP, 22% CF, Aker Biomarine, Norway.
3: Soycomil P: 63% CP. 0.8% CF. ADM. The Netherlands.
4: VITAL: 83.7% CP. 1.6% CF. ROQUETTE Frères. France.
5: Corn gluten meal: 61% CP. 6% CF. COPAM. Portugal.
6: Wheat meal: 10.2% CP; 1.2% CF. Casa Lanchinha. Portugal.
7: 20 PREMIX Lda. Portugal: Vitamins (IU or mg/kg diet): DL-alpha 9ocoferol acetate. 100mg; sodium menadione bisulphate. 25mg; retinyl acetate. 20.000 IU; DL-cholecalciferol. 2.000 IU; thiamin. 30mg; riboflavin. 30mg; pyridoxine. 20mg; cyanocobalamin. 0.1mg; nicotinic acid. 200mg; folic acid. 15mg; ascorbic acid. 500mg; inositol. 500mg; biotin. 3mg; calcium panthotenate. 100mg; choline chloride. 1.000mg. betaine. 500mg. Minerals (g or mg/kg diet): copper sulfate. 9mg; ferric sulfate. 6mg; potassium iodide. 0.5mg; manganese oxide. 9.6mg; sodium selenite. 0.01mg; zinc sulfate.7.5mg; sodium chloride. 400mg; excipient wheat middlings.

8: 21.8% phosphorus, 18.4% calcium, Fosfitalia, Italy.
9: Sigma Aldrich, USA.
10: 98.1%CF (16% EPA; 12% DHA), Sopropêche, France.
11: Henry Lamotte Oils GmbH. Germany.

2.2. Rearing system and feeding trial design

Juveniles of European seabass, with mean initial weight of 7.26 ± 0.06 g, were obtained from Sonríonansa, S.L. hatchery (Cantabria, Spain) and allocated in Riasearch Lda. facilities (Murtosa, Portugal). The trial consisted in assessing the health condition of seabass after the inclusion of different percentages of Salicornia in the feed. For that, fish were divided by twelve 350-L tanks (80 fish per thank), corresponding to 3 tanks per condition/diet (Figure 2), and a RAS with 18 m^3 with a water renewal of 1 tank per hour. During the experimental period, water parameters were measured once a day using commercial probes and maintained as follows: temperature at 21.6 ± 0.2 ºC, dissolved oxygen at 6.4 ± 0.6 mg L^{-1}, salinity at 18.2 ± 0.2, pH at 7.5 ± 0.2 and nitrogen compounds below 0.1 mg L^{-1}.

Figure 2 - Schematic representation experimental conditions and tanks allocated. C - Control group (standard feed); S2.5 - Incorporation of 2.5% of Salicornia; S5 - Incorporation of 5% of Salicornia; S10 - Incorporation of 10% of Salicornia

A feeding trial with a total duration of 2 months, and an intermediate sampling after 1 month, was performed, involving the diets above described (see point 2.1.). Animals were fed to satiation. At each sampling moment, 5 fish were randomly collected from each thank (15 fish per condition/diet). Fish were anesthetized with 0.2 mg L-1 tricaine methanesulfonate (MS-222; buffered with NaHCO3) for approximately 10 min, and blood was drawn from the posterior cardinal vein using heparinized (27 mg mL^{-1} heparin) glass Pasteur pipettes, and collected in 2-mL microtubes. Thus, one microtube, containing 0.002 mL of blood diluted in 1 mL of chilled PBS (pH=7.4; 0.01M), constituted the cell suspension for comet assay, and other, with the remaining blood volume, was assigned for antioxidants analysis. Aliquots for comet assay were kept cold up to further procedures, while the aliquots for antioxidants determination were immediately frozen in liquid nitrogen. Blood smears were immediately prepared for ENA and EMI assays. Immediately after sampling, fish were sacrificed by cervical transection.

2.3. Cytogenetic and biochemical evaluations

2.3.1. Evaluation of antioxidant system status

Blood cell lysis and fractioning

The whole blood samples were lysed through homogenization, using a Potter-Elvehjem homogenizer, approximately in a 1:11 v/v ratio (volume of blood: buffer volume) of chilled 0.1 M phosphate buffer (pH 7.4). The lysate was collected with a pipette and separated into two aliquots, one for LPO and other to obtain the PMS (post-mitochondrial supernatant) after centrifugation. Both aliquots were immediately frozen in liquid nitrogen and stored at -80ºC until analyses or further procedures.

To obtain PMS, the lysate was centrifuged (Eppendorf 5415R centrifuge) at 12000 rpm for 20 min, at 4 °C. PMS was then separated into two different aliquots, frozen in liquid nitrogen and stored at -80 ºC until use.

Measurement of antioxidants and peroxidative damage

All measurements were carried out in a SpectraMax 190 microplate reader, at 25 ºC.

Total protein content was determined in serum, through the Biuret method (Gornall et al. 1949), using bovine serum albumin as a standard.

CAT activity was assayed in PMS by the method of Claiborne (1985), with slight modifications. Briefly, the assay mixture consisted of 0.190 mL phosphate buffer (0.05 M, pH 7.0) with hydrogen peroxide (H_2O_2; 0.010 M) and 0.010 mL of PMS, in a final volume of 0.2 mL. Change in absorbance was measured in appropriated UV-transparent microplates (UV-Star® flat-bottom microplates, Greiner Bio-One GmbH, Germany), recorded at 240 nm and CAT activity was calculated in terms of µmol H_2O_2 consumed min^{-1} mg^{-1} protein using a molar extinction coefficient (ε) of 43.5 M^{-1} cm^{-1}.

SOD activity was assayed in PMS with a Ransod kit (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodo- phenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye determined at 505 nm. SOD activity was then measured by the degree of inhibition of this reaction, considering that one unit of SOD causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD unit's mg $protein^{-1}$.

GST activity was determined in PMS with CDNB (1-chloro-2,4- di- nitrobenzene) as a substrate, according to the method of Habig et al. (1974). The assay mixture consisted in 0.1 mL of PMS and 0.17 mL of phosphate buffer (0.2 M, pH 7.9) and GSH (0.0018 M). The reaction was initiated by addition of 0.03 mL of CDNB (0.01 M), and the increase in absorbance was recorded at 340 nm. The enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein (ε=9.6mM^{-1} cm^{-1}).

For GSH_t content determination, PMS was precipitated with trichloroacetic acid (TCA 12%) for 1 h and then centrifuged at 12000g for 5 min at 4 °C. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using

GR excess, whereby the sulfhydryl group of GSH reacts with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) and produces a yellow coloured 5-thio-2-ni-trobenzoic acid (TNB) (Baker et al., 1990; Tietze, 1969). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the GSH concentration in the sample. The assay mixture consisted in 0.2 mL sodium phosphate buffer (0.143 M, pH 8), EDTA (0.0063 M), DTNB (0.001 M) and NADPH (0.00034 M), added to 0.04 mL of deproteinated PMS. The reaction was initiated with 0.04 mL of GR (8.5 UmL^{-1}). Formation of TNB was measured at 415 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg $protein^{-1}$ (ε=14.1×103 M^{-1} cm^{-1}).

As an estimation of LPO, the quantification of thiobarbituric acid reactive substances (TBARS) was carried out in the previously prepared lysate according to the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) and adapted by Wilhelm Filho et al. (2001a; 2001b). Briefly, 0.005 mL of butylatedhydroxytoluene (BHT; 4% in methanol) and 0.045 mL of potassium phosphate buffer (0.05 M, pH 7.4) were added to 0.075 mL of lysate and mixed well to prevent oxidation. To 0.05 mL of this mixture, 0.25 mL of TCA (12%) were added and vortexed, and 0.225 mL of Tris–HCl (0.06 M) and DTPA (0.0001 M) (pH 7.4) and 0.25 mL of thiobarbituric acid (TBA; 0.73%) were added. This mixture was heated for 1 h in a water bath set at 100 ºC and then cooled to room temperature and centrifuged (Eppendorf 5415R) at 15,700 g for 5 min. The absorbance of each sample supernatant was measured at 535. LPO was expressed in nmol of TBARS formed mg $tissue^{-1}$ using a molar extinction coefficient of 1.56 x 105 M^{-1} cm^{-1}.

2.3.2. DNA and chromossomal integrity

Comet assay

The alkaline comet assay was performed based on the method described by Collins (Collins 2004), with slight modifications according to Guilherme et al. (2010)

and Shaposhnikov et al. (2010). All slides were freshly prepared. Briefly, 20 µL of cell suspension (using the whole blood previously diluted in PBS) was resuspended in 70 µL of low melting point agarose (1 %; dissolved in PBS). Twelve drops (gels) with 6 µL of cell suspension were placed on a pre-coated glass slide with 1 % of normal melting-point agarose (1 %; dissolved in distilled water), as two rows of 6 gels (6 groups of two replicates), without coverslips, containing approximately $1.5x10^3$ cells/gel. Gels were kept for 5 min at 4 °C to let agarose polymerise and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1 % Triton X-100, pH 10) for 1 h. Thereafter, slides were gently placed in a horizontal electrophoresis tank, filled with freshly electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), for alkaline treatment. DNA was then allowed to unwind for 20 min. Electrophoresis was performed under 1.04 V cm^{-1} for 15 min. DNA unwinding and electrophoresis (as well as the preceding lysis) were carried out in the dark, at 4 °C. Once finished the electrophoresis, slides were washed in PBS (10 min), distilled water (10 min), and then gels were fixed for 10 min in absolute ethanol.

Slides were stained with ethidium bromide (20 g L^{-1}) and analysed on Leica DM2000 fluorescence microscope (x 100 magnification). Images were captured with the camera Leica DFC7000 T. Tail DNA % was determined with OpenComet software (Gyori et al. 2014) with a minimum of 100 nucleoids per sample. This metric for the comet assay is the most accepted in use for determining DNA breaks (Kumaravel et al. 2009; Møller 2018; Møller et al. 2020). Automatically generated scores corresponding to multiple nuclei, non-cellular debris, or obstructions were manually removed from the analysis.

ENA assay

The assay was carried out in mature peripheral erythrocytes, according to the procedure of Pacheco and Santos (1996). Briefly, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under 1000× magnification (microscope Olympus BX50), to evaluate the relative

frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN). Results were expressed as the sum of frequencies for all the categories observed (K + L + S + V + MN).

2.3.3. EMI determination

The EMI was determined according with Maceda-Veiga et al. (2010), with the modifications proposed by Castro et al. (2018). Briefly, 10 microscopic fields were randomly selected per slide (one slide per fish; the same slides used for the ENA assay) and photographed under 400x magnification (microscope Olympus BXB50). Then, in each microscopic field, 25 random cells were analysed with ImageJ software, measuring the minor axis of the nuclei and the major axis of the cells (A and B, respectively; see Figure 3). EMI was calculated for each cell by dividing A by B values, to a total of 250 cells. From the values of the ratio, cells were then categorized into one of the 10 maturity classes: [0.0 ≤ class 1 < 0.1]; [0.1 ≤ class 2 < 0.2]; [0.2 ≤ class 3 < 0.3]; [0.3 ≤ class 4 < 0.4]; [0.4 ≤ class 5 < 0.5]; [0.5 ≤ class 6 < 0.6]; [0.6 ≤ class 7 < 0.7]; [0.7 ≤ class 8 < 0.8]; [0.8 ≤ class 9 < 0.9]; [0.9 ≤ class 10 ≤ 1], where the class 1 represents erythrocytes with the higher maturity level and class 10 corresponds to cells with lower maturity status. Finally, average values for the frequency (%) of cells observed in each maturity class were represented for each experimental group.

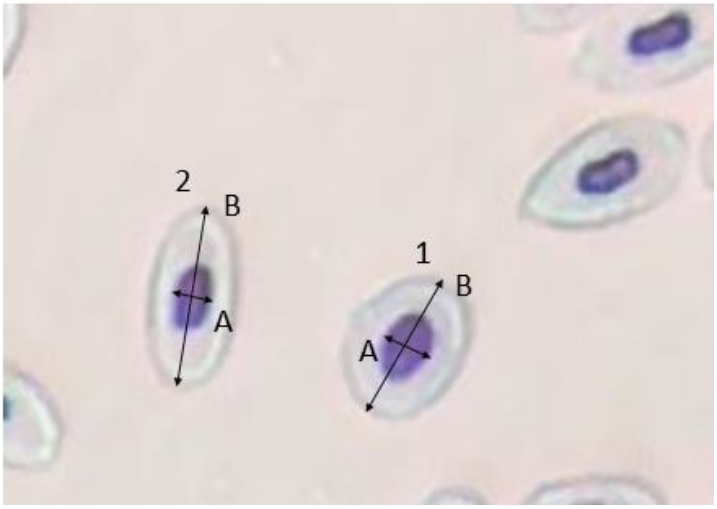

Figure 3-*Dicentrarchus labrax* peripheral erythrocytes (with nuclear normal shape). Elucidating measurements performed for the calculation of erythrocyte maturity index (EMI). Erythrocytes in earlier (1) and later (2) maturity stages are represented. Minor axis of the nucleus (A); Major axis of the cell (B).

2.4. Statistical analyses

The statistical analysis was conducted on the free trial of Statistica 10.0 software (StatSoft, Inc., USA), considering each sampling moment individually. For EMI, data were, first, transformed using the arcsine square root transformation, and then tested for normality (Shapiro-Wilk test and graphical analysis) and homogeneity of variances (Levene's test). Since the data didn´t fulfil the assumptions for normality, a non-parametric test was executed (Kruskal-Wallis ANOVA) and, when detected differences, a post-hoc Dunn´s test was applied. For the rest of the parameters, no transformation was performed. When the assumptions for normality and homogeneity were fulfilled, it was performed a parametric one-way ANOVA followed by Dunnet test, and, when the assumptions failed, a non-parametric test was executed (Kruskal-Wallis ANOVA) followed by the post-hoc Dunn´s test. In all the analyses, differences between means were considered significant when $p < 0.05$ (Zar, 1996).

Chapter 3

Results

3. Results

3.1. Antioxidant defences *vs*. oxidative damage

No significant differences were detected between the experimental groups (in both sampling moments) for CAT and SOD activities (Figures 4A and 4B, respectively).

Regarding GPX activity, significant differences were only detected in the second month, with all the supplemented groups (S2.5, S5 and S10) showing higher levels in comparison with the control group, but no differences among them (Figure 4C).

GST activity displayed different variation patterns for the two trial durations (Figure 4D). Hence, after 1 month, all the supplemented groups depicted lower levels when compared to the respective control, with the highest supplementation group (S10) showing also significantly lower GST activity in comparison with the other supplemented groups (S2.5 and S5). Differently, on month 2, groups S2.5 and S5 showed GST activity significantly elevated in comparison to the control, with the highest level reported for S5 (significantly higher than S2.5).

Concerning GSH_t, time-related profiles were also found (Figure 4E). Thus, in the first month, the groups S5 and S10 revealed significantly higher contents in comparison with the control group (as well as with S2.5), while, after 2 months, those groups displayed significantly lower contents in comparison with the control group (as well as with S2.5).

In terms of lipid peroxidation (LPO), significant differences were detected neither in the first and nor second month (Figure 4F).

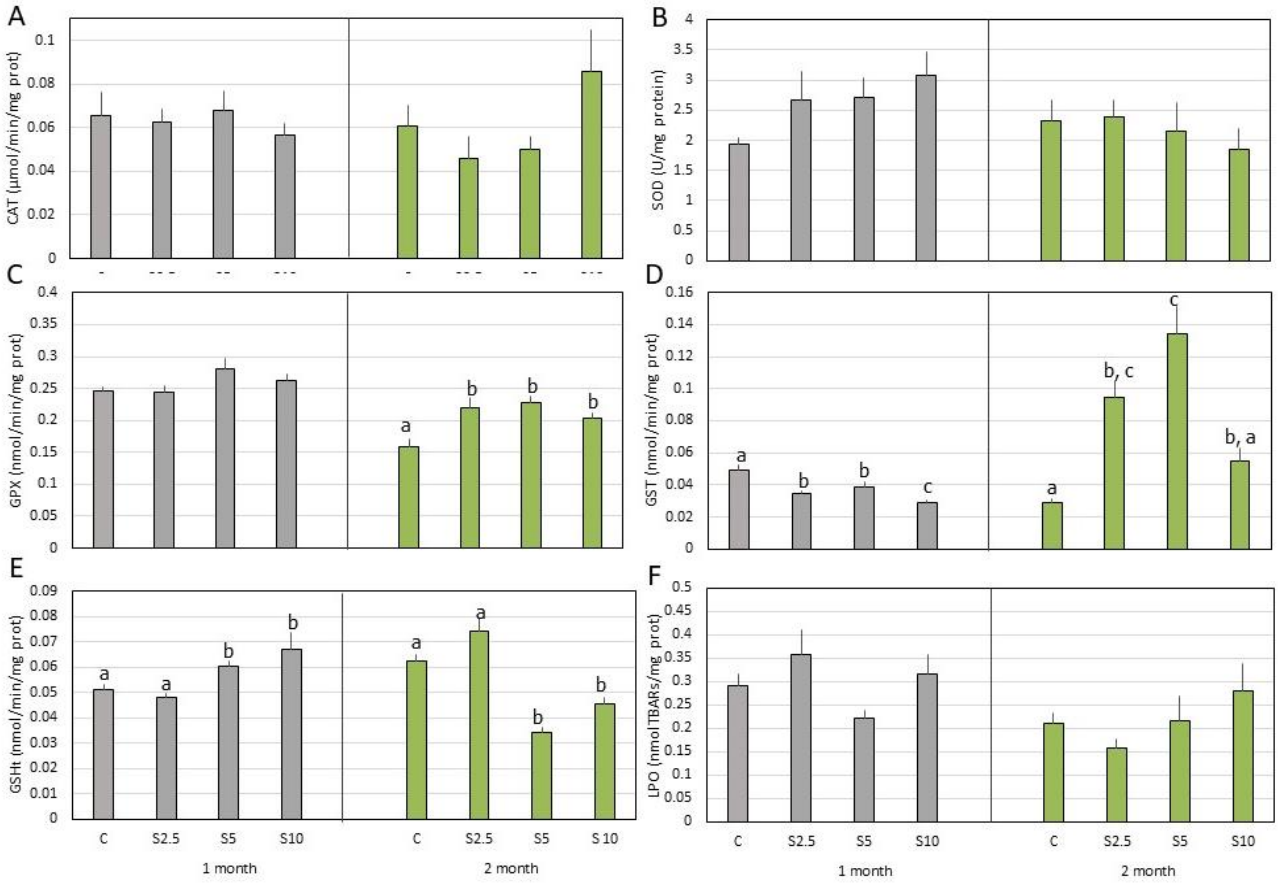

Figure 4- Mean values of blood oxidative stress parameters, namely (A) catalase (CAT), (B) superoxide dismutase (SOD), (C) glutathione peroxidase (GPx), (D) glutathione-S-transferase (GST) activities, and (E) total glutathione content (GSH_t), as well as (F) lipid peroxidation (LPO), following 1 and 2 months of dietary supplementation. Experimental groups concern: control (C), fed with standard feed, and Salicornia supplemented diets (S2.5, S5 and S10, corresponding to 2.5%, 5% and 10% supplementation, respectively). Bars represent standard errors. Different lower case corresponds to statistically significant differences ($p<0.05$).

3.2. DNA and chromosomal integrity

Comet assay

Significant differences in terms of tail DNA% were detected in both months, but translating contrasting variation profiles. In the first month, the group S5 revealed significantly higher DNA damage when compared to the control group. In the second month, all the supplemented groups (S2.5, S5 and S10) showed lower values of tail DNA% in comparison with the control group, with the lowest percentage (highest DNA integrity) reached by S10 (also significantly lower than S2.5) (Figure 5).

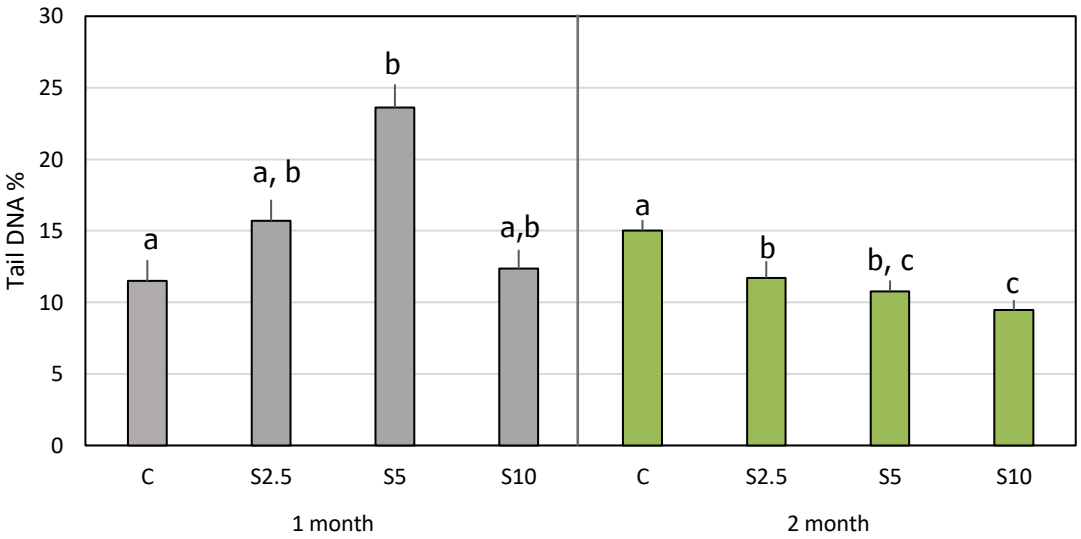

Figure 5- DNA damage measured by comet assay, and expressed as percentage of DNA in the tail, in blood cells of the sea bass, following 1 and 2 months of dietary supplementation. Experimental groups concern: control (C), fed with standard feed, and Salicornia supplemented diets (S2.5, S5 and S10, corresponding to 2.5%, 5% and 10% supplementation, respectively). Bars represent standard errors. Different lower case corresponds to statistically significant differences ($p<0.05$).

ENA assay

During the two months of the trial, no significant differences were detected for the ENA frequency (Figure 6).

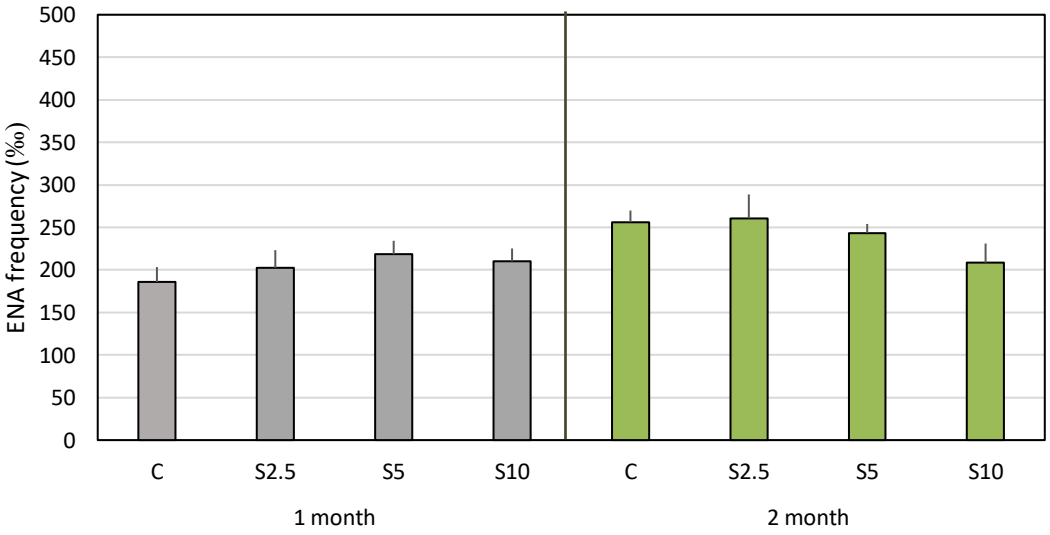

Figure 6-Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) evaluated in peripheral erythrocytes of European seabass, following 1 and 2 months of dietary supplementation. Experimental groups concern: control (C), fed with standard feed, and Salicornia supplemented diets (S2.5, S5 and S10, corresponding to 2.5%, 5% and 10% supplementation, respectively). Bars represent standard errors.

3.3. EMI assay

The cell frequency for each maturity class is depicted in the figure 7 (7A for 1-month trial; 7B for 2-month trial). No cells on classes 8, 9, and 10 were observed, so only frequencies of cells from classes 1 to 7 were illustrated. The most representative class in both sampling moments, regardless of the experimental group, was class 3.

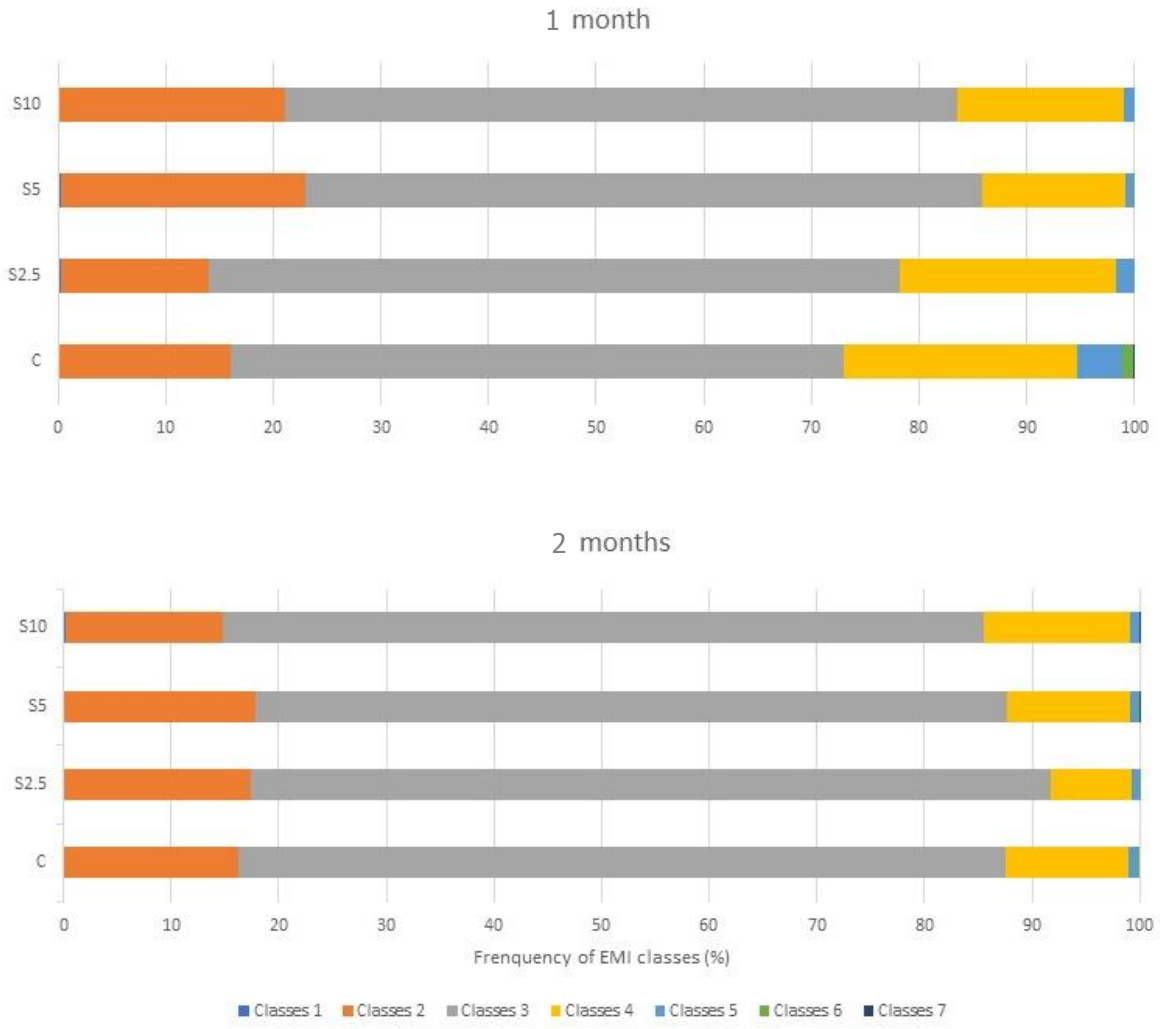

Figure 7-Representation of frequency (%) of the classes 1 to 7 of the Erythrocyte Maturity Index (EMI) evaluated in peripheral erythrocytes, following 1 and 2 months of dietary supplementation. Experimental groups concern: control (C), fed with standard feed, and Salicornia supplemented diets (S2.5, S5 and S10, corresponding to 2.5%, 5% and 10% supplementation, respectively). Bars represent standard errors. Different lower case corresponds to statistically significant differences ($p<0.05$).

Significant differences between the groups were detected only for class 5 and in the first month of the experimental trial. The group feed with 5% *Salicornia* (S5) revealed a lower frequency of these cells when compared to the control group (Figure 8).

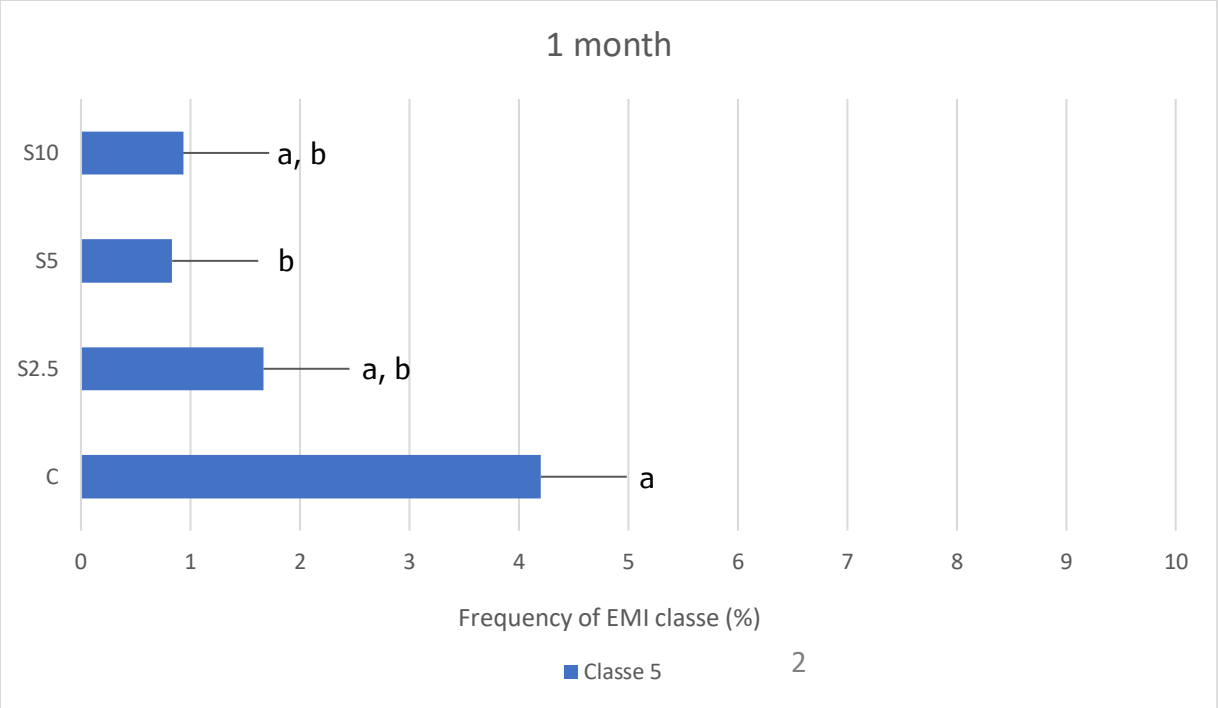

Figure 8-Representation of the frequency (%) of class 5 of the EMI in the first month. Experimental groups concern: Control (C), fed with standard feed, and Salicornia supplemented diets (S2.5, S5 and S10, corresponding to 2.5%, 5% and 10% supplementation, respectively). Bars represent standard errors. Different lower case corresponds to statistically significant differences ($p<0.05$).

Chapter 4

Discussion

4. Discussion

DNA and chromosomal integrity in fish can be affected by several factors, associated either with endogenous (*e.g*. ROS produced under stress conditions) or exogenous (*e.g*. waterborne contaminants) pressures (Pereira et al., 2019). In both situations, specific mechanisms can be mobilized to cope with those challenges and protect genome integrity.

In the last decades, developments in fish nutrition towards more economical and environmentally sustainable FF in fish farms have been made; however, research in this area has been mainly focused on the animal growth and feed intake. In more recent years, studies towards the DNA and chromosomal integrity, as well the antioxidant system modulation, have been performed, like in Pereira et al. (2019) where the introduction of a macroalgae mixture to the feed of *Sparus aurata* showed an increase of genoprotection.

The following discussion will focus on how Salicornia can affect antioxidant mechanisms, and the protection in relation to each type of genetic damage, since ENA and comet assays can both provide independent data and should be adopted as complementary endpoints (Pereira et al, 2019). First, will be discussed the antioxidant system. Then, the primary damage (measured as comet assay) or more persistent alterations (chromosomal damage measured as ENA assay) and, finally, the effect in the haematological dynamics (measured through EMI).

4.1. Evaluation of antioxidants and genoprotective properties

4.1.1. Antioxidant system modulation

Both GSHt content and GST activity showed an interference of the *Salicornia* supplementation, but translating contrasting time-related patterns differences. Furthermore, the results observed for the antioxidant system were different in both months, with no differences detected in CAT and SOD activities, and changes occurring

only in the glutathione and glutathione-dependent enzymes, representing an improvement of antioxidant status due to supplementation.

Regarding specifically GSHt, in the first month, the diets with 5 and 10% supplementation displayed elevated levels. A possible explanation for this alteration could rely on the concomitant decrease of GST activity. GSTs are important enzymes that can catalyse in conjugation with GSH to electrophilic substrates, producing compounds that can be less reactive and more soluble, and, apart from their essential functions in intracellular transport and biosynthesis, they have a critical role in defence against oxidative damage (Pereira et al., 2019). In a mirror way, in the second month, GSHt content of the higher supplemented groups (S5 and S10) showed decreased values. Two possibilities may be taking place, the first one involving the contribution of an exogenous source (due to dietary supplementation) of non-enzymatic compounds, as suggested by (Perreira et al., 2019), saving endogenous resources via a lower expression/synthesis of these low molecular weight scavengers and non-enzymatic antioxidants. A second explanation involves the occurrence of a lower pro-oxidant challenge caused by protective action of the supplementation (Marques, 2019). However, the results of enzymatic activities (in specific the GST and GPX activities) displayed an increase in the groups supplemented with Salicornia, evidencing a low pro-oxidative challenge, but the result of the LPO (measured as TBARS) showed no damage.

4.1.2. Strengthening DNA and chromosomal integrity

Regarding the non-specific DNA damage evaluated through the comet assay, after one month of Salicornia supplementation, only the group S5 presented lower DNA integrity. This effect is in line with a previous work with *Ulva rigida* supplementation (Marques et al., 2021) suggest that certain food, beverages, extracts, or isolated compounds can have a slight genotoxic action. However, the same was not observed in the ENA results, therefore indicating a slight and temporary damage from the supplementation with 5% of *Salicornia*.

After two months, all the groups with Salicornia supplementation have shown an improvement in DNA condition with emphasis for the S10 group that showed the most significantly improvement, although the results indicate that a supplementation of 2.5% is sufficient to reinforce DNA integrity. These results from the comet and ENA assays are corroborated by the results from the LPO.

4.1.3. Erythrocyte population dynamics

Concerning the fluctuation on *D. labrax* erythrocyte dynamics resulting from the Salicornia supplementation, the analysis of the nucleo-cytoplasmatic ratios only signalised effects attributed to the diet supplemented with 5% in the second month. A similar result was shown in Marques et al. (2019) where differences were detected in class 5 of the EMI in *Sparus aurata* in the diet supplemented with algae, also in this study the same most frequent class observed was class 3. So, according to Marques et al. (2019), this type of results could indicate a slight rejuvenation of the circulating erythrocyte population. In the same period and group (S5) differences were detected in DNA damage, this could be a indicator of a peculiar physiological condition of the European seabass to a temporary challenge. In the second month, the results showed no differences, indicating that this rejuvenation was temporary.

4.2. Contribution to the SDGs and improvement of aquaculture practices

Previous works on the European seabass have demonstrated that plant base meals does not negatively affect growth (Peixoto et al., 2016; Torrecillas et al., 2017). In the current context, the results of AQUACOMBINE Project report, 2020, have demonstrated no differences in growth of this species feed with different percentages of *Salicornia ramosissima*. In addition, the present results showed that the incorporation of *S. ramosissima* did not show toxicity or any health impairment, and, on the contrary, it showed advantages, namely in the improvement of DNA integrity and antioxidant protection.

In a major context, halophytes like *S. ramosissima* can have a role to play in the 2030 Agenda as a whole or as mean to achieve some sort of blue biotechnology that can contribute to the SDGs. The culture of *S. ramosissima* requires some investigation to achieve the so-called blue biotechnology "built on the same scientific and technological principles as other fields of biotechnology, but the source, process and/or final product is aquatic in some respect. The seafood industry for example, and consequently the challenge of feeding the world population, has experienced unprecedented breakthroughs through blue biotechnology and has been one of the areas where this technology has made the biggest progress towards the UN SDGs" (Vieira et al., 2020).

Sustainable Development Goals that are important to this dissertation can be divided between three groups. The first one related to the people, the second one related to economy and the last one related to the environment. Although we can directly or indirectly link Salicornia to the 17 SDGs, 8 can be considered more important in the context of this dissertation: 1. No poverty; 2. Zero hunger; 3. Good health and well-being; 6. Clean water and Sanitation; 8. Decent work and economic growth; 9. Industry, innovation and infrastructure; 12. Responsible consumption and production; 14. Life below water.

The first SDG can be related to the Salicornia industry creating new jobs associated directly to culture productions and biorefineries and indirectly through the local development of the communities and the regions associated. The second and third SDG are zero hunger, and good health and well-being, and thus, their association with Salicornia is direct since it is seen as one of the solutions for the ongoing increase of the demand for biological products and as new emerging food alternative for human consumption (Barreira et al., 2017; Ksouri et al., 2012). The sixth SDG can be associated to the production of Salicornia in a aquaponic system (that by itself can reduce water consumption by 90%) it would work as a biofilter, purifying water (Spradlin & Saha, 2022).

In terms of the economic value of Salicornia to the SDGs, its production, including in aquaponic systems (improving the circular economy) and the need to innovate responding to the demand, can generate blue biotechnology associated with new and more efficient facilities contributing to the eight and ninth SDGs.

Environmentally, the twelfth SDG is associated with the growing of ecological awareness, creating pressure for the stakeholders and governments to regulate and encourage the production and the products like *Salicornia.* The fourteenth SDG is life below water, making this SDG the most obviously connectable, once Salicornia is a saltmarsh organism that can be used in a more sustainable and efficient way, like the use of halophytes in an aquaponics system where they function as a biofilter reducing the environmental impacts of wastewater from aquaculture reutilizing up to 95% of nitrogen and 85% of phosphorus that would normally be discharge (Spradlin & Saha, 2022).

Aquaculture, farming, and bioprocessing of *Salicornia ramossisima*, can help desalinize areas and can be easily combined with sustainable management of natural areas and use otherwise marginal lands to create value and jobs in rural, remote, and salt affected areas (Vizetto-Duarte et al., 2019). Salicornia can improve aquaculture practices for that was discussed above and presents serval benefits for the future in economic, environmental, social, and political areas (Lima et al., 2020).

Socially/ Politically

- Creation of jobs directly or indirectly
- Production of compounds to increase human and animal health
- Reduction of inequality between urban and rural lands
- The project contributes to at least 8 of the 17 of United Nations 2030 Agenda for Sustainable Development

Economically

- Besides the intrinsic value of the halophyte's plans, we can add the bio products (whole botanical extract, protein, and biogas)

- Reduction of cost of aquaculture due to FLEXRACK systems

Environmentally

- Improvement of circular economy and recycling nutrients
- Reduce the negative impacts of traditional aquaculture and low water consumption
- Increase of carbon sequestration

Chapter 5

Conclusions

5. Conclusions

Summarizing the main findings and providing an answer to the objectives of the present dissertation, it was demonstrated that a diet supplemented with *Salicornia ramosissima*, in percentages of 2.5, 5 and 10%, showed no toxicity, since the general physiological condition of the animals was not negatively affected, namely as DNA/chromosomal integrity and LPO levels.

Furthermore, the supplemented diets exhibited genoprotective properties in the European seabass blood cells. This beneficial action was observed through the comet assay and specifically on the second month of Salicornia supplementation, where an improvement of DNA integrity was discernible. This positive impact was detected in all the supplementation levels, but with greater acuity at the highest level (10%). In addition, an enhancement of the antioxidant defences was apparent, namely involving the regulation of glutathione and glutathione-dependent enzymes.

The Salicornia as a product have the potential to growth economically, environmentally and socially, since the aquaculture, farming, and bioprocessing can produce benefits and developments in the industries associated with its production while accomplishing several SDGs.

Overall, these results seem to be promising towards the incorporation of halophytes like Salicornia in aquafeeds, offering a potential strategy to reduce cost of aquaculture while strengthening fish fitness, and thus, to improve aquaculture practices.

Chapter 7

References

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