POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Health promoting effects of Salicornia ramosissima biomass in diets for European seabass (Dicentrarchus labrax)

Francisco Manuel Ribeiro Cruz

2022

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Acknowledgments

After one year and five months since the beginning of this dissertation, I now end my academic journey with a pioneering project. Which makes me feel very proud and grateful for the participation.

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Sadly during this dissertation, precisely in the dawn of the 12th January 2021 my godfather died, a relative that provided me a great support for my objectives and this fascination for this area. I had dark moments of almost giving up. But I had great support from all my family and friends. I owe a huge thanks to all of them, but mainly to my parents and my godmother. I also owe a special thanks to Gisele, even if she was far away she supported me throughout this phase of the dissertation and helped me a lot to get back on my feet in this worst moment, and to Evelyn for the same reason. Lastly, I thank once again to Ana Couto and Marina Machado for the support and concern for this loss.

Finally I dedicate this dissertation to all of you but especially to my godfather, ending this text in memory of him.

> “Hoje partiu mar sereno, sem nenhuma ondulação, deixando para trás uma tormenta que foi bastante sufrida. Mas porém terminada.
> Agora segue rumo a um novo cais, que vai para lá dos céus.
> Neste momento a saudade é imensa, mas sei para tu vais, estarás muito melhor.” (Francisco Cruz, 12/01/2021)
> Amo-te Padrinho

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Resumo

O projecto AQUACOMBINE pretende demonstrar a produção em aquacultura combinada com a produção de halófitas, através da utilização dos princípios de economia circular, onde os resíduos são recuperados e utilizados dentro do sistema para criar valor interno e novos produtos, para além de evitar desperdícios. Todas as partes da biomassa halófita serão utilizadas para produzir múltiplos produtos tais como alimentos, rações, extractos botânicos e compostos bioactivos puros, bem como biogás dos resíduos finais para produzir energia e um resíduo rico em nutrientes para trazer nutrientes essenciais (por exemplo, fosfatos) de volta às terras de cultivo. As halófitas, como do género Salicornia são plantas tolerantes ao sal actualmente utilizadas para temperos e em saladas na dieta humana, cujo género não só apresenta um grande potencial como planta de cultura em solos altamente salinos, mas também valor na medicina tradicional exibindo atividades biológicas e farmacológicas. O presente estudo foi concebido para a avaliação dos efeitos da inclusão da *Salicornia ramosissima* J. Woods no estado de saúde de uma espécie com elevado interesse comercial, o robalo (*Dicentrarchus labrax*). Foi observado que a inclusão de Salicornia (fracção não usada para consumo humano) até 10% da ração de robalo, não compromete o crescimento e o estado imunitário dos peixes após um período de alimentação de 62 dias. Mas quando os peixes foram desafiados com uma bactéria inactivada, demonstrou uma resposta inflamatória concomitante à *Photobacterium damselae* subsp. *piscicida* inativada. Verificou-se um aumento da concentração de leucócitos na cavidade peritoneal em peixes alimentados com 10% de inclusão de salicornia, em comparação com a dieta controlo. Assim, podemos concluir que a inclusão de Salicórnia numa dieta do robalo, até 10%, pode ser feita com sucesso, uma vez que não demonstrou alterações no sistema imunitário dos peixes. Além disso perante um estimulo imunitário esse mesmo nivel de inclusão potencia a resposta inflamatória aumentando o recrutamento celular para o local da infecção.

Palavras-Chaves: Halófitas; compostos bioactivos; inflamação; bactéria.

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Abstract

The AQUACOMBINE project aims to demonstrate aquaculture production combined with halophyte production by using circular economy principles, where waste is recovered and used within the system to create internal value and new products, in addition to avoiding waste. All parts of the halophyte biomass will be used to produce multiple products such as food, feed, botanical extracts and pure bioactive compounds, as well as biogas from the final waste to produce energy and a nutrient-rich residue to bring essential nutrients (e.g. phosphates) back to farmland. The halophytes such as Salicornia genus are salt-tolerant plants currently used for condiments and on salads in the human diet, the genus of which not only has great potential as a crop plant in highly saline soils, but also value in traditional medicine exhibiting biological and pharmacological activities. The present study was designed to evaluate the effects of the inclusion of *Salicornia ramosissima* J. Woods on the health status of a species of high commercial interest, the European seabass (*Dicentrarchus labrax*). It was observed that the inclusion of Salicornia (fraction not used for human consumption) up to 10% of the diet, did not compromise the growth and immune status of the fish after a feeding period of 62 days. But when the fish were challenged with an inactivated bacterium, it demonstrated an inflammatory response concomitant with the inactivated *Photobacterium damselae* subsp. *piscicida*. An increased concentration of leukocytes was found in the peritoneal cavity in fish fed 10% of Salicornia inclusion compared to the control diet. Thus, we can conclude that the inclusion of Salicornia in a sea bass diet, up to 10%, can be done successfully, as it showed no changes in the fish immune system. Moreover, upon immune stimulation, the same inclusion level enhanced the inflammatory response by increasing cell recruitment to the infection focus.

Keywords: Halophytes; bioactive compounds; inflammation; bacteria.

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Abbreviations List

AA – Amino acids

ANFs – Antinutritional factors

APA – Portuguese Aquaculture Association

BM – Blood meal

FA – Fatty acids

FAO – Food and Agriculture Organization

FM – Fish meal

HBSS – Hanks' Balanced Salt Solution

HG – Haemoglobin

HT – Haematocrit

MCH – Mean corpuscular haemoglobin

MCHC – Mean corpuscular haemoglobin concentration

MCV – Mean corpuscular volume

PBM – Poultry by-product meal

Phdp – Photobacterium damselae subsp. piscicida

PP – Plant protein

PUFA – Polyunsaturated fatty acids

RBC – Red blood cells

RT-qPCR – Quantitative reverse transcription Polymerase Chain Reaction

SM – Shrimp meal

TSA – Tryptic soy agar

TSB – Tryptic soy broth

UPLC – Ultra-high performance liquid chromatography

WBC – White blood cells

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1. Introduction

1.1. Aquaculture

Aquaculture has become an essential activity in recent years for the assurance of human access to a quality protein source. Also, because of the continuous human population growth, the food needs exponentially increase and FAO points to the fact that fish will continue to be the main protein source for most communities. (FAO, 2021). Fish and other products resulting from aquaculture, have high nutritional value and are a symbol of universal health (FAO, 2021). According to the FAO report, fishing was overtaken in the 1990s by aquaculture (Figure 1), mainly explained by the increase in the world population and the per capita consumption of aquatic products. With that, an increase of 23 million tonnes annually of fish supply was required and aquaculture peaked. In 2009, fish consumption was already accounted for 16.6% of all animal protein and 6.5% of the total protein consumed by the world population. However, the aquaculture sector is still rising since, FAO predicts that in 2020-2030 the aquaculture production, only in the Mediterranean and Black Sea, will exceed 4 600 000 tonnes (FAO,2020).

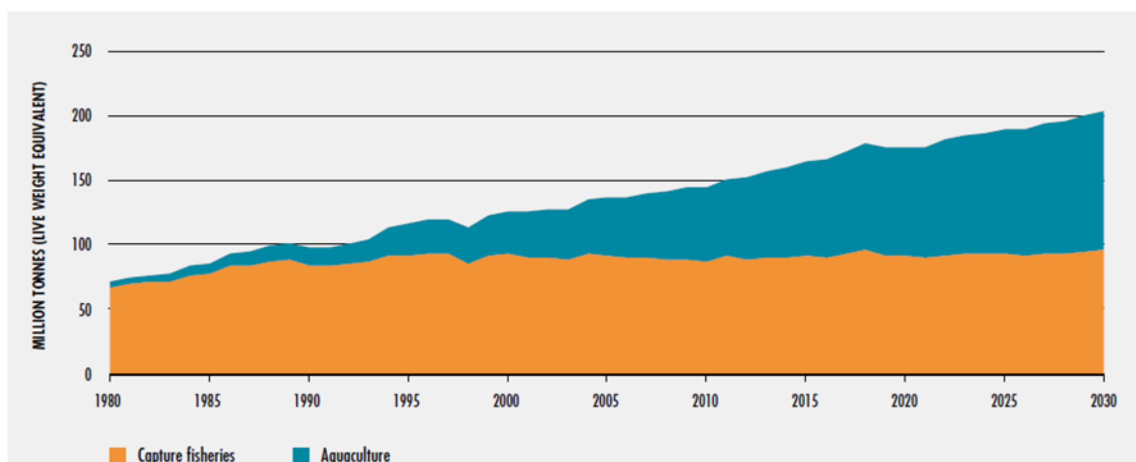

Figure 1: World production by capture fisheries and aquaculture, 1980-2030 (Source: FAO, 2020 SOFIA report).

However, this exponential growth presents new challenges with regard to the sector sustainability that includes the cultural and social responsibility of the farm operations as well as the creation of an environmentally and economically friendly industry. Another important factor to take into account is the needed balance between productivity and animal welfare in the production system (FAO, 2020).

Since 2007, Mediterranean fisheries have suffered a decline of about 15 % (FAO, 2021). In the area, aquaculture is composed of several types of culture, and includes bivalve, crustacean and marine species. Current data indicate Greece as the largest producer, followed by Turkey, Spain, Italy and France (APA, 2014). However, although these countries have a great facility in the aquaculture process due to their geographical location, they are also susceptible to various factors such as diseases, environmental conditions and scientific

and technological limitations (Ramalho & Dinis, 2010). In Portugal, aquaculture in the 1980s was quite low, with freshwater bivalves and trout (*Oncorhynchus mykiss*) aquaculture as the main products and sustained by the use of traditional techniques such as clay tanks built in old containment basins and fish reservoirs, and in estuaries or lagoons for extensive shellfish aquaculture. For a long time, these techniques were seen as small familiar practices. From the 1990s onwards, these techniques were modified due to the stimulation of aquaculture production in the European Union. At the same time, an overall increase in marine aquaculture took place, followed by a period of some fluctuation, with total production reaching 7 987 tonnes in 2008 (Ramalho & Dinis, 2010). Despite all, Portugal aquaculture industry is still characterised as traditional and requires some modernisation. To this date, turbot (*Scophthalmus maximus*), rainbow trout (*Oncorhynchus mykiss*), sole (*Solea senegalensis*), European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are being produced mainly in intensive or semi-intensive farming systems, such as earthen ponds (APA, 2014). Production in aquaculture sagged in 2019 at 14,291 tonnes, with a value of €118.3 million. These values translate an increase of 2.6% in quantity and an increase of 18.5% in value, when compared with the values of the previous year (DGRM, 2021; INE, 2021). Production in brackish and marine waters remained the most important, corresponding to about 93.4% of total production. The production of fish in brackish and marine waters, in turn, represented 46.6% of total production, and of this, 82.4% corresponded to the production of sea bream and turbot (DGRM, 2021; INE, 2021).

European seabass (*Dicentrarchus labrax*, Linnaeus, 1758), is naturally found from the North Atlantic of Norway and the British Isles, to the South in Morocco, Canary Islands, Mediterranean and Black Sea. This species is able to survive in extreme salinity conditions and even in fresh water of coastal areas, estuaries and brackish water lagoons (Moretti et al., 1999). Seabass production has been increasing since the 1990s and 95% of its production comes from aquaculture (Bjørndal & Guillen, 2017). Seabass was historically farmed in coastal lagoons and tidal reservoirs before the rush to develop mass production of juveniles in the late 1960s. As the first non-salmonid marine species to be commercially farmed in Europe, is currently the most important commercial fish widely farmed in Mediterranean areas. Although seabass is still farmed in seawater lakes and ponds, most production comes from sea cage farming (FAO, 2020). In aquaculture, genetic variability can be controlled by brood stock selection and it is adapted to small portions of food, but in periods controlled by operators, so that there is no competition for food (Kocher & Kole, 2008).

1.2. Dietary protein sources

Fishmeal (FM) is considered one of the most nutritious and balanced source of protein for commercial aquafeeds production, particularly for carnivorous species (Glencross et al., 2007). Nowadays, the demand of such ingredient is quite high while its supply is limited, leading to its high prices. As a result, the seek of aquafeeds production sector, for practical, high quality and economically viable alternative protein ingredients has been recognized as an important way to establish a sustainable aquaculture industry (Naylor et al., 2009). Alternative animal protein ingredients, such as spray-dried blood meal (BM), shrimp meal (SM) and poultry by-product meal (PBM) were described as viable economical

protein sources and considered as potential alternatives in aquaculture animal feed due to their high protein content, low carbohydrate content and lack of anti-nutritional factors when compared to plant-derived ingredients (Allan et al., 2000).

Insects have also been studied as an alternative protein source for aquatic animals feeds production. Their short life cycles, ability to grow on a wide range of substrates, high productivity, high feed conversion factors (Berggren et al., 2019), combined with the relatively good nutritional profiles make them a high potential source of protein for aquatic feeds formulation. In fact, they have received increasing attention in many countries. Additionally, the nutritive composition of insects can be increased by combining insect meals with complementary nutrient profiles or by manipulating the substrate used as a nutrient source to improve fatty acid content, digestibility, and even palatability (Henry et al., 2015). A number of insect species has already been approved as food grade substrates (Wang et al., 2017) since they are considered non-pathogenic, non-pathogen vectors, and non-invasive. Research has been mainly focused on the black soldier fly (*Hermetia illucens*), the common housefly (*Musca domestica*), and the yellow mealworm (*Tenebrio molitor*) (Wang et al., 2017).

Despite the abovementioned protein sources, most of the research and industry focus has been directed to plant protein (PP) sources, due to their low price, high protein content and acceptable amino acid composition (Santigosa et al., 2011; Al-Thobaiti et al., 2018). Such studies have showed that plant protein sources such as soybean, barley, canola, maize, cottonseed, pea/lupin and wheat can successfully replace FM (Brinker & Friedrich, 2012; Ibrahem & Ibrahim, 2014). However, the use of PP presents some constraints as they contain many antinutritional factors (ANFs) (e.g. phytic acid, protease inhibitors, lectins, saponins, antivitamins and allergens (Hua et al., 2019)) that affect fish growth (Zhou et al., 2017; Yin et al., 2020) and cause pathological damage (Wang et al., 2017; Gu et al., 2018). As a result, current PP can replace FM at a level corresponding to slightly more than 50% of total dietary protein if high quality FM is used, whereas some lower inclusions appear to be necessary for lower quality FM to achieve optimal growth and feed utilization (Mundheim et al., 2004; Albrektsen et al., 2006). Regardless, the replacement of FM with PP sources has resulted in cost savings and resource savings (De Santis et al., 2015; Trejo-Escamilla et al., 2016).

In addition, an important factor that determines the nutritional quality of vegetable proteins is the potential regulatory activity of bioactive peptides contained in the amino acid sequence. In fact, it has been shown that some peptides contained in the primary sequence of proteins, released by enzymatic hydrolysis during food production processes, may exert important biological functions (Toldrá et al., 2018), as they can bind to receptors belonging to cells involved in specific metabolic processes. Depending on the amino acid sequence, these peptides can exhibit immunomodulatory, antimicrobial, antioxidant and antithrombotic actions (Mohanty et al., 2016).

Regardless the protein source used, nutrient utilization and growth performance are factors often used to evaluate such ingredients efficiency in aquafeeds, while changes in lipid metabolism are usually neglected. In fish, lipids act as an important source of energy, and lipid sources support several physiological, developmental and reproductive processes (Tocher, 2003). However, it has been shown that replacing FM with alternative protein ingredients could regulate lipid metabolic pathways and ultimately lead to hepatic lipid

deposition (steatosis) in many fish species (Couto et al., 2016; Kokou et al., 2017; Yin et al., 2018). On the other hand, the relationship between diet and immune function has also been shown to be complex, not only with respect to many physiological processes, including apoptosis and inflammation, but also with respect to physiological functions and animal welfare (Kiron, 2012). Previous studies have confirmed that alternative protein ingredients in the diet influenced immune responses and immune parameters in some fish species, such as seabass (Campos et al., 2017), turbot (Wang et al., 2016) and seabream (Kokou et al., 2012).

1.3. Halophytes

Halophytes are edible plants that can tolerate high salinity soils. These species represent about 1% of the world flora and are often plants rich in protein, fiber, amino acids and vitamins (Brown et al., 1999; Ventura & Sagi, 2013). Their cultivation in soil and water conditions unsuitable for conventional crops can result in an alternative agriculture (Marques et al., 2017), such as the case of marine aquaponics. These plants possess a wide range in n-3 polyunsaturated fatty acids (PUFA) and are even richer in these nutrients when compared to other conventional crops commonly used for human consumption (Ventura & Sagi, 2013). Moreover, they can be characterized by their salty taste when consumed in salads, lead to the growing interest in the use of these new ingredients in gourmet cuisine and the creation of new markets for the commercialization of these products (El Shaer, 2010).

Commercialization of halophyte plants is commonly found in populations located close to salt marsh habitats, but can also be grown outdoors or in greenhouses, as a suitable and environmentally friendly alternative minimizing the impact on wild harvests. Nowadays, it is already well established that exposure to different abiotic factors, can promote major changes in the metabolism of the plant and consequently in its chemical composition (Sampaio et al., 2016). The growing conditions of this plant species can influence the fatty acid (FA) composition and membrane lipids and thus promote significant differences in the nutritional value of these "sea vegetables" (Sui et al., 2010; Ventura et al., 2011; Sui & Han, 2014). Within the halophyte species, the recently identified lipidome of *Salicornia ramosissima* (Hook. f.) J. Woods and *Halimione portulacoides* (L.) Aellen [*Syn. Atriplex portulacoides L.*] revealed the presence of quite important lipids with good nutritional values and bioactive compounds (Maciel et al., 2018).

Salicornia comes from the Latin word "salt" because it is one of the halophyte species quite tolerant to high salinities (Lv et al., 2012; Volkov, 2015). There are more than 150 scientific names of plant species in the genus Salicornia, and only 64 of these are already reported as accepted species names. Among these are*: S. alpini, S. ambigua, S. andina, S. arabica, S. bigelovii, S. blackiana, S. brachiata, S. capensis, S. decumbens, S. decussata, S. disarticulata, S. dunensis, S. europea L., S. fruticosa L., S. hispanica, S. maritima, S. ramosissima*. Majority of them are identified in the largest database of halophytes, the eHaloph-Halophytes database (Santos et al., 2016).

Salicornia genetic composition determines its physiological tolerance to high salinity and its rich composition in the production of bioactive metabolites, mostly associated with antioxidant systems and repair mechanisms (Kong et al., 2008; Kim et al., 2011; Duarte et

al., 2013). *Salicornia ramosissima* green tips are commonly used as a salt substitute for salads and gourmet cuisine, presenting a good nutritional value suitable for human consumption. However, apart from the green tips, the woody part of the plant is considered waste.

The main pigments associated with the reddish color of Sarcocornia are betacyanins and other phenolic compounds (Costa et al., 2006; Davy et al., 2006; Duarte et al., 2013), that have shown anti-stress properties (Soares et al., 2008; Severo et al., 2011; Duarte et al., 2013; Pinheiro et al., 2017). The different concentrations of these compounds may result in varying antioxidant properties (Oh et al., 2007; Surget et al., 2015; Ferreira et al., 2018). They also present a rich lipid profile with fatty acids in its composition (El Shaer, 2010). Similarly, Salicornia seeds are rich in varied nutrients and minerals such as potassium, sucrose, glycerol, unsaturated oleic and linoleic fatty acids (Austenfield, 1986), and these in turn can be used for teas (Kang et al., 2011). In traditional medicine, is commonly used as a potential remedy for ameliorating diseases such as constipation, obesity, diabetes and cancer (Ksouri et al., 2012). Its compounds have various pharmacological activities, including antioxidant, anti-inflammatory, and immunomodulatory (Rhee et al., 2009). In the world of aquaculture, these halophytic plants are utilized in wastewater treatment and in integrated multi-trophic aquaculture (Cárdenas-Pérez et al., 2021; Custódio et al., 2021) and they are able take of inorganic nitrogen compounds from the water (Quintã et al., 2015). However, there are still unknown studies on the effect of Salicornia in fish diets (Cárdenas-Pérez et al., 2021).

1.4. Immune system

Fish have a highly developed immune system (Press & Evensen, 1999) divided into two parts, according to their specificity and speed of response (Parkin & Cohen, 2001). Initially, the non-specific immune mechanism (innate system) acts regardless of the type of invader and temperature. The rapid action of these mechanisms prevents the attackers from settling and multiplying in the host, although there is the possibility of damaging healthy tissue due to the lack of specificity (Parkin & Cohen, 2001). It then proceeds to a specific defense that takes time to work and is temperature dependent (Ellis, 2001). At the cellular level, the immune response is composed by lymphocytes, monocytes, macrophages, granulocytes (e.g. neutrophils) and thrombocytes (Ellis, 1999). As the first cellular defense, monocytes/macrophages and neutrophils interact through phagocytosis processes that begin soon after injury or infection (Corbel, 1975; Ellis, 1999). This group of phagocytic cells can also release antibacterial peptides, complement factors, cytokines and acute phase proteins (Chistiakov et al., 2007). Also, within this group of phagocytic cells, humoral components are produced and released as they prevent settlement and adhesion of the pathogen, encompassing a wide range of antimicrobial agents such as lysozyme, anti-proteases, complement factors and many others (Chistiakov et al., 2007). Skin, gills and intestine are the mucosal tissues with direct association to the immune system. As the main barriers, mucosal tissues provide physical and chemical protection (Kiron, 2012), with the help of cellular and humoral components (Kiron, 2012). Accumulations of the innate immune cells are found in the mucosal tissues where pathogens can enter the body. The various cells interact with each other producing a coordinated response for the elimination of

pathogens (Ellis, 1999). When the external barriers are broken, an inflammatory response is initiated to resolve the infection, repairing the damage done and establishing a new homeostasis (Corbel; 1975). Within this range of the innate immune system two soluble components stand out, the anti-proteases and the enzyme lysozyme. Anti-proteases inhibit proteases released by bacteria, thereby decreasing protein digestion and the source of amino acids (Ellis, 2001). Lysozyme is an enzyme capable of degrading the peptidoglycan membrane of the bacterial cell wall. With the same capacity as this enzyme, we have the complement factors that are activated by lipopolysaccharides (LPS), present mainly in Gram-negative bacteria, leading bacteria to bacterial lysis, this factor is also responsible for the recruitment of phagocytic cells (Ellis, 2001).

Moreover, there are two types of lymphocyte cells, the B and T cells, which recognize infection and develop the adaptive immune response (Uribe et al., 2011). Besides antibodies production (Cerezuela, et al., 2016; Magnottir, 2010), B lymphocytes are responsible for the humoral response, when activated by the antigen the B cells proliferate and differentiate into memory cells, which will fix this response for a long period of time (Magnadottir, 2010; Uribe et al., 2011).

1.5. Project AQUACOMBINE

The AQUACOMBINE project aims to demonstrate combined aquaculture and halophyte production (saline tolerant plant cultivation) using circular economy principles, where waste is recovered and used within the system to create both internal value and new products, in addition to waste avoidance. Waste is used within the system to create both internal value and new products. Excess nutrients from fish production will be used as fertilizer for the halophyte plants and filtered through a microbial water treatment system to allow recirculation of the water back to the aquaculture tanks. All parts of the halophyte biomass will be used to produce multiple products such as food, feed, botanical extracts and pure bioactive compounds, as well as biogas from the final waste to produce energy and a nutrient-rich residue to bring essential nutrients (e.g. phosphates) back to the farmland. This combination of aquaculture, cultivation and bioprocessing can help desalinate salt-affected areas and can easily be combined with sustainable management of natural areas and/or use of marginal land to create value and jobs in rural, remote and salt-affected areas. Bioprocessing will create added value to combined agriculture and diversify products.
The team includes industrial partners along the entire value chain, from raw material production (Alpha-Aqua, RIASEARCH, and Les Douceurs du Marais), technology providers (Celabor, Envirohemp), exploitation partners (Riasesarch, Naturfarm, and Thise dairy) as well as farmer and end-user organizations (Alentejo's Regional Develop and Food Processing Initiative). The project is further supported by universities and research centers in order to carry out a full (economic, environmental and social, (CIIMAR and University of Aveiro)) evaluation of the integrated system, as well as a business plan and a set of policy options.

1.6. Thesis specific objective

The present Thesis will focus on the evaluation of Salicornia ability to be included in fish feeds formulation. Specifically, the work was designed to assess the effect of *Salicornia ramosissima* non-food fraction dietary inclusion, on health condition and inflammatory response of European seabass, fed during 62 days. Fish immune condition during the feeding period and the concomitant inflammatory response to inactivated *Photobacterium damselae* subsp. *piscicida* (*Phdp*) was evaluated.

2. Material and Methods

2.1. Heat-inactivation of *Photobacterium damselae* subsp. *piscicida*

Photobacterium damselae subsp. *piscicida* (*Phdp*), strain PP3, was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria were routinely cultured at 22 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories) supplemented with NaCl to a final concentration of 1% (w/v) (TSB-1 and TSA-1, respectively) and stored at 80 °C in TSB supplemented with 15% (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, stocked bacteria were cultured for 48 h at 22 °C on TSA-1 and then inoculated intoTSB-1 and cultured overnight at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × *g* for 30 min, re-suspended in sterile HBSS and adjusted to 3.4×10^5 colony forming units (cfu) per ml. Bacteria were then killed by heat exposure (60 °C) for 2 h. Loss of bacterial viability following heat-exposure was confirmed by plating resulting cultures on TSA-1 plates and failing to see any bacterial growth.

2.2. Experimental diets

Fresh *Salicornia ramosissima* was collected from Praia da Areia Branca, (Bunheiro-Murtosa, Portugal) between the 3rd and 15th of June of 2020. From the 21.5 kg of collected *Salicornia ramosissima*, the whole plant excluding the green tips and the roots was dried in Riasearch Lda research facilities (Murtosa, Portugal). The resulting 6 kg of *Salicornia ramosissima* were then sent to Sparos, Lda, where they were grinded and incorporated into the feeds. Four diets (Table 1) were formulated and manufactured by Sparos Lda (Olhão, Portugal). The control diet (CTRL) was formulated to include an indispensable AA profile meeting the ideal pattern estimated for European seabass (Kaushik, 1998). At the expenses of wheat meal, *Salicornia ramosissima* whole plant was incorporated at 3 different levels: 2.5, 5 and 10% of feed weight (SAL2.5, SAL5 and SAL10, respectively).

Main ingredients were ground (below 250µm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 1.5mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45ºC. Formulation of experimental diets is presented in Table 1. Bromatological analysis of experimental diets was performed following the Association of Official Analytical Chemists procedures (AOAC, 2006). Briefly, dry matter was determined by drying samples at 105ºC in an oven until constant weight; ash, by incineration at 450 ºC for 16 h in a muffle furnace; crude protein content (N x 6.25), by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit; lipid content, by petroleum ether extraction (Soxtec HT System) and gross energy, by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

Diets were analyzed for total AA content (Table 2). Diet samples were hydrolysed in 6M HCl at 116ºC for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high performance liquid chromatography (UPLC) in a Waters reversed-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. The resultant peaks were analyzed with EMPOWER software (Waters, USA). Tryptophan was determined by HPLC, after alkaline hydrolysis (Silliker Portugal, S.A.).

Table 1. Ingredients and chemical composition of the experimental diets.

Ingredients (%)	CTRL	SAL2.5	SAL5	SAL10
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 premix[7]	1.00	1.00	1.00	1.00
Monocalcium phosphate[8]	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.

Table 2. Proximate composition of the experimental diets

As fed basis (% feed)	CTRL	SAL2.5	SAL5	SAL10
Crude protein	50.0	50.0	50.0	50.0
Crude fat	16.0	16.0	16.0	16.0
Fiber	1.1	2.3	3.5	5.9
Starch	**12.3**	**10.7**	**9.2**	**6.1**
Ash	9.0	9.8	10.5	12.0
Gross Energy, MJ/kg feed	21.0	20.8	20.7	20.5
Arginine	3.15	3.14	3.14	3.12
Histidine	1.29	1.29	1.28	1.27
Isoleucine	2.06	2.05	2.04	2.04
Leucine	3.99	3.98	3.96	3.94
Lysine	2.95	2.95	2.94	2.93
Threonine	2.04	2.03	2.02	2.01
Tryptophan	0.48	0.48	0.48	0.49
Valine	2.35	2.35	2.34	2.33
Methionine	1.10	1.10	1.10	1.10
Cysteine	0.62	0.62	0.61	0.60
Methionine + Cysteine	1.72	1.72	1.71	1.70
Phenylalanine	2.43	2.42	2.41	2.39
Tyrosine	1.85	1.85	1.85	1.85
Phenylalanine + Thyrosine	4.28	4.28	4.26	4.24
Taurine	0.21	0.21	0.21	0.21
Aspartic acids + Asparagine	3.82	3.81	3.80	3.79
Glutamic acid + Gluatmine	8.66	8.59	8.50	8.38
Alanine	2.70	2.69	2.68	2.67
Glycine	3.10	3.10	3.09	3.07
Proline	3.15	3.13	3.10	3.06
Serine	2.31	2.30	2.29	2.27
Total Phosphorus	1.27	1.28	1.28	1.28
Calcium	1.50	1.50	1.50	1.50
Calcium/phosphorus	1.20	1.20	1.20	1.20
Sodium	0.80	0.90	0.90	1.00
Magnesium	0.20	0.20	0.30	0.30
Potassium	0.80	0.80	0.80	0.80
Copper. mg/kg	13.50	13.70	13.90	14.20
Iron. mg/kg	85.20	90.80	96.40	107.70
Iodine. mg/kg	4.10	4.10	4.10	4.10
Manganese. mg/kg	20.40	20.60	20.80	21.20
Selenium. mg/kg	1.80	1.80	1.80	1.80
Zinc. mg/kg	57.70	58.30	58.90	60.10
Vit A. IU/kg	30876.00	30876.00	30876.00	30876.00
Vit E. mg/kg	101.40	101.40	101.40	101.40
Vit C. mg/kg	1000.00	1000.00	1000.00	1000.00
Biotin. mg/kg	3.40	3.40	3.40	3.40
Choline. mg/kg	1915.40	1915.40	1915.40	1915.40
Inositol. mg/kg	500.10	500.10	500.10	500.10
Betaine. mg/kg	630.20	630.20	630.20	630.20

2.3. Trial design

European seabass juveniles with mean initial weight of 7.26 ± 0.06 g were obtained from SONRÍONANSA, S.L. farm (Cantabria, Spain) and allocated in Riasearch Lda. research facilities (Murtosa, Portugal). Nine hundred and sixty fish were randomly distributed to 12 tanks of 350 L (80 individuals per tank) that composed an 18 m^3 RAS system with a water renewal of 1 tank per hour. Water parameters were measured daily using commercial probes. During the experimental period, temperature was maintained at 21.6 ± 0.2 °C, dissolved oxygen at 6.4 ± 0.6 mg L^{-1}, salinity at 18.2 ± 0.2 g L^{-1}, pH at 7.5 ± 0.2 and nitrogen compounds below 0.1 mg L^{-1}.

2.3.1. Feeding trial

To evaluate the effects of halophytes containing diets in European seabass juveniles growth and health performance a long-term (34 and 62 days) trial was performed and each diet was distributed in triplicate tanks (feeding trial). At the indicated times, fish were sorted and euthanized (overdose of anesthetic, 2-phenoxyethanol) for tissue sampling. From five individuals per tank (15 per experimental group), blood was collected from the caudal vessels and plasma was isolated for the assessment on innate immune parameters and head-kidney was sampled for expression analysis of key immune related genes through RT-qPCR. Moreover, from three individuals per tank (9 per treatment) blood was used for haematological profile evaluation. Additionally, at 62 days sampling point, peritoneal exudates from three fish per tank (9 per treatment) were collected for assessment of cell proliferation.

2.3.2. Inflammatory trial

With the aim of assessing the modulatory roles of specific phytochemicals in the inflammatory processes, the inflammatory response was further studied at the end of the 62 days feeding period (time-course trial). Twelve fish per tank were re-allocated in a similar recirculation system at Interdisciplinary Centre of Marine and Environmental Research facilities (CIIMAR, Matosinhos, Portugal) and the individuals were submitted to an inflammatory challenge by intra-peritoneal injection with heat-inactivated *Phdp* strain PP3 (100 µl, 3.4 x 10^5 CFU ml^{-1}). After that, 3 individuals from each tank (9 per treatment) were sampled for blood, head-kidney and peritoneal exudates at 4, 24, 48 and 72 hours after the challenge period in order to assess cell recruitment, plasma immune mechanisms and immune-related genes.

2.4 Haematological profile and peritoneal exudates

The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts, as well as haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) assessments. Subsequently, the mean corpuscular volume (MCV), mean

corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also calculated.

Peritoneal cells were only collected from fish sampled at 62 days of feeding and at 4-, 24-, 48- and 72-hours post-inflammation (time-course). Briefly, following fish euthanasia and blood collection, 2 ml of cold Hank's balanced salt solution (HBSS) supplemented with 30 units heparin ml^{-1} was injected into the peritoneal cavity. Then, the peritoneal area was slightly massaged in order to disperse peritoneal cells in the injected HBSS. The i.p. injected HBSS containing suspended cells was collected and total peritoneal leucocytes counts were performed with a haemocytometer.

2.5 Plasma humoral parameters

Lysozyme activity was measured using a turbidimetric assay as described by Costas et al 2011. A solution of *Micrococcus lysodeikticus* (0.5mg ml^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared. In triplicates, 15 µl of plasma was added to a microplate and 250 µl of the above suspension was pipetted to give a final volume of 265 µl. The reaction was carried out at 25◦C and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a SynergyHT microplate reader. Serial diluted, lyophilized hen egg white lysozyme (Sigma) in sodium phosphate buffer (0.05 M, pH 6.2), was used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth, 1997 . In triplicates, 15 µl of plasma was diluted with 135 µl of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of 20mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µl of 5mM H2O2 were added. After 2min, the color-change reaction was stopped by adding 50 µl of 2M sulphuric acid and the optical density was read at 450 nm in a Synergy HT microplate reader. Wells without plasma were used as blanks. The peroxidase activity (units ml^{-1} plasma) was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 optical density (OD).

The anti-protease activity was determined as described by Machado et. al, 2015. Ten ml of plasma was incubated with the same volume of a trypsin solution (5 mg ml^{-1} in NaHCO3, 5mg ml^{-1}, pH 8.3) for 10 min at 22 °C in polystyrene microtubes. To the incubation mixture, 100 ml of phosphate buffer (NaH2PO4, 13.9 mg ml^{-1}, pH 7.0) and 125 ml of azocasein (20 mg ml^{-1} in NaHCO3, 5 mg ml^{-1}, pH 8.3) were added and incubated for 1 h at 22 °C. Finally, 250 ml of trichloroacetic acid was added to each microtube and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 × g for 5 min at room temperature. Afterwards, 100 ml of the supernatant was transferred to a 96 well-plate that previously contained 100 ml of NaOH (40 mg ml^{-1}) per well. The OD was read at 450 nm in a SynergyHT microplate reader. Phosphate buffer in place of plasma and trypsin served as blank whereas the reference sample was phosphate buffer in place of plasma. The percentage inhibition of trypsin activity compared to the reference sample was calculated. All analyses were conducted in duplicates

The bactericidal activity assay was performed using Phdp strain PP3. Bacteria were cultured in TSB-1 (Difco Laboratories) and exponentially growing bacteria were resuspended in sterile HBSS and adjusted to 1×10^6 cfu ml^{-1}. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of cfu following incubation at 22°C confirmed bacterial concentration of the inoculum. Plasma bactericidal activity was then determined following the method described by Machado et al., 2015 . Briefly, 20 µl of plasma was added to duplicate wells of a U-shaped 96-well plate. HBSS was added to some wells instead of plasma and served as positive control. To each well, 20 µl of Phdp (1×10^6 cfu ml^{-1}) was added and the plate was incubated for 2.5 h at 25°C. Twenty-five µl of 3-(4, 5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg ml^{-1}; Sigma) was then added to each well and incubated for 10 min at 25 °C to allow the formation of formazan. Plates were then centrifuged at 2,000 × g for 10min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan resulting from the reduction of MTT in direct proportion to the number of viable bacteria present, was measured at 560 nm. Viable bacteria was expressed as percentage, calculated from the difference between the dissolved formazan in samples and the one formed in the positive controls (100 %). The bactericidal activity was calculated as the percentage of non-viable bacteria.

2.6 Data analysis

All results are expressed as mean ± standard deviation (mean ± SD). Data was analysed for normality and homogeneity of variance and, when necessary, transformed before being treated statistically. Data from the feeding trial was analysed by one-way NOVA, with diet as factor and followed by Tukey post-hoc test to identify differences in the dietary treatments in each sampling point. Data from the time-course trial was analysed by Two-way ANOVA, with diet and time as factors and was followed by Tukey post-hoc test to identify differences among treatments. To evaluate the activation of the inflammatory mechanisms, the sampling point 62 days was used as time 0h during the time-course data analysis, as they represent unstimulated animals prior to inflammation.

3. Results

3.1. Feeding trial

Blood was collected from 9 fish from each group (3 per replicate) after 34 and 62 days of feeding the experimental diets. The WBC, RBC, HT, HG, MHC, MCV and MCHC were found unaltered with feeding time. Similarly, no statistically significant differences were found between diets in the haematological parameters evaluated (Table 3). Plasma humoral parameters were analysed for lysozyme, peroxidase, anti-protease activity and bactericidal activity, for the dietary treatments after 34 and 62 days, which did not show statistical differences among the treatments in study (Figure 2).

3.2. Inflammatory response

Most changes were observed among the sampling times after inflammation. A general decrease of several haematological parameters, as total RBC, HT, HG, MCV, MCH and MCHC (Table 4), accompanied by the decrease of plasma peroxidase activity (Figure 3-B) were observed. On the other hand, an increase of both peripheral (WBC) and peritoneal leucocytes (peritoneal WBC) concentrations at 4 and 24 h (Table 4) and plasma bactericidal activity (Figure 3-D) were observed in response to inflammation.

Regarding the effect of dietary incorporation of *Salicornia ramosissima* on the inflammatory response, fish fed ST5 showed a decrease of HT and MCV values compared to CTRL and CTRL plus ST10, respectively. Oppositely, fish fed ST10 showed higher plasma lysozyme concentration compared to ST2.5, while at 4 h after stimulus ST10 presented a higher concentration of peritoneal leucocytes compared to fish fed CTRL.

Table 3: Peripheral white blood cells (WBC), red blood cells (RBC), haematocrit (HT), haemoglobin (HG), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) in European seabass fed dietary treatments during 34 and 62 days.

Parameters	Dietary treatments							
	34 Days				62 Days			
	CTRL	ST2.5	ST5	ST10	CTRL	ST2.5	ST5	ST10
WBC, × 10^4 μl^{-1}	9.27 ±1.89	8.94 ±2.03	8.62 ±1.44	2.50 ±1.39	7.29 ±1.26	7.60 ±1.07	7.42 ±1.31	7.53 ±0.81
RBC, × 10^6 μl^{-1}	2.77 ±0.42	2.60 ±0.32	2.62 ±0.58	0.35 ±0.37	2.91 ±0.44	3.35 ±0.87	3.47 ±1.07	3.02 ±0.66
HT, %	35.38 ±3.87	34.78 ±1.69	32.67 ±2.91	34.56 ±2.50	35.44 ±3.30	35.33 ±4.40	32.71 ±8.78	37.00 ±3.68
HG, g dl^{-1}	1.54 ±0.30	1.46 ±0.24	1.50 ±0.29	1.50 ±0.35	1.46 ±0.74	1.51 ±0.59	1.49 ±0.49	1.47 ±0.35
MCH, pg $cell^{-1}$	5.78 ±1.83	5.66 ±0.95	5.91 ±1.29	6.95 ±2.06	5.33 ±3.12	4.96 ±2.77	4.94 ±2.57	5.11 ±1.56
MCV, μ m^3	129.24 ±15.52	135.88 ±19.93	131.44 ±35.03	129.58 ±18.17	124.03 ±16.21	113.10 ±32.83	91.14 ±19.47	128.03 ±28.34
MCHC, g 100 ml^{-1}	4.54 ±1.13	4.21 ±0.77	4.57 ±0.59	5.33 ±1.15	4.14 ±2.10	4.40 ±1.87	5.14 ±3.53	4.03 ±1.11

One-way ANOVA		
	34 Days	62 Days
	p-value	*p-value*
WBC	ns	ns
RBC	ns	ns
HT	ns	ns
HG	ns	ns
MCH	ns	ns
MCV	ns	ns
MCHC	ns	ns

Values are presented as mean ± SD (n=9). One-way ANOVA ($p \leq 0.05$).

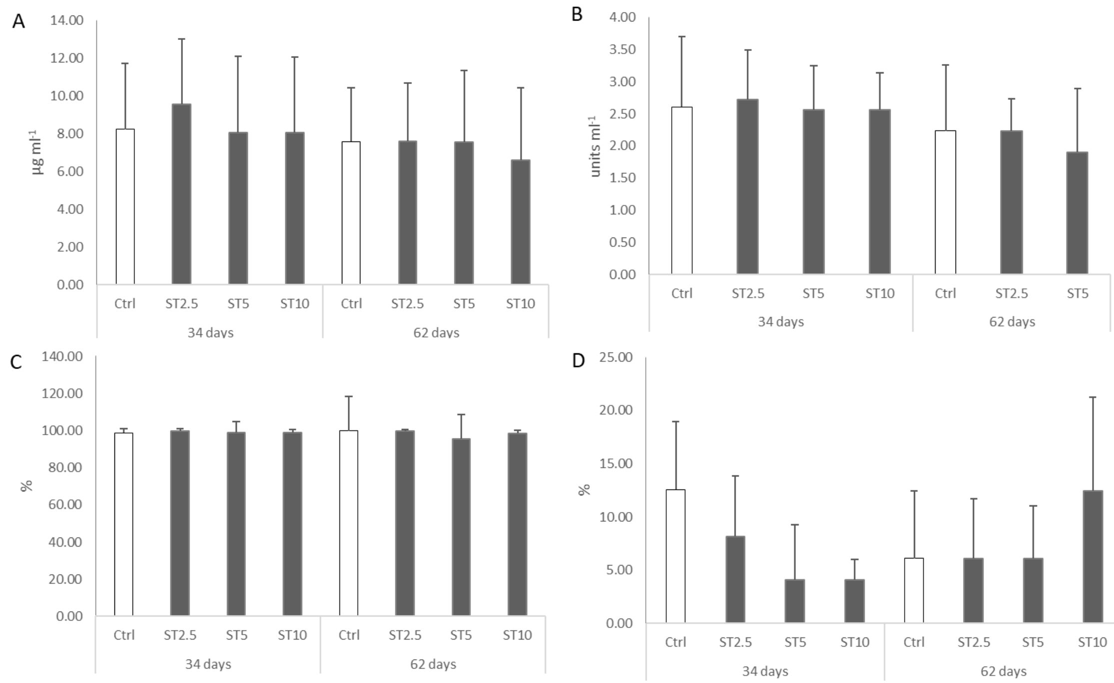

Figure 2: Plasma lysozyme (A), peroxidase (B), anti-proteases activity (C) and bactericidal activity (D) In European seabass fed dietary treatments during 34 and 62 days. Values are presented as mean ± SD (n=9). One-way ANOVA (p ≤ 0.05).

Table 4: Peripheral white blood cells (WBC), red blood cells (RBC), haematocrit (HT), haemoglobin (HG), mean corpuscular haemoglobin (MHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and peritoneal WBC in European seabass fed dietary treatments for 62 days and sampled at 0h, 4, 24, 48 and 72 hours after inflammatory stimulus.

Parameters	Dietary treatments							
	T0h (60 days)				4 hours			
	CTRL	SAL2.5	SAL5	SAL10	CTRL	SAL2.5	SAL5	SAL10
WBC, × 10^4 μl^{-1}	7.29±1.26	7.60±1.07	7.42±1.31	7.53±0.81	5.77±1.16	5.36±1.64	5.36±1.09	6.38±1.55
RBC, × 10^6 μl^{-1}	2.91±0.44	3.35±0.87	3.47±1.07	3.02±0.66	2.65±0.16	2.88±0.46	2.88±0.34	2.66±0.67
HT, %	35.44±3.30	35.33±4.40	32.71±8.78	37.00±3.68	31.22±2.78	24.25±3.40	24.25±3.70	27.56±4.37
HG, g dl^{-1}	1.46±0.74	1.51±0.59	1.49±0.49	1.47±0.35	1.36±0.59	1.35±0.31	1.35±0.66	1.14±0.38
MCH, pg cell^{-1}	5.33±3.12	4.96±2.77	4.94±2.57	5.11±1.56	5.19±2.26	4.67±1.13	4.67±2.18	4.99±3.22
MCV, μ m^3	124.03±16.21	113.10±32.83	91.14±19.47	128.03±28.34	118.57±14.67	85.94±25.23	85.94±9.72	107.61±19.61
MCHC, g 100 ml^{-1}	4.14±2.10	4.40±1.87	5.14±3.53	4.03±1.11	4.35±1.82	5.23±1.11	5.23±2.62	4.43±2.23
Peritoneal WBC, × 10^4 μl^{-1}	1.73±0.77 C	0.88±0.36 C	1.02±0.28 C	0.76±0.35 B	6.17±1.50 bAB	7.47±2.07 abA	7.51±3.20 abA	9.77±2.96 aA

Parameters	Dietary treatments							
	24 hours				48 hours			
	CTRL	SAL2.5	SAL5	SAL10	CTRL	SAL2.5	SAL5	SAL10
WBC, × 10^4 μl^{-1}	8.94±2.23	7.14±2.79	8.39±2.17	7.22±2.20	7.29±2.30	6.50±2.11	6.51±1.43	6.44±1.21
RBC, × 10^6 μl^{-1}	2.90±0.44	2.90±0.46	2.95±0.46	2.64±0.58	3.04±0.37	3.03±0.46	2.78±0.54	2.76±0.41
HT, %	31.00±3.00	28.56±2.65	29.33±5.33	29.56±3.34	32.88±3.14	32.29±1.98	31.29±2.85	31.63±2.29
HG, g dl^{-1}	1.15±0.33	1.29±0.48	1.24±0.36	1.07±0.19	1.01±0.21	1.06±0.18	0.93±0.32	1.26±0.34

MCH, pg cell^{-1}	4.02±1.08	4.55±2.29	4.32±1.41	4.23±0.98	3.33±0.70	3.57±0.68	3.48±1.18	4.54±1.00
MCV, μ m^3	108.99±15.93	101.95±27.70	100.93±18.64	117.23±28.79	109.41±8.83	102.06±12.56	114.94±14.37	115.27±18.00
MCHC, g 100 ml^{-1}	3.74±1.24	4.57±1.56	4.30±1.26	3.65±0.66	3.13±0.69	3.38±0.49	3.01±1.21	4.00±1.15
Peritoneal WBC, × 10^4 μl^{-1}	7.02±2.02 A	7.47±1.84 A	7.34±2.24 A	8.48±2.62 A	4.76±1.44 ABC	4.77±1.38 AB	5.27±1.96 AB	3.60±1.72 B

Parameters	Dietary treatments			
	72 hours			
	CTRL	SAL2.5	SAL5	SAL10
WBC, × 10^4 μl^{-1}	6.76 ± 2.00	8.39 ± 2.21	6.31 ± 1.75	8.03 ± 2.69
RBC, × 10^6 μl^{-1}	2.47 ± 0.39	2.72 ± 0.35	2.61 ± 0.31	2.67 ± 0.30
HT, %	33.67 ± 4.85	30.00 ± 3.84	29.57 ± 5.55	30.71 ± 3.19
HG, g dl^{-1}	0.84 ± 0.15	0.91 ± 0.19	1.03 ± 0.23	0.95 ± 0.13
MCH, pg cell^{-1}	3.45 ± 0.84	3.34 ± 0.58	3.95 ± 0.85	3.60 ± 0.57
MCV, μ m^3	135.09 ± 12.74	113.85 ± 13.74	110.42 ± 10.11	118.55 ± 13.70
MCHC, g 100 ml^{-1}	2.53 ± 0.51	2.97 ± 0.66	3.65 ± 0.85	3.21 ± 0.60
Peritoneal WBC, × 10^4 μl^{-1}	3.11 ± 1.66 BC	2.72 ± 0.69 B	3.94 ± 1.10B	2.31 ± 1.11 B

Two-way ANOVA

	Time	Diet	Time × Diet	Time					Dietary treatments			
				T0h (62 days)	4 hours	24 hours	48 hours	72 hours	CTRL	ST2.5	ST5	ST10
WBC	<0.001	ns	ns	AB	CD	A	BC	AC	-	-	-	-
RBC	< 0.001	ns	ns	A	B	B	AB	B	-	-	-	-
HT	< 0.001	0.005	ns	A	C	C	B	B	a	ab	b	ab
HG	<0.001	ns	ns	A	AB	BC	BC	C	-	-	-	-
MCH	0.002	ns	ns	A	AB	ABC	BC	C	-	-	-	-
MCV	0.046	<0.001	ns	AB	B	AB	AB	A	a	ab	b	a
MCHC	<0.001	ns	ns	ABC	A	ABCD	CD	D	-	-	-	-
Peritoneal WBC	<0.001	ns	0.008	B	A	A	C	D	-	-	-	-

Values are presented as mean ± SD (n=9). Two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among times while lower case letters stand for differences among diets.

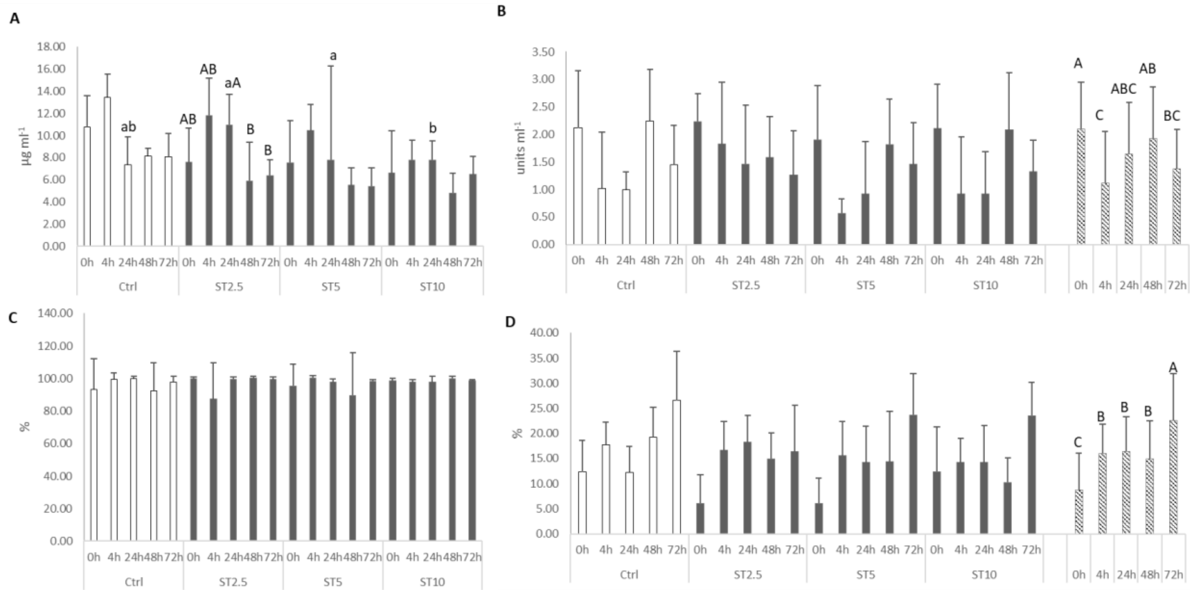

Figure 3: Plasma (A) lysozyme, (B) peroxidase, (C) anti-proteases activity and (D) bactericidal activity in European seabass fed dietary treatments at 63 days (0h), 4, 24, 48 and 72 hours after inflammatory stimulus. Values are presented as mean ± SD (n=9). Two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among times while lower case letters stand for differences among diets.

4. Discussion

The reasoning behind Salicornia dietary inclusion on fish diets is supported by their ability to substitute FM use as an alternative protein source but also by the fact that they can present the benefit of offering fish specific phytochemicals with immune-related benefits. Hence, the effect of the dietary inclusion of *Salicornia ramosissima* non-food fraction was evaluated on health condition of European seabass after a feeding period of 34 and 62 days. Thereafter, the immune response to an inflammatory insult was evaluated after 62 days. Therefore, the present work was designed to assess the effect of *Salicornia ramosissima* non-food fraction dietary inclusion, on health condition and inflammatory response of European seabass. Fish immune condition during the feeding period and the concomitant inflammatory response to inactivated *Photobacterium damselae* subsp. *piscicida* (*Phdp*) was evaluated.

In a first approach, and aiming to evaluate the dietary inclusion of Salicornia on seabass immune status, no clear differences were observed on fish haematological profile and humoral parameters in plasma. It is important to mention that the capacity of halophytes dietary incorporation to modulate fish immune response may be dependent on the presence of an obvious immune stimuli, which was absent during the feeding period. Therefore, we believe that Salicornia may succeed as an alternative ingredient for European seabass, despite the few observed alterations, at least from an immune system standpoint. The good immune condition of fish fed Salicornia- incorporated diets is further supported by the similar zootechnical performance of fish fed the different dietary treatments (data not presented) and denotes an opportunity for Salicornia dietary inclusion for European seabass juveniles, up to 10 % of feed. Thus, we can conclude that dietary inclusion of Salicornia biomass in a sea bass diet, up to 10% can be done successfully as it does not appear to compromise the immune status of the fish. Nonetheless, we see some signs of immune awareness but no signs of immune response. As can be seen in the bactericidal activity where we have an increased activity in the ST10 diet after 62 days of feeding (Figure 2 D).

Furthermore, it is of common knowledge that the dietary integration of vegetable protein sources is responsible for the increase of dietary carbohydrates, that are not well utilized by carnivorous species. In fact, in the present study the inclusion of Salicornia at the expenses of wheat meal resulted in a significant decrease of dietary starch from 12.3 % in the CTRL to 6.1 % of feed in the ST10. The presented values are found well below the defined maximum limit considered for the specie (Bojevik et al., 2014), with regards to fish growth and feed efficiency. Nonetheless, Machado *et al., 2019* has previously failed to see a significant impact in fish immune status of high carbohydrate levels (17.5% of DW) in a plant-based diet. This seems also be the case in present study.

As previously mentioned, Salicornia dietary inclusion could also present the added value of offering fish specific phytochemicals with immune-stimulating properties as hydroxycinnamic acids, phenolic acids, phenols, fatty acids, flavonoids and others (Bodas et al., 2012). A clear response to the inflammatory insult was then observed. It was perceived a general depression of several haematological indicators and plasma peroxidase activity. This was accompanied, in all dietary treatments, by a rapid increase of the leucocytes

concentration found in the peritoneal inflamed cavity at 4 and 24 h, followed by a decrease. More importantly, the dietary inclusion of *Salicornia ramosissima* during a 62 days period led to a significant modulation of the inflammatory-related cells. A clear improvement of the total leucocytes number found in the peritoneal cavity was observed, 4 h after injection, in fish fed ST10 compared to CTRL. A differential count of the peripheral and local leucocytes would be required to better explain these results as they migrate to the site of inflammation. As soon as inflammation is localized, neutrophils migrate immediately and then, monocytes and macrophages cells appear (Quade & Roth, 1997), according to the observations of Reite & Evensen, who state that this type of cell migration can be considered biphasic in teleost fish (Reite & Evensen, 2006). The main task of these cells is the recognition and elimination of invading pathogens (Scombes & Fletcher, 1992; Quade & Roth, 1997). According to the study of Machado et al., 2021, what led to the increase in these phagocytic cells was the increased methionine content, as dietary methionine was used as a strategy to enhance innate immunity (Machado et al., 2021). However, an isolation of bioactive compounds from salicornia would be needed to see if it has the amino acid in question present, and if the content of it is significant for there to be this increase. Since all these cells are found in the body cavity of fish, with macrophages also found in the gills and neutrophils being stored in smaller numbers in the haematopoietic organs (Scombes & Wang, 2012; Machado et al., 2021). With this neutrophils are then rapidly recruited as needed, degrading invading agents with their antimicrobial capabilities (Do Vale et al., 2002).

Takin into account the assumption that Salicornia dietary incorporation could represent an immune-related advantage for European seabass, the present study failed to see such outcome in a non-stimulation situation. However, the authors see of value such results since, the inclusion up to a 10 % of feed, of the non-food fraction of *Salicornina ramosissima* collected from Praia da Areia Branca (Bunheiro-Murtosa, Portugal) did not compromise fish immune status after a feeding period of 62 days and believe that such response may be dependent on the presence of an obvious immune stimuli. In fact, when fish were challenged with an inactivated bacteria and a look was given to the inflammatory mechanisms a clear improvement of the leucocyte response was noticed. Considering all, the authors denote the opportunity for a practical Salicornia dietary inclusion for European seabass juveniles, up to 10 % of feed, and its ability to improve European seabass inflammatory response. Moreover, since the authors believe that the observed immunomodulation could be the result of the presence of a singular or a group of bioactive compounds with immune stimulating properties, studies should be performed on the matter.

5. Future approaches

In the future, differential leucocyte counts in blood and peritoneum may be performed to better assess the inflammation response. However, mortality assays should be performed, as this was not possible in the present study. For a better conclusion on these results, it would be necessary to differentiate the compounds behind the observed effects and further studies should be conducted which are in fact already being prepared (both in vivo and in vitro) with the extracted bioactive compounds. As the effects of these compounds on some living beings are already known, they can be used as a basis for comparison.

6. Additional lab work performed

6.1 Differential cell counting

Immediately after blood collection, blood smears were performed from homogenized blood and air dried. After fixation with formol-ethanol (10% of 37% formaldehyde in absolute ethanol) detection of peroxidase was carried out in order to facilitate identification of neutrophils. Blood smears were then stained with Wright's stain (Haemacolor; Merck). Slides were examined (1000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. Absolute value ($\times 10^4$ ml^{-1}) of each cell type was calculated according to the total blood WBC count.

Cytospins preparations were made with a THARMAC Cellspin apparatus and stained as indicated above for blood smears. Lymphocytes, macrophages and neutrophils in the peritoneal exudates were differentially counted, and the percentage of each cell type was established after counting a minimum of 200 cells per slide. Concentration ($\times 10^4$ ml^{-1}) of each leucocyte type was also calculated.

6.2 Digestibility trial

While this study was being performed, a digestibility trial was also carried out. Work description is here described despite results were not included in this thesis.

The trial was conducted in a RAS equipped with 12 fiberglass tanks of 60 L water capacity designed according to the Guelph system; each tank supplied at the outlet with a fecal sedimentation column. Sixteen fish with an average of 60.2 ± 0.8 g were assigned to each tank and diets were randomly assigned to triplicate groups of these fish. During the trial, the fish were hand-fed twice daily, 7 days a week, until apparent visual satiation. The fish were adapted to the rearing conditions and diets for 5 days. Subsequently, feces were collected daily for the time required to collect sufficient feces to perform the required analyses, pooled for each tank, and frozen at -20ºC until analysis. Rearing conditions were similar to those described for the growth test.

6.3 Chemical Analyses

Bromatological analysis of experimental diets, carcasses and faeces was performed following the procedures of the Association of Official Analytical Chemists (AOAC, 2000). Briefly, dry matter was determined by drying samples at 105ºC in an oven to constant weight; ash, by incineration at 450ºC for 16 h in a muffle furnace; crude protein content (N x 6. 25), by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit; lipid content, by petroleum ether extraction (Soxtec HT System) and gross energy, by direct combustion in an adiabatic pump calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Yttirum was determined by atomic absorption spectrometry with flame atomization analysis after microwave digestion with nitric oxide (Reis et al., 2008).
The crude protein content of a sample is obtained by determining the nitrogen in the sample by the Kjeldahl method and multiplying the value obtained by the factor 6.25. The application of this factor assumes that a protein contains on average 16% nitrogen (100:16=6.25), although the nitrogen content of proteins can vary between 12 and 19%. The Kjeldahl method consists of three main stages:

1) Digestion of the sample with concentrated acid in the presence of catalysts, leading to the production of ammonium sulphate;

2) Vapors drag distillation in a basic medium and collection of the ammonia produced in an acid medium;

3) Quantification of the ammonia by titration with diluted acid.

Lipid content is determined by extracting the lipids from a finely ground sample with light petroleum, continuously for about six hours in a suitable extraction apparatus (Soxhlet-type extractor). Crude fat is defined as the dry, non-volatile residue remaining after evaporation in an oven of the extract obtained by treating the sample with anhydrous ether for an appropriate period of time. This extract contains all the ether-soluble substances in addition to the real fats (carotenoids, chlorophylls, fat-soluble vitamins, etc.). In certain raw materials or compound feeds with a high fat content this method is not able to quantify the total fat. In order to determine the total fat it is necessary to carry out a prior digestion of the sample with hydrochloric acid.

The gross energy is the chemical energy of a sample which is estimated from the heat produced by burning a sample completely in a pressurized oxygen atmosphere. The products of combustion are carbon dioxide, water, sulphuric acid, nitrogen and ash. In an adiabatic calorimeter (i.e., where heat is not lost from the system), during the combustion of the sample the heat produced is transmitted to a mass of water of known weight (in a container) which surrounds the container (calorimetric pump) containing the sample, and this temperature rise correlates with the energy value of the sample. In an adiabatic calorimeter there is a sleeve with water that completely surrounds the mass of water in the inner container, and which prevents the heat loss by conduction to the outside of the system.

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