U.PORTO

MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS

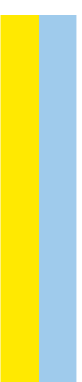
ESPECIALIDADE EM AQUACULTURA E PESCAS

EVALUATION OF ALGAL BIOMASSES IN DIETS FOR GILTHEAD SEABREAM (*SPARUS AURATA)* JUVENILES - EFFECTS ON IMMUNE CONDITION AND GENERAL PERFORMANCE

Rute Luís de Almeida Mateus

M

2020



U.PORTO

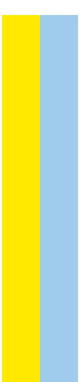
Rute de Almeida Mateus. EVALUATION OF ALGAL BIOMASSES IN DIETS FOR GILTHEAD SEABREAM (*SPARUS AURATA)* JUVENILES - EFFECTS ON IMMUNE CONDITION AND GENERAL PERFORMANCE

M.ICBAS **2020**

FEVALUATION OF ALGAL BIOMASSES IN DIETS FOR GILTHEAD SEABREAM (*SPARUS AURATA)* JUVENILES - EFFECTS ON IMMUNE CONDITION AND GENERAL PERFORMANCE

Rute Luís de Almeida Mateus

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



RUTE LUÍS DE ALMEIDA MATEUS

EVALUATION OF ALGAL BIOMASSES IN DIETS FOR GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES- EFFECTS ON IMMUNE CONDITION AND GENERAL PERFORMANCE

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos- Especialidade de Aquacultura e Pescas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientador – Doutor Benjamín Costas
Categoria – Investigador Auxiliar
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental

Co-Orientador – Professor António Afonso
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Eventualmente,

havemos de chegar onde queremos estar

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

Rute Luís de Almeida Mateus

Rute Luís de Almeida Mateus

Acknowledgements

After two years since the beginning of this dissertation, I finish my academic journey involved in a theme in which I identify much and also employed in the aquaculture industry, which I always wanted. For that, I feel extremely proud, fulfilled and thankful.

I would like to start by thanking professor Afonso for his enthusiasm and kindness and for promoting all this work by listening to me and introducing me to Benjamín. A big thanks to Benjamín for his orientation, efforts, and accessibility which make him a great leader. He gave me the opportunity to join a group full of competent, friendly and helpful people where I grew and learned a lot. A special thanks to Bruno for his guidance, teachings, help and advices that were vital for the elaboration of this thesis and for that I am extremely grateful.

I am thankful to have met my “peixinhos” friends with whom I shared so many joys, friendship and experiences and whom I hope I will continue to count. I also thank my best friends for always accompanying me in life.

To all the professors, friends and colleagues that I met and helped me in the way, thank you. Everything becomes much easier with the right people by our side.

Finally, but always the first, I want to give a big thanks to my family for not only the love but also the strength, motivation and sacrifice. I am especially and forever grateful to my parents, who always did everything they could to see me succeed in life. Everything I am and accomplish is also yours.

This work was supported by VALORMAR project (POCI-01-0247-FEDER-024517) funded by Compete 2020, Lisboa 2020, Algarve 2020, Portugal 2020 and the European Union through FEDER.

Abstract

Fish susceptibility to stress and diseases are one of the major constraints to the development of aquaculture. Therefore, there is an emerging need for new prophylactic and sustainable measures capable of improving fish health. Algae species are a great source of bioactive compounds with potential to be used as novel functional feed ingredients.

The present study aimed to screen the potential of algal biomasses from both micro (*Tetraselmis striata*, *Phaeodactylum* sp. and *Nannochloropsis* sp.) and macroalgae (*Gracilaria* sp. and *Ulva rigida*) as functional feed ingredients and its effects on the immune status and general growth performance of gilthead seabream juveniles, a species of great importance for the Mediterranean aquaculture.

For such purpose, six isonitrogenous (45% protein) and isolipidic (18% fat) diets were formulated without algae inclusion: CTRL; and with 2% algae inclusion: STRIATA (*Tetraselmis striata*), PHAEO (*Phaeodactylum* sp.), NANNO (*Nannochloropsis* sp.), GRAC (*Gracilaria* sp.) and ULVA (*Ulva rigida*). Gilthead seabream (*Sparus aurata*) juveniles (11.58 ± 0.72 g) were fed the experimental diets for a period of 4 weeks in which seven fish per tank (21 fish per dietary treatment) were sampled at the end of the 1st, 2nd and 4th week of trial. Of these, weight was recorded, and the haematological profile as well as several immune parameters were analyzed.

GRAC dietary treatment appears to positively influence the concentration of circulating lymphocytes compared to those of fish fed PHAEO dietary treatment, after 1 week of trial. In addition, our dietary treatments did not compromise the growth performance and feed utilization of gilthead seabream.

In summary, it is suggested that an inclusion of 2 % *Gracilaria* sp. in gilthead seabream diets seems to benefit peripheral lymphocytes concentration hence contributing to strengthen the adaptive arm of the immune system of this species in a short-term basis. Also, dietary treatments showed suitability to be included in fish feed formulations. This study raises new preliminary and considerable data available for future investigations.

Key words: aquaculture, immunomodulation, algae species, cellular immunity, growth performance, nutritional immunology

// Resumo

A susceptibilidade dos peixes ao stress e doenças são um dos principais constrangimentos ao desenvolvimento da aquicultura. Portanto, existe uma necessidade emergente de novos métodos profiláticos e sustentáveis capazes de melhorar a saúde dos peixes. As espécies de algas são uma grande fonte de compostos bioactivos com potencial para serem usados como novos ingredientes funcionais.

O presente estudo teve como objetivo avaliar o potencial de biomassas de micro (*Tetraselmis striata*, *Phaeodactylum* sp. e *Nannochloropsis* sp.) e macro- algas (*Gracilaria* sp. e *Ulva rigida*) como ingredientes funcionais e os seus efeitos no estado imunológico e no desenvolvimento geral de juvenis de dourada, uma espécie de grande importância para a aquicultura mediterrânica.

Para tal, foram formuladas seis dietas isoazotadas (45% proteína) e isolipidícas (18% gordura) sem inclusão de alga: CTRL; e com 2 % inclusão de alga: STRIATA (*Tetraselmis striata*), PHAEO (*Phaeodactylum* sp.), NANNO (*Nannochloropsis* sp.), GRAC (*Gracilaria* sp.) e ULVA (*Ulva rigida*). As douradas juvenis (11,58 ± 0.72 g) foram alimentadas durante um período de 4 semanas e sete peixes por tanque (21 peixes por tratamento) foram amostrados no final da 1ª, 2ª e 4ª semana. O peso dos mesmos foi apontado e vários parâmetros imunes foram analisados.

A dieta GRAC pareceu influenciar positivamente a concentração de linfócitos circulantes em comparação com os dos peixes alimentados com a dieta PHAEO, após uma semana de ensaio. Adicionalmente, as nossas dietas não comprometeram o desempenho de crescimento e a utilização do alimento pela dourada.

Em suma, é sugerido que uma inclusão de 2% *Gracilaria* sp. nas dietas de dourada parece beneficiar a concentração de linfócitos periféricos e consequentemente, contribuir para fortalecer o braço adaptativo do sistema imunológico desta espécie num curto período de tempo. Além disso, as nossas dietas mostraram aptidão para serem incluídas nas formulações de ração de peixe. Este estudo levanta novos dados preliminares e consideráveis disponíveis para futuras investigações.

Palavras-chave: aquacultura, imunomodulação, espécies de algas, imunidade celular, desempenho de crescimento, imunologia nutricional.

Contents

Acknowledgements I
Abstract III
Resumo IV
List of figures VII
List of tables VIII
Introduction 1
1. World Aquaculture 1
1.1. Current status 1
2. Gilthead seabream (*Sparus aurata*) 2
2.1. Habitat and biology 2
2.2. Production 3
3. Fish immune system 4
3.1. Innate immunity 5
3.1. Adaptive immunity 7
4. Health as an aquaculture constraint 9
5. Algae and immunonutrition 9
5.1. Microalgae 11
5.2. Macroalgae 13
Objectives 15
Graphical Abstract 16
Material and Methods 17
1. Fish rearing conditions 17
2. Experimental diets 18
3. Feeding trial and tissue sampling 20
4. Haematological assays 21
4.1. Preparation and examination of stained blood smears 21
5. Humoral immune parameters 22
5.1. Plasma immunoglobulin M 22
5.2. Bactericidal activity 22
5.3. Anti-protease activity 23
5.4. Peroxidase activity 24
6. Growth parameters and feed utilization 24
7. Data analysis 25

Results 26
1. Growth and feed parameters.. 26
2. Haematological assays 26
3. Humoral immune parameters... 29
Discussion 31
Conclusion and future perspectives 34
References 35

List of figures

Figure 1- Comparison between fisheries and aquaculture production along with population growth. Adapted from (FAO, 2018, 2020) 1

Figure 2- Gilthead seabream, Sparus aurata (Linnaeus, 1758) 3

Figure 3- Production of gilthead seabream by farming countries in 2018 (values in thousand tonnes). Online data from (Eurostat, 2020) 4

Figure 4- Scheme of general mechanisms involved in immune responses. Adapted from (Castro & Tafalla, 2015). Created with Mind the graph 9

Figure 5- General algae applications of algae suitable for aquaculture (Charoonnart et al., 2018; Rizwan et al., 2018; Shields & Lupatsch, 2012) 10

Figure 6- Main image- Trial room in CIIMAR; Corner images- Some daily procedures: Measurement of dissolved oxygen and temperature (at the top); Verification of ammonium and nitrites levels (at the bottom) 17

Figure 7- Experimental design of the feeding trial 20

Figure 8- Sampling procedures: removal of organs (left); blood collection (right) 21

Figure 9- Identification of leucocytes in gilthead seabream´s blood smear. Image captured with microscope at 1000x, without scale 21

Figure 10- U-shaped 96-well microplates used for bactericidal activity assay: precipitate before (left) and after dissolution with DMSO (right) 23

Figure 11- IgM in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 29

Figure 12- Bactericidal activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 29

Figure 13- Antiprotease activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 30

Figure 14- Peroxidase activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 30

List of tables

Table 1- Resume of the main functions of innate mechanisms in fish 6

Table 2- Resume of the main functions of adaptive mechanisms in fish 8

Table 3- Formulation and proximate analysis of the experimental diets 18

Table 4- Algae species included in the experimental diets 19

Table 5- White blood cells (WBC) and red blood cells (RBC) of juvenile gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 26

Table 6- Effect of experimental diets on growth and feed parameters of juvenile gilthead seabream in the 4th week of trial. Values are expressed as mean ± SD (n= 3) 27

Table 7- Relative proportion and absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of juvenile gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6) 27

Introduction

1. World Aquaculture

1.1. Current status

Aquaculture has experienced a blue revolution since 1960 (Ahmed & Thompson, 2019) and continues to grow worldwide. This industry had an average annual growth rate of 4.5 percent between 2011 to 2018 and achieved a considerable total fish production of 82 million tonnes in 2018 (Figure 1) which was valued at USD 250 billion (APROMAR, 2019). The world's leading fish farmers are the Asian countries responsible for over 92 percent of the world aquaculture production (APROMAR, 2019).

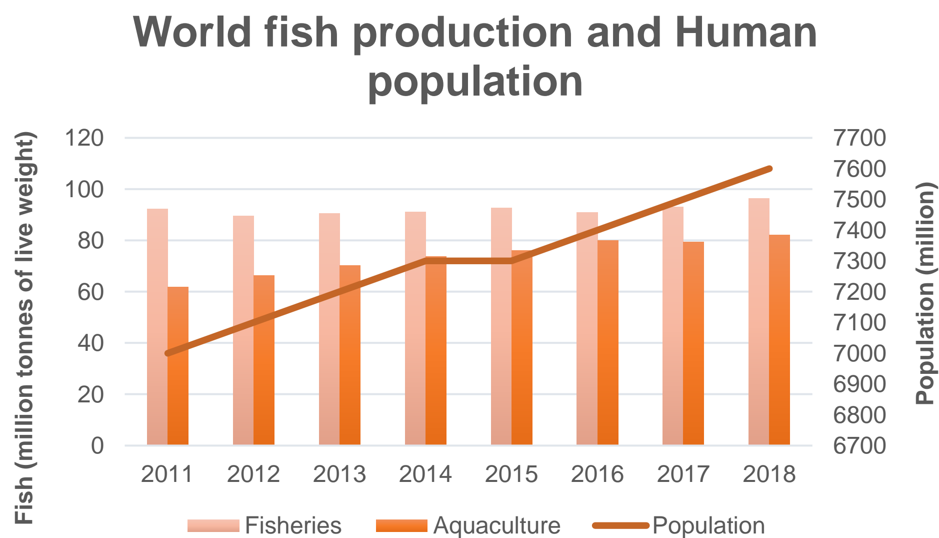


Figure 1- Comparison between fisheries and aquaculture production along with population growth. Adapted from (FAO, 2018, 2020)

In contrast to blue revolution, fisheries have been facing a stagnation since the 1980's due to a reduction in fish stocks caused mainly by overexploitation of the wild bioresources. Additionally, global population is rising (Figure 1) and is expected to reach about 9 billion people in 2050 which in accordance to their high consumption of fish products (20 kg per capita in 2017) will lead to a 60-80% higher requirements of food production then presently (FAO, 2018, 2020).

Currently, aquaculture promises to have further progression in the light of its rapid development, and also due to the present situation of fisheries and to the high nutritional demand of the growing population.

The European Union (EU) is reported as the world main importer of seafood products. Moreover, in 2017, the EU's fish production represented the eighth largest worldwide and counted with a total production of 1,37 million tonnes of live weight which was estimated to worth EUR 5,06 billion (European Commission, 2019). Hence, aquaculture is mentioned as a driver for the European economy with potential for innovation and growth and therefore promoting Blue Growth. The most prominent member states producers were Spain, The United Kingdom, France, Italy and Greece (EUMOFA, 2017; European Commission, 2017).

Despite mollusks being the main species produced in European aquaculture, the most valuable species are Atlantic salmon and Rainbow trout as well as the marine fish Gilthead seabream and European seabass. In an overview, aquaculture has a predominant activity in southern Europe, also known as Mediterranean Europe (EUMOFA, 2017).

Portugal is a southwestern country of the EU that has the largest European consumption of fish products per capita (about 57 Kg per year) (EUMOFA, 2017; European Commission, 2017). Moreover, despite having one of the widest European Exclusive Economic Zone (EEZ) of about 1,7 million Km^2 bathed by Atlantic Ocean (Lopes, 2016), the aquaculture sector remains typically traditional in extensive and semi-intensive systems in estuaries, lagoons and intertidal zones (Ramalho & Dinis, 2010). In 2018, Portuguese fish farming reached a production of around 14 tonnes which generated a corresponding value of EUR 96,8 million. Of these, the main produced species were mussels and clams as well as rainbow trout, gilthead seabream, seabass and turbot (Instituto Nacional de Estatística, 2020).

In brief, aquaculture is a worldwide activity with good prospects for the near future since it brings great economic and social value and is supported by world governmental and political instruments. For such purpose, much of the development in fish farming is achieved through scientific research as a way for evolution in knowledge and innovation.

2. Gilthead seabream (*Sparus aurata*)

2.1. Habitat and biology

The gilthead seabream (S*parus aurata*) is a marine teleost that belongs to the Sparidae family. This fish species has a characteristic oval and compressed body and its coloration is silver-gray with a black spot at the beginning of the lateral line. Also, it has a typical golden band between the eyes that is bordered by two dark areas and the fork and tips of the caudal fin are limited with black (Figure 2) (Basurco et al., 2011).

Figure 2- Gilthead seabream, *Sparus aurata* (Linnaeus, 1758)

This is a benthopelagic and euryhaline species found typically in coastal marine habitats (Basurco et al., 2011) usually in subtropical areas such as the Mediterranean Sea and in the Atlantic Ocean (Sola et al., 2007). It is mainly carnivorous but may also be accessorily herbivorous (Basurco et al., 2011). Under normal conditions of social and environmental factors, the spawning period occurs from December to April in waters temperature between 13 and 17 degrees (Basurco et al., 2011; Colloca & Cerasi, 2019).

In relation to the nutritional value, 100 gram of gilthead seabream fillet is a good source of energy (energetic value of 167 Kcal/ 698 Kj), with a significant omega-3 content of eicosapentaenoic acid (EPA) (425,1 mg) and docosahexaenoic acid (DHA) (1207 mg). Also, it has a good vitamin (e.g. vitamin A, B12, B6 D and niacin) and mineral content (potassium, phosphorus, sodium, magnesium and calcium (Docapesca, 2017).

2.2. Production

Gilthead seabream is traditionally produced alongside with European seabass (*Dicentrarchus labrax*) because of the close relationship between species and similarities in production (Basurco et al., 2011). Farming of these species was triggered in the 1980's due to the achievement of better production techniques such as hatchery methods, broodstock management, larval culture, adequate feeding and hygiene procedures (Basurco et al., 2011).

These farmed fish are economic valuable species mainly for the Mediterranean aquaculture since they account together around 20% of the total value of EU aquaculture production. In 2018 gilthead seabream generated a production of about 168,9 thousand

tonnes of live weight valued at around EUR 304 million (Figure 3) (EUMOFA, 2019; Eurostat, 2020).

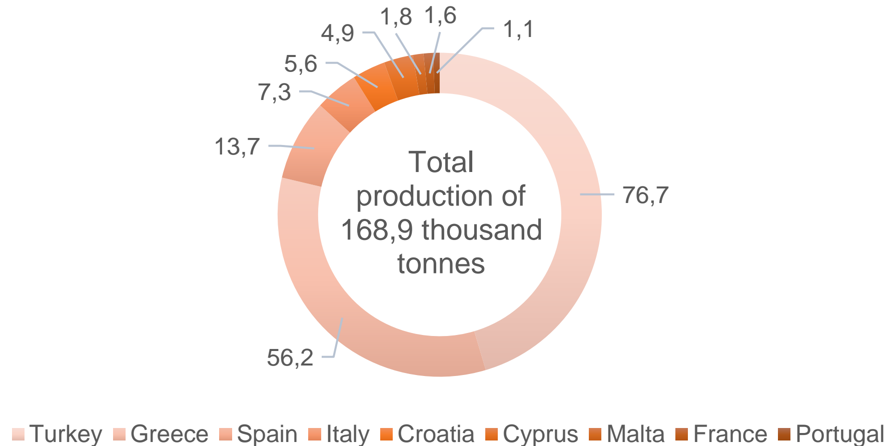


Figure 3- Production of gilthead seabream by farming countries in 2018 (values in thousand tonnes). Online data from (Eurostat, 2020)

Gilthead seabream can be cultured in various structures as is the case of inshore extensive and semi-intensive systems using earth ponds, in intensive land-based systems (e.g. raceways and tanks) as well as in offshore sea cages. In detail, it takes 18 to 24 months for this fish to reach a weight of 400 g, and the commercial size varies between 250 g and more than 1.5 Kg (Basurco et al., 2011; Colloca & Cerasi, 2019).

In regard to Portuguese aquaculture, gilthead seabream is the third most produced fish and in 2018 reached a production of EUR 6,8 million (APROMAR, 2019).

As a result of the considerable importance of gilthead seabream to marine aquaculture, there has been an intensification in the investigation of several welfare and biological aspects of this species. Namely its physiology, nutrition, immunity, growth, performance, reproduction and genetics (Basurco et al., 2011).

3. Fish immune system

Fish live in aquatic environments that are also the habitat of a wide range of opportunistic and pathogenic microorganisms. Therefore, the mechanisms of their immunity

have evolved in order to increase the immune response and adapt to that environment (Uribe et al., 2011).

Their immune system is physiologically comparable to that of higher vertebrates. Therefore, they have physical barriers, cellular and humoral immune mechanisms as well as lymphoid organs whose main functions are related to immunological defense (Tort et al., 2003). For instance, head-kidney, spleen, thymus and the mucosal-associated lymphoid tissue (MALT) are key players in orchestrating the fish immune response. The head-kidney of fish is equivalent to the bone marrow of vertebrates and is responsible for the haematopoiesis of the majority of immune cells (Uribe et al., 2011). The spleen plays an important role in immunological memory since its main functions are the macrophages phagocytosis of antigens (antigen presentation), blood filtration, erythrocyte destruction and antibody production, whereas thymus is responsible for the development of T lymphocytes (Castro & Tafalla, 2015; Uribe et al., 2011). The MALT is present in skin, gills and gastrointestinal tract and these mucosal barriers are considered the first line of defense against pathogens providing physical and chemical protection (Castro & Tafalla, 2015; Uribe et al., 2011).

There are two fundamental branches of the immune system: the innate immunity which is characterized by a rapid non-specific response, and the adaptive or acquired immunity that can lead to a slower but highly specific response. Although in general, innate response precedes the adaptive response, both are related and work in cooperation to reestablish homeostasis after injury or infection (Lieschke & Trede, 2009; Whyte, 2007).

The specialization of mechanisms involved in fish immunity and the intensity of the immune response depends on factors like fish species, health condition, genetics, stage of development, weight, sex and other external factors (Tort et al., 2003).

3.1. Innate immunity

Fish are poikilothermic organisms that have an immune system that relies mostly in the innate immune response (Tort et al., 2003). This is mainly because of some limitations in their adaptive response such as being influenced by external factors like temperature, having a low repertoire of antibodies as well as slow proliferation, maturation and memory of lymphocytes (Appenheimer & Evans, 2018; Ellis, 2001; Magnadóttir, 2006; Whyte, 2007).

In short, the innate immune mechanisms comprise physical barriers, cellular components and humoral compounds (Table 1).

Table 1- Resume of the main functions of innate mechanisms in fish

	Parameter	Main function	Reference
Physical	Mucosal barriers (mucus, scales, epithelium)	- Act as a 1st layer that blocks or limit the pathogenic invasion - Provided with antimicrobial humoral mechanisms (e.g. lectins, lysozymes, complement proteins, bacterial peptides and immunoglobulins)	(Castro & Tafalla, 2015)
Cellular	Natural killer cells (NKC)	- Acts on innate response by killing virous infectious or cancerous cells	(Castro & Tafalla, 2015; Whyte, 2007)
	Phagocytic cells	- Key cells of the innate immunity inducers of adaptive responses - Granulocytes: o Neutrophils: among the 1st cells to arrive at inflammation sites, kills pathogen by phagocytosis and by releasing their cytoplasmic granules; signals to the activation and maturation of other immune cells o Eosinophils and basophils: produce proinflammatory mediators - Monocytes and macrophages: monocytes are recruited to the inflammation site and differentiate into macrophages that produces oxygen and nitrogen radicals, increases phagocytosis and proinflammatory cytokines; macrophages present antigen to T lymphocytes	(Castro & Tafalla, 2015; Secombes & Fletcher, 1992; Thompson, 2017; Uribe et al., 2011)
Humoral	Lysozymes	- Lytic enzymes that hydrolysate peptidoglycan of bacteria cell wall - Anti-inflammatory and antiviral properties - Opsonin, activates the complement system and do phagocytosis	(Magnadóttir, 2006; Saurabh & Sahoo, 2008)
	Protease inhibitors	- Molecules that act against pathogen proteases interrupting their colonization	(Uribe et al., 2011)

Natural antibodies	- Mostly IgM - Essential to innate immunity, provides rapid protection against pathogens or cancerous cells due to direct lytic activity - Enhancement of adaptive immunity by activation of complement system	(Castro & Tafalla, 2015; Whyte, 2007)
Antimicrobial peptides (AMPs)	- Small peptides able to directly destroy pathogen or tumor cells	(Castro & Tafalla, 2015)
Complement	- Central mechanism of immune response: defense against infection (through opsonization, chemotaxis, activation of leukocytes and lysis of bacteria/ cells); interface between innate and adaptive immune response (principally by classical pathway); haemolytic activity (alternative pathway) - Activated by three pathways: o Classical: by antibody binding to cell surface of pathogen o Alternative: by direct connection to pathogens or tumor cells o Mannose- binding lectin: by linking lectin opsonins in pathogen cells surface	(Uribe et al., 2011; Walport, 2001a, 2001b)

3.1. Adaptive immunity

The adaptive immunity has the unique characteristic of create immune memory, being responsible for a highly specific antigen recognition that leads to a stronger and more effective immune response (Thompson, 2017). This is, after a 1st exposure to an antigen, B and T lymphocytes can differentiate simultaneously in immune effector cells, immune memory cells and also intermediate other immune mechanisms (Table 2). Therefore, after an repeated exposure to the same antigen, the secondary immune response is faster and more effective due to the major repertoire of immune specific cells created in the 1st exposure (Janeway et al., 2001; Ratajczak et al., 2018).

Table 2- Resume of the main functions of adaptive mechanisms in fish

	Parameter	Main function	Reference
Cellular	B lymphocytes	- Key cells of the adaptive immunity that produce immunoglobulin/ antibodies (mainly IgM) that neutralize antigens	(Castro & Tafalla, 2015; Whyte, 2007)
	T lymphocytes	- Key cells of the adaptive immunity that coordinate the immune response: o T cytotoxic cells destroy infected or cancerous cells o T helper cells mediate B lymphocytes by production of cytokines or by antigen presentation; activates T cytotoxic cells	

In this way, the immune response starts usually when a pathogen crosses the first line of innate physical defense of fish and is not eliminated by some immune humoral parameters that are present in these mucosal tissues (e.g. lysozymes, complement, antimicrobial peptides and immunoglobulins). Therefore, by penetrating the epithelium, it is recognized as pathogen associated molecular patterns (PAMPs) by pattern recognition receptors/proteins (PRR/PRP) present in immune cells (e.g. macrophages) that induce humoral and cellular components through release of cytokines which are proinflammatory mediators. This leads in one hand, to the recruitment of immune cells to the local of infection along with the action of humoral parameters for development of the inflammatory process. On the other hand, after antigen presentation to adaptive immune cells, they simultaneously differentiate either into effector cells that will act in that primary response, or become memory cells that may act on posterior secondary responses (Figure 4) (Castro & Tafalla, 2015).

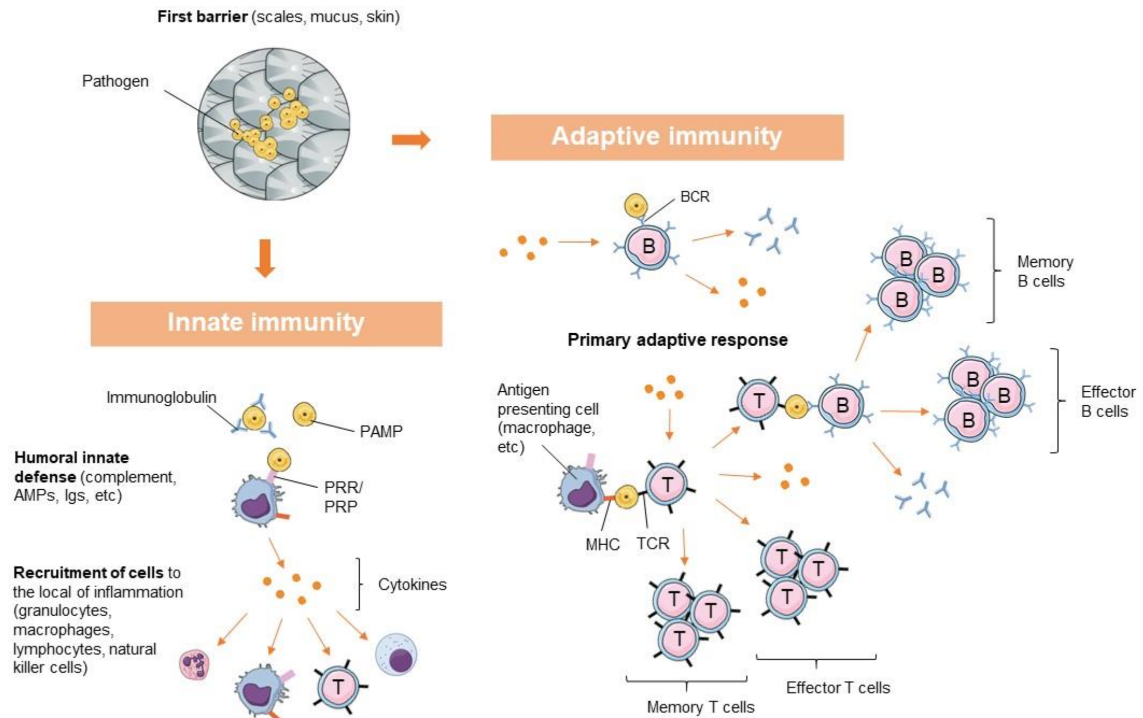


Figure 4- Scheme of general mechanisms involved in immune responses. Adapted from (Castro & Tafalla, 2015). Created with Mind the graph

4. Health as an aquaculture constraint

Fish's health is one of the major concerns in aquaculture not only regarding security and safety of the product that is available for human consumption but also because diseases lead to significant economic losses (Borrego et al., 2017). The need for an overall increase in fish production has led to an intensification of the farming methods which can inevitably expose fish to some physical, social and environmental stressful situations that may weaken their normal condition (Panagiota & Malandrakis, 2018; Tort, 2011).

Therefore, much of the investigation in aquaculture involves the search for sustainable prophylactic methods capable of improving fish welfare and health, and so the overall performance of fish (Oliva-Teles, 2012).

5. Algae and immunonutrition

Immunonutrition is a prominent scientific area that aligns nutrition and immunology to support fish health and growth in aquaculture. This type of approach can be applied for a certain period of time, specially before the occurrence of probable outbreaks, as is the

case of expected increases of temperature or long transportations or handlings. Thus, along with the nutritional ingredients that are essential in fish feeds, functional feed additives such as probiotics, prebiotics and immunostimulants may be included (Calder, 2003; Oliva-Teles, 2012). Naturally, these compounds have bioactive and phytochemical properties capable of enhancing fish immunity and consequently, improve health and diseases resistance (Dawood et al., 2017; Oliva-Teles, 2012).

In aquaculture, the exploitation of algal species as a natural source of functional feed ingredients is a recent field that seems promising. This because both micro and macroalgae have a wide repertoire of bioactive compounds as is the case of polyunsaturated fatty acids (PUFAs), proteins, carbohydrates, antioxidants, vitamins, carotenoids, pigments, sterols and minerals (Fu et al., 2017; Guedes & Malcata, 2012; Holdt & Kraan, 2011; Paiva et al., 2017).

Additionally, some advantages of using algae products are their high- quality nutrient profile, primary position in aquatic food chains, proved beneficial effects on growth and immunity of some species, natural biodiversity and availability as well as their ability to grow in many environments and to accumulate or secrete metabolites (Figure 5) (Raposo et al., 2013; Sathasivam et al., 2019; Shields & Lupatsch, 2012; Tredici et al., 2009).

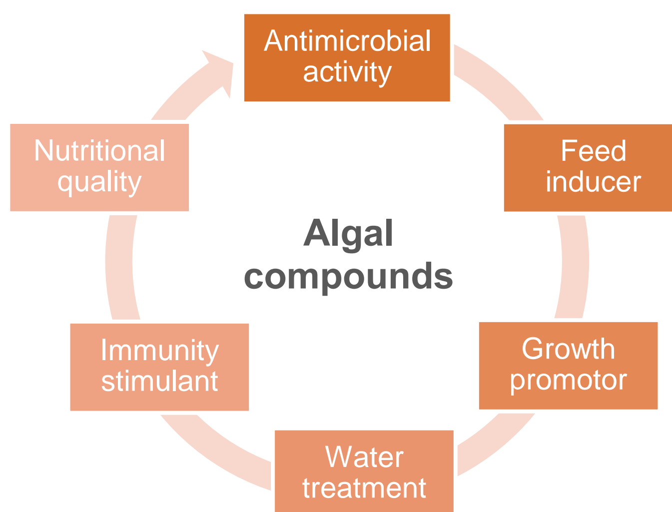


Figure 5- General algae applications of algae suitable for aquaculture (Charoonnart et al., 2018; Rizwan et al., 2018; Shields & Lupatsch, 2012)

Therefore, algal components are nowadays applied in a wide range of areas since biofuel production till wastewater treatments, cosmetic and medical outputs as well as for animal and human nutrition (Rizwan et al., 2018). However, despite all the valuable products that may come from algae species, the algal biotechnology is still evolving and the

high prices involved in its large scale production (especially in the microalgae industry) is something to be improved, since it is the main constraint to its development (Pulz & Gross, 2004).

Nonetheless, these algal constituents can be included in fish feeds in the form of micronutrient extracts or in total as a raw biomass. The latter is the most predominant technique since it allows the total conservation of the functional ingredients as it is developed by drying technologies (Lerat et al., 2018).

5.1. Microalgae

Microalgae have long been used in aquaculture, especially in hatcheries when used for "green water" techniques, for live feed, or as an enriching source for rotifers which are essential for the larval phase development of marine fish (Charoonnart et al., 2018; Shields & Lupatsch, 2012). Also, pigments from these organisms are typically used in salmonids industry for coloring fish flesh (El-Kassas & El-Sheekh, 2016). Moreover, microalgae are increasingly been used specially for the production of biofuels, resulting in a waste by-product useful to be applied in other fields as is the case of nutraceuticals (German-Báez et al., 2017).

5.1.1. *Tetraselmis sp.*

The *Tetraselmis* genus comprises a range of green flagellates that inhabit marine and freshwater environments. Typically, these species are planktonic photoautotrophs and thus represent the primary producers in aquatic ecosystems (Norris et al., 1980).

These organisms have a rich content of polyunsaturated fatty acids and so, are widely used in aquaculture for feeding mollusks and enrichment of larvae feeds (Fabregas et al., 2001). Moreover, *Tetraselmis* species are a great source of functional elements as is the case of vitamin E, carotenoids, chlorophyll and phenolic compounds, being proposed as a food supplement in animal and human nutrition (Carballo-Cárdenas et al., 2003; Pérez-López et al., 2014; Sansone et al., 2017).

Regarding the literature, there are studies demonstrating that the sulphated polysaccharides as well as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α- linolenic acids isolated from *Tetraselmis* sp. had anti-inflammatory and preventive activities for cardiovascular diseases (Sathasivam et al., 2019; Talero et al., 2015). Also, pigments like the β-carotene and lutein present in these organisms showed to have antioxidant and anti-cancer properties as well as beneficial effects in prevention of liver

fibrosis, cardiovascular and skin diseases (Bonilla-Ahumada et al., 2018; Custódio et al., 2012; Sansone et al., 2017; Sathasivam et al., 2019).

In relation to fish immunonutrition, extracts derived from *Tetraselmis* sp. demonstrated to inhibit the pathogenic action of *Vibrio anguillarum* and other wide group of bacteria in Atlantic salmon (Austin et al., 1992) which was consistent with other in vitro studies (Austin & Day, 1990; Kokou et al., 2012; Makridis et al., 2006). Also, *T. suecica* and *T. chuii* microalgae were suggested as probiotics and immunostimulants when given alone or in combination with other microalgae in European sea bass (*Dicentrarchus labrax*) and gilthead seabream diets (Cerezuela et al., 2012b; Irianto & Austin, 2002; Messina et al., 2019).

5.1.2. *Phaeodactylum sp.*

This microalga inhabits both marine and fresh water ecosystems and has worldwide dispersion. Moreover, it is a rich source of protein, carbohydrates, EPA, and fucoxanthin and has been widely used not only in aquaculture feeds but also as a model organism for genetic engineering (Borowitzka, 2018; German-Báez et al., 2017; Prestegard et al., 2015).

Previous studies demonstrated the bioactivity and antioxidant content of this species (German-Báez et al., 2017) as well as the anti-cancer activity since it induced the apoptosis of leukemia cells (Prestegard et al., 2009; Samarakoon et al., 2014). Also, constituents of this microalga demonstrated to have antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Vibrio* sp. (Desbois et al., 2008; Kwak et al., 2014; Wang et al., 2018).

Regarding fish immunonutrition, *P. tricornutum* exhibited potential as an immunostimulant for gilthead seabream when given single or in combination with other micro-algae and -organism (Cerezuela et al., 2012a; Cerezuela et al., 2012b). Besides, it was noticed that this microalga enhanced the external pigmentation and aspect of gilthead seabream which is also favorable for the consumer acceptance (Ribeiro et al., 2017).

5.1.3. *Nannochloropsis sp.*

Nannochloropsis is a genus of photosynthetic algae whose majority of species inhabit marine environments but may also be freshwater. Typically, these organisms are unicellular and have a great content of EPA, vitamin E and some pigments like chlorophyll a, zeaxanthin, canthaxanthin and astaxanthin. Therefore, they are nowadays used in hatcheries for larvae feeding (Durmaz, 2007; Lubián et al., 2000; Maruyama et al., 1986).

There are studies that indicate the antioxidant activity of *Nannochloropsis* species (Ebrahimzadeh et al., 2018; Millao & Uquiche, 2016). Also, omega-3 fatty acids along with docosapentaenoic acid (DPA) and sterols derived from *N. oculata* revealed positive anti-inflammatory effects in vivo and vitro studies (Nauroth et al., 2010; Sanjeewa et al., 2016). In regard to the antibacterial activity, these organisms showed to inhibit the growth of a wide range of bacterial species (Duraiarasan et al., 2014), including *Vibrio* sp. (Kokou et al., 2012).

In brief, there are studies that reveal the potential of *Nannochloropsis* species for immunonutrition, however, the literature is scarce with regard to their role in fish immunity. Despite that, there is a study that proposes *Nannochloropsis gaditana* as an immunostimulant for gilthead seabream (Cerezuela et al., 2012b).

5.2. Macroalgae

Macroalgae are multicellular photoautotrophic and benthic organisms that usually inhabit marine environments. They have great genetic diversity amongst species and are organized in three main phyla, namely Chlorophyta, Phaeophyta and Rhodophyta (Rindi et al., 2012).

These organisms have long been applied in multiple fields as is the case of Asian gastronomy, Chinese medicine, pharmaceutics, fertilizers, phycocolloides industries (e.g. agar, carrageenan and alginates), biofuel production, cosmetics and as functional feeds (Paiva et al., 2017).

Regarding aquaculture, macroalgae are recently used for integrated multi-trophic aquaculture (IMTA) as primary producers and as feed supplement in fish diets (Carvalho & Pereira, 2014; Wan et al., 2018).

5.2.1*. Gracilaria sp.*

Typically, these macroalgae constitute a great source of arachidonic acid and lipids (e.g. prostaglandins, steroids and cholesterol) which are a key player in phagocytosis and antigen presentation (Montero et al., 2010). Also, these algae species have phycocolloids that are used as a source for agar extraction and other secondary metabolites (Almeida et al., 2011).

In the literature, there are studies that suggest compounds from *Gracilaria* species as an stimulant of phagocytosis activity in mouse (Yoshizawa et al., 1996). Also, this macroalgae showed not only antiviral effects against herpes virus (Mazumder et al., 2002) but also antimicrobial activity for a range of microorganisms including *Pseudomonas*

aeruginosa and *Escherichia coli* (Kızılkaya et al., 2006; Maftuch et al., 2016). Additionally, these algal species exhibited antioxidant activity (Francavilla et al., 2013; Murugan & Iyer, 2012; Souza et al., 2011) and anticancer action in mice (Patra & Muthuraman, 2013).

In relation to fish immunity, there are studies that suggest the defensive role of the antioxidant properties of *Gracilaria* sp. for oxidative stress in gilthead seabream (Magnoni et al., 2017) and their modulatory role in the resistance to bacterial infections in European seabass (Peixoto et al., 2019). Also, it was observed that the inclusion up to 5% of this species in rainbow trout´s feeds stimulated some components of the innate immunity but levels above that percentage impaired growth (Araújo et al., 2016).

5.2.2. *Ulva sp.*

Typically, these organisms belong to the phylum Chlorophyta and are very rich in ulvan which is a sulfated polysaccharide component of their cell wall (Morelli et al., 2017).

Regarding their bioactivity, there are studies that highlighted compounds of *Ulva* species (e.g. phenol content, ulvans, vitamin E and carotenes) as a great source of antioxidant properties (Ganesan et al., 2011; Qi et al., 2005; Yildiz et al., 2011). These macroalgae showed anti-inflammatory (Jin et al., 2006; Okai & Higashi-Okai, 1997), anti-cancer (Ahmed & Ahmed, 2014), anticoagulant (Adrien et al., 2019), and immunostimulant activity (Jiao et al., 2010; Leiro et al., 2007). Also, *Ulva rigida* demonstrated antimicrobial effects against fish pathogenic bacteria *Vibrio anguillarum* and *Pseudomonas anguilliseptica* (Bansemir et al., 2006).

There are several studies that highlight *Ulva* species as a valuable dietary ingredient in fish diets (Emre et al., 2013; Silva et al., 2015; Valente et al., 2006), however literature regarding fish immunity is scarce. Nonetheless, it was observed that polysaccharides from *Ulva rigida* positively enhanced the innate immune response of turbot and grey mullet, as well as the growth and antioxidant activity of the latter species (Akbary & Aminikhoei, 2017; Castro et al., 2006).

Objectives

Several studies highlight algal constituents as a source of bioactive compounds with proved beneficial effects for the immune system of various species. Nevertheless, there is scarce information regarding the specific action on the immune mechanisms specially when algae biomasses are used as functional feed ingredients for fish nutrition.

The aim of the present study is to screen the potential of algal biomasses from both micro (i. e. *Tetraselmis striata*, *Phaeodactylum* sp. and *Nannochloropsis* sp.) and macroalgae (i. e. *Gracilaria* sp. and *Ulva rigida*) as functional feed ingredients and its effects on the immune status of gilthead seabream juveniles, a species of great importance for the Mediterranean aquaculture.

The development of functional feeds based on algal biomasses could serve as new prophylactic measures that provide an alternative to the use of chemotherapeutics and antibiotics, thus contributing to the sustainable development of aquaculture and to better farming results in gilthead seabream production.

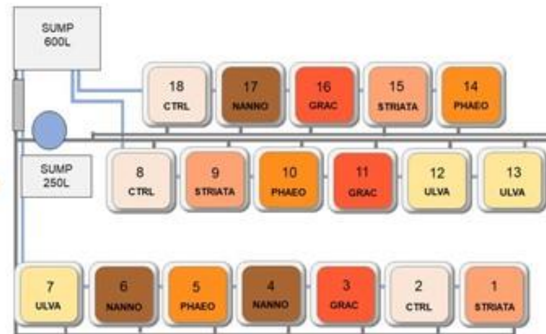
Graphical Abstract

Evaluation of algal biomasses in diets for gilthead seabream (*Sparus aurata*) juveniles- effects on immune condition and general performance

Dietary treatments and design

2 %
CTRL
STRIATA
PHAEO
NANNO
GRAC
ULVA

Sparus aurata



Samplings

1 week *2 week* *4 week*

Haematological profile- WBC, RBC, neutrophils, thrombocytes, lymphocytes and monocytes
Humoral immune parameters- IgM, bactericidal activity, anti-protease and peroxidase
Growth and feed indicators- weight gain, RGR, FCR, survival and feed intake

Conclusions

- GRAC diet could stimulate the proliferation of lymphocytes after 1 week of feeding
- The experimental diets could be included in fish feeds without compromising growth and feed utilization
- This dissertation provides preliminary and considerable data able for future studies

Material and Methods

1. Fish rearing conditions

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Gilthead seabream juveniles were obtained from a certified hatchery (Sonrionansa, Santander, Spain). The trial was performed at BOGA (Bioterium of Aquatic Organisms), the animal experimentation facilities of CIIMAR (Interdisciplinary Centre of Marine and Environmental Research) in Matosinhos, Portugal. Prior to the trial, fish were maintained under standard culture conditions for a quarantine period of two weeks.

Thereafter, fish (initial body weight 11.58 ± 0.72 g) were randomly distributed into 18 tanks (250 L) in a recirculation seawater system (Temperature: 22.4 ± 1 °C; Salinity: 35.2 ± 0.7 ppt; Photoperiod: 12h dark, 12h light; Dissolved oxygen: 7.0 ± 0.2 mg/L, Water flow: 4 L/min) (Figure 6). Daily, mortalities and total given feed per tank as well as water quality were verified and parameters were recorded (temperature, salinity, oxygen saturation, levels of ammonium and nitrites). Whenever necessary, water renovations and system cleanings were performed.

Figure 6- Main image- Trial room in CIIMAR; Corner images- Some daily procedures: Measurement of dissolved oxygen and temperature (at the top); Verification of ammonium and nitrites levels (at the bottom)

2. Experimental diets

Experimental feeds were formulated and produced by Sparos Lda. (Olhão, Portugal) and were in accordance to the nutritional requirements of the species (Table 3).

As a result, six isonitrogenous (45% protein) and isolipidic (18% fat) diets were formulated. Diet CTRL served as control whereas five other diets consisted of control diet with a 2% inclusion of different algae: Diet STRIATA (*Tetraselmis striata*); Diet PHAEO (*Phaeodactylum* sp.); Diet NANNO (*Nannochloropsis* sp.); Diet GRAC (*Gracilaria* sp.); Diet ULVA (*Ulva rigida*) (Table 4).

The algal species that were incorporated in the experimental diets were supplied by Portuguese algae producers Allmicroalgae (STRIATA), NECTON (PHAEO and NANNO) and ALGAPLUS (GRAC and ULVA).

Table 3- Formulation and proximate analysis of the experimental diets

Ingredients (%DM)	CTRL	STRIATA	PHAEO	NANNO	GRAC	ULVA
Fishmeal 60[1]	10.00	10.00	10.00	10.00	10.00	10.00
Feather meal[2]	5.00	5.00	5.00	5.00	5.00	5.00
Porcine blood meal[3]	2.00	2.00	2.00	2.00	2.00	2.00
Poultry meal 65[4]	25.00	25.00	25.00	25.00	25.00	25.00
Porcine gelatin[5]	2.00	2.00	2.00	2.00	2.00	2.00
Wheat gluten[6]	2.50	2.50	2.50	2.50	2.50	2.50
Corn gluten meal[7]	5.00	5.00	5.00	5.00	5.00	5.00
Soybean meal 48[8]	12.00	12.00	12.00	12.00	12.00	12.00
Sunflower meal[9]	5.00	5.00	5.00	5.00	5.00	5.00
Wheat meal[10]	12.90	10.90	10.90	10.90	10.90	10.90
Potato starch (gelatinised)[11]	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil[12]	6.90	6.90	6.90	6.90	6.90	6.90
Rapeseed oil[13]	2.30	2.30	2.30	2.30	2.30	2.30
Palm oil[14]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin and mineral premix[15]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin E (50%)[16]	0.03	0.03	0.03	0.03	0.03	0.03
CELATOM FP1SL (diatomite)[17]	0.50	0.50	0.50	0.50	0.50	0.50
Antioxidant[18]	0.20	0.20	0.20	0.20	0.20	0.20
Sodium propionate[19]	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine[20]	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine[21]	0.17	0.17	0.17	0.17	0.17	0.17
L-Taurine[22]	0.10	0.10	0.10	0.10	0.10	0.10
Proximate analyses (% dry weight)						
Ash	8.31	9.07	8.85	8.79	9.04	9.07

Fat	8.88	9.65	9.33	9.18	9.35	9.53
Protein	45.88	46.00	47.97	47.69	47.68	46.79
Phosphorus	1.63	1.73	1.66	1.56	1.62	1.57
Energy (Kg/g)	21.20	21.12	21.68	22.03	22.50	21.52

[1]Fishmeal by- products (CP: 59.2%, CF: 9.9%), CONRESA, Spain

[2]Feather meal (CP: 82.9%, CF: 11.2%), SONAC BV, The Netherlands

[3]Porcine blood meal (CP: 89.1%, CF: 0.4%), SONAC BV, The Netherlands

[4]Poultry meal (CP: 62.4%, CF: 14.5%), SAVINOR UTS, Portugal

[5]Porcine gelatin, Lapi Gelatins, Italy

[6]Wheat gluten VITAL (CP: 80.4%, CF: 5.8%), Roquette, France

[7]Corn gluten meal (CP: 61.2%, CF: 5.2%), COPAM, Portugal

[8]Soybean meal dehulled solvent extracted (CP: 47.4%, 2.6%), CARGILL, Spain

[9]Sunflower meal solvent extracted (CP: 29.1%, CF:1.8%), Ribeiro e Sousa Lda, Portugal

[10]Wheat meal (CP: 11.7%, CF: 1.6%), Molisur, Spain

[11]Potato starch, Pregeflo P100 90% starch (CP: 0.4%, CF: 0.1%), Roquette, France

[12]Fish oil (CF: 98.1%, EPA:16%, DHA: 12%), Sopropêche, France

[13]Rapeseed oil (CF:98.2%), JC Coimbra, Portugal

[14]Palm oil, Henry Lamotte Oils GmbH, Germany

[15]Vitamin and mineral premix (Vitamins (IU or mg/Kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat gluten, DSM Nutritional Products, Switzerland

[16] Vitamin E ROVIMIX E50, DSM Nutritional Products, Switzerland

[17]CELATOM FP1SL (diatomite), Angelo Coimbra S.A., Portugal

[18]Antioxidant VERDILOX, Kemin Europe NV, Belgium

[19]Sodium propionate, Disproquímica, Portugal

[20]L-Lysine 99% Lysine, Anjinomoto EUROLYSINE S.A.S., France

[21]DL-Methionine Rhodimet NP99 99% Methionine, ADISSEO, France

[22]L-Taurine 98% Taurine, ORFFA, The Netherlands

Table 4- Algae species included in the experimental diets

Experimental diets	Algae biomasses
CTRL	Without algae inclusion
STRIATA	With 2% inclusion of *Tetraselmis striata*
PHAEO	With 2% inclusion of *Phaeodactylum* sp.
NANNO	With 2% inclusion of *Nannochloropsis* sp.
GRAC	With 2% inclusion of *Gracilaria* sp.
ULVA	With 2% inclusion of *Ulva rigida*

3. Feeding trial and tissue sampling

The feeding trial had a total duration of 4 weeks (from May 5 until June 3, 2018). Dietary treatments were randomly assigned to triplicate groups of 110 fish per tank and a total of 10 animals were taken for proximal composition (Figure 7).

Fish were fed by hand, three times a day with a four-hour interval between feeding (except on weekends which was given a single feeding). At the beginning of the trial fish were fed 2 % of fish body weight, with adjustments being made along the trial until fish apparent satiety.

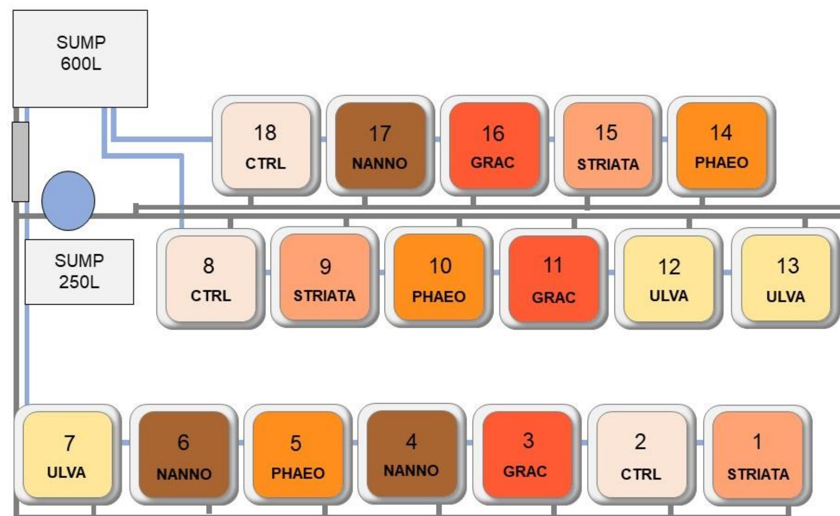


Figure 7- Experimental design of the feeding trial

Fish were sampled at the end of the 1st, 2nd and 4th weeks of the feeding trial. Seven fish per tank (21 fish per treatment) were randomly sampled and euthanized by overexposure to the anesthetic 2-phenoxyethanol (1 mg/L). Fish were weighed and three fish per tank were collected for whole body composition, while the remainder four fish per tank were used for blood and tissues (head-kidney, liver and total gut) collection (Figure 8).

Blood was collected from the caudal vein using heparinized syringes centrifuged at 10,000 × g during 10 min at 4 ºC to obtain plasma samples. Tissue samples were immediately frozen at -80 ºC until further analyses.

Figure 8- Sampling procedures: removal of organs (left); blood collection (right)

4. Haematological assays

4.1. Preparation and examination of stained blood smears

The haematological profile proceeded in accordance to Machado et al. (2015). Blood smears were prepared immediately after blood collection air dried and fixed for 1 minute with formol-ethanol (10% formaldehyde solution in absolute ethanol) and stained with Wright's stain (Haemacolor; Merck). To allow neutrophil detection through peroxidase activity, procedures were carried out as described by Afonso et al. (1998). Finally, slides were observed in the microscope under oil immersion (1000x) and whenever possible, a total of 200 leucocytes were counted and then classified as neutrophils, thrombocytes, lymphocytes and monocytes (Figure 9). The relative percentage and absolute value (x10^4/mL) of each cell type was calculated.

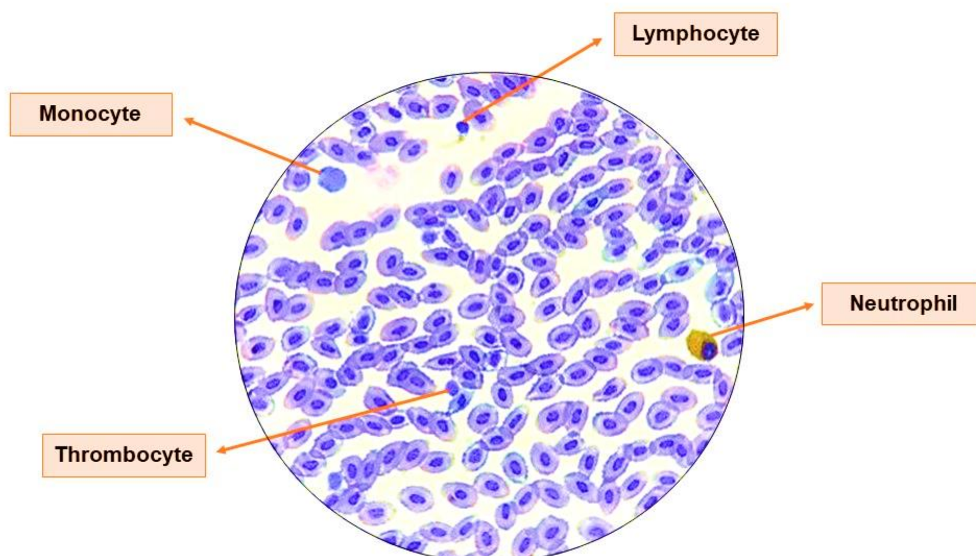


Figure 9- Identification of leucocytes in gilthead seabream´s blood smear. Image captured with microscope at 1000x, without scale

5. Humoral immune parameters

5.1. Plasma immunoglobulin M

The indirect enzyme-linked immunosorbent assay (ELISA) was used to measure total IgM levels, as described by Cerezuela et al. (2016), with some modifications. For such purpose, plasma was diluted 1:100 with sodium carbonate (50 mM, pH= 9.6) and 100 µL of diluted samples were pipetted in triplicate into a flat-bottomed 96-well plate. Samples were left to incubate overnight at 4 ºC. Thereafter, wells were blocked with 300 µL of blocking buffer (5% low fat milk in T-TBS (0.1% Tween 20)) and incubated for one hour at room temperature followed by three consecutive rinses with T- TBS (0.1 % Tween 20). Then, the primary antibody (monoclonal anti-IgM, Aquatic Diagnostics, UK) was diluted 1:200 with blocking buffer and 100 µL were added to each well. After new incubation of one hour at room temperature, plates were rinsed as previously described. Afterwards, the secondary antibody (monoclonal anti-IgG, SIGMA) was diluted 1:1000 in blocking buffer and 100 µL were added to each well. Plates were once again incubated at room temperature for one hour and washed as previously described. Then, 100 µL of "TMB substrate solution for ELISA" (1 volume of solution A plus 1 volume of solution B) was added to the wells and incubated for 5 minutes at room temperature with the samples. Finally, 100 µL of sulphuric acid (2M) were added to the wells to stop reaction. Samples optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek). Wells with sodium carbonate (50 mM, pH=9.6) instead of plasma were used as blanks.

5.2. Bactericidal activity

The bactericidal activity of plasma was determined by using *Edwardsiella tarda* strain ACC53.1. Bacteria were cultured for 48 hours at 25 ºC on tryptic soy agar (TSA; Difco Laboratories) and then it was resuspended and inoculated into tryptic soy broth (TSB; Difco Laboratories). Both mediums were supplemented with NaCl to a final concentration of 1 % (w/v) (TSA-1 and TSB-1, respectively). The bacteria in TSB-1 medium were then cultured at the 25 ºC for 24 hours, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × g for 30 minutes, resuspended in sterile HBSS and adjusted to 1 × 10^6 cfu/mL. The confirmation of bacterial concentration in the inoculum was achieved by counting the number of cfu after plating serial dilutions of the suspension into TSA-1 plates and incubating at 25 ºC.

Plasma bactericidal activity was determined following the method of Machado et al. (2018). Briefly, 20 µL of sample were mixed with 20 µL of *E. tarda* (1 × 106 cfu.mL^{-1}) in triplicate in a U-shaped 96-well plate, that was then incubated for 2.5 h at 25 ºC (20 µL of

TSB were added instead of plasma to 3 wells and served as positive control). Afterwards, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg.mL^{-1}; Sigma) were added to each well and incubated for 10 min at 25 ºC to allow the formation of formazan precipitates. Plates were then centrifuged at 2,000 × g for 10 min and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

$$\%\ viable\ bacteria = \frac{Abs. of\ sample \quad \times 100}{Abs. of\ positive\ control}$$

$$\%\ non\ viable\ bacteria = 100 - \%\ viable\ bacteria$$

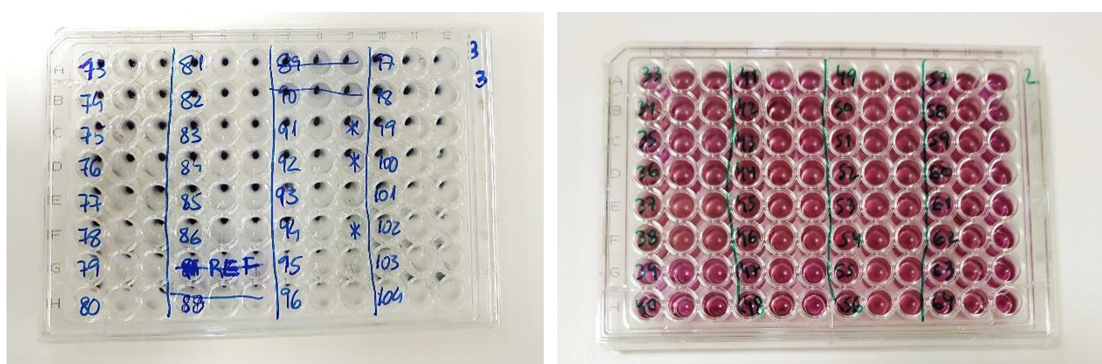


Figure 10- U-shaped 96-well microplates used for bactericidal activity assay: precipitate before (left) and after dissolution with DMSO (right)

5.3. Anti-protease activity

The anti-protease activity was determined as described by Machado et al. (2015) with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 mg.mL^{-1} in $NaHCO_3$, 5 mg.mL^{-1}, pH 8.3) for 10 min at 22 °C in 1.5 mL microtubes. After incubation, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 mg.mL^{-1}, pH 7.0) and 125 µL of azocasein solution (20 mg.mL^{-1} in $NaHCO_3$, 5 mg.mL^{-1}, pH 8.3) were added and incubated for 1 h at 22 °C. Finally, 250 µL of trichloroacetic acid were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 × g for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (40 mg.mL^{-1}). The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer in place of plasma and trypsin

served as blank, whereas the reference sample was phosphate buffer in place of plasma. Sample inhibition percentage of trypsin activity was calculated as follows:

$$\%\, non\ inhibited\ trypsin = \frac{Abs.of\ sample \quad \times 100}{Abs.of\ reference}$$

$$\%\, inhibited\ trypsin = 100 - \%\, non\ inhibited\ trypsin$$

5.4. Peroxidase activity

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). Briefly, 10 µL of plasma was diluted with 140 µL of HBSS without Ca^{2+} and Mg^{2+} in 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µL of 5 mM H_2O_2 were added to the wells. The reaction was stopped after 2 min by adding 50 µL of 2 M H_2SO_4 and the optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek). Wells without plasma were used as blanks. The peroxidase activity (units.mL^{-1} plasma) was determined defining that one unit of peroxidase produces an absorbance change of 1 OD.

6. Growth parameters and feed utilization

To understand the growth and feed utilization of fish after 4 weeks of trial, initial and final weight was measured as well as weight gain, relative growth rate, feed conversion ratio, survival and feed intake was calculated.

The total weight gain of fish per dietary treatment was obtained by the next formulation, in grams:

$$Weight\ gain = Final\ wet\ weight - Initial\ wet\ weight$$

The daily growth of fish was calculated using the relative growth rate (RGR) and expressed as percentage increase of weight per day, as following (Hardy & Barrows, 2003):

$$RGR = \left(e^{\frac{\ln(Final\ weight) - \ln(Initial\ weight)}{Days\ of\ feeding}} - 1\right) \times 100$$

The ability of feed formulations to support weight gains was perceived using the feed conversion ratio (FCR) and expressed in grams, as following (Hardy & Barrows, 2003):

$$FCR = \frac{Feed\ intake}{Weight\ gain}$$

The percentage of fish that survived to the trial was given by the following formula:

$$\%\ Survival = \frac{Final\ fish\ + Sampled\ fish}{Initial\ fish} \times 100$$

To have a better perception of the feed intake per day, it was considered the next formulation, expressed in percentage of grams (Ali et al., 2016):

$$\%\ Feed\ intake = \frac{Feed\ intake}{Average\ body\ weight\ \times Days\ of\ feeding} \times 100$$

7. Data analysis

All results were expressed as mean ± standard deviation (mean ± SD). All data were tested for normality (Shapiro- Wilk test) and homogeneity of variance (Levene's test). When data did not meet the assumptions, data were transformed before analysis. Then, data were analyzed by One- way analysis of variance (ANOVA) with diets as the fixed factor and when this test showed statistical significance, the post-hoc HSD Tukey was performed. When data did not meet the ANOVA assumptions after transformation, the non-parametric Kruskal Wallis test was used instead. All statistical analyses were performed using the computer package SPSS 26 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

Results

1. Growth and feed parameters

There were no significant differences in the final body weight, weight gain, relative growth rate (RGR), feed conversion ratio (FCR), survival and feed intake among diets in the 4th week of trial (Table 6).

2. Haematological assays

Total white (WBC) and red (RBC) blood cells concentrations are displayed in Table 5. Results showed no significant differences in WBC and RBC concentrations among seabream fed the experimental diets irrespective of the sampling point.

The relative proportion and absolute values of the different circulating leucocytes are present in Table 7. No statistical differences were observed for the relative proportion of the leucocyte cells and absolute values of circulating thrombocytes, monocytes, neutrophils and lymphocytes at the end of the 2nd and the 4th week of feeding, among dietary treatments. However, at the end of the the 1st week, fish fed GRAC dietary treatment exhibited significantly higher concentration of lymphocytes when compared to fish fed PHAEO diet. In the following weeks it was observed that lymphocyte values remained similar among different dietary treatments. /

Table 5- White blood cells (WBC) and red blood cells (RBC) of juvenile gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

	WBC (×10⁴/μl)			**RBC (×10⁶/μl)**		
	1 week	2 week	4 week	1 week	2 week	4 week
CTRL	3.50 ± 0.71	3.52 ± 0.45	4.05 ± 0.43	1.55 ± 0.15	1.57 ± 0.23	1.38 ± 0.32
STRIATA	3.48 ± 0.44	3.20 ± 0.43	3.85 ± 0.68	1.36 ± 0.26	1.41 ± 0.17	1.32 ± 0.31
PHAEO	3.25 ± 0.58	3.67 ± 0.63	3.68 ± 0.84	1.65 ± 0.24	1.42 ± 0.22	1.42 ± 0.16
NANNO	3.53 ± 0.49	3.40 ± 0.77	3.37 ± 0.64	1.47 ± 0.28	1.50 ± 0.36	1.18 ± 0.20
GRAC	3.52 ± 0.44	3.55 ± 0.59	3.73 ± 0.78	1.71 ± 0.20	1.38 ± 0.18	1.41 ± 0.28
ULVA	3.98 ± 0.70	3.82 ± 0.51	3.25 ± 0.39	1.60 ± 0.29	1.27 ± 0.24	1.15 ± 0.11

Table 6- Effect of experimental diets on growth and feed parameters of juvenile gilthead seabream in the 4th week of trial. Values are expressed as mean ± SD (n= 3)

Indicators	**Initial weight (g)**	**Final weight (g)**	**Weight gain (g)**	**RGR (%/day)**	**FCR (g)**	**Feed intake (%g/day)**	**Survival (%)**
CTRL	11.67 ± 0.72	20.20 ± 1.39	8.54 ± 1.99	1.98 ± 0.44	1.27 ±0.27	2.28 ± 0.08	100.00 ± 0.00
STRIATA	11.99 ± 0.88	18.71 ± 1.58	6.72 ± 1.57	1.60 ± 0.35	1.31 ± 0.05	2.36 ± 0.10	100.00 ± 0.00
PHAEO	11.67 ± 0.72	18.65 ± 1.11	6.98 ± 1.73	1.69 ± 0.41	1.21 ± 0.09	2.25 ± 0.07	100.00 ± 0.00
NANNO	11.25 ± 0.00	19.22 ± 1.65	7.97 ± 1.65	1.92 ± 0.32	1.27 ± 0.26	2.33 ± 0.10	99.70 ± 0.52
GRAC	11.67 ± 0.72	20.32 ± 0.88	8.66 ± 0.59	2.00 ± 0.15	1.15 ± 0.11	2.19 ± 0.07	100.00 ± 0.00
ULVA	11.25 ± 1.25	19.57 ± 1.70	8.32 ± 0.57	2.00 ± 0.13	1.14 ± 0.09	2.24 ± 0.17	100.00 ± 0.00

Table 7- Relative proportion and absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of juvenile gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

1 week		**CTRL**	**STRIATA**	**PHAEO**	**NANNO**	**GRAC**	**ULVA**
Thrombocytes	(% WBC)	50.11 ± 13.96	54.13 ± 10.18	60.21 ± 5.20	51.08 ± 9.49	45.67 ± 11.57	48.83 ± 12.41
	($\times 10^4$ µl)	1.77 ± 0.61	1.89 ± 0.47	1.98 ± 0.49	1.84 ± 0.54	1.61 ± 0.47	2.02 ± 0.82
Lymphocytes	(% WBC)	44.97 ± 12.95	42.78 ± 10.25	34.46 ± 5.79	46.08 ± 8.64	51.25 ± 11.21	44.67 ± 10.65
	($\times 10^4$ µl)	1.55 ± 0.41 ^{ab}	1.49 ± 0.44 ^{ab}	1.10 ± 0.17 ^{b}	1.60 ± 0.19 ^{ab}	1.80 ± 0.43 ^{a}	1.71 ± 0.19 ^{ab}
Monocytes	(% WBC)	1.83 ± 1.31	1.67 ± 0.90	1.90 ± 0.86	1.33 ± 1.14	2.08 ± 0.73	1.40 ± 0.66
	($\times 10^4$ µl)	0.05 ± 0.04	0.05 ± 0.02	0.06 ± 0.04	0.04 ± 0.04	0.07 ± 0.03	0.05 ± 0.02
Neutrophils	(% WBC)	3.00 ± 1.95	1.42 ± 0.89	2.06 ± 1.40	1.50 ± 0.76	1.00 ± 0.50	3.40 ± 1.36
	($\times 10^4$ µl)	0.10 ± 0.08	0.05 ± 0.03	0.07 ± 0.06	0.05 ± 0.03	0.03 ± 0.02	0.12 ± 0.04

NOTE: Different letters mean significant differences among dietary treatments (one-way ANOVA, P≤ 0.05).

2 week		CTRL	STRIATA	PHAEO	NANNO	GRAC	ULVA
Thrombocytes	(% WBC)	61.65 ± 8.44	64.69 ± 4.40	62.95 ± 14.68	60.16 ± 6.46	59.18 ± 7.52	72.75 ± 3.40
	($\times 10^4$ µl)	2.16 ± 0.41	2.06 ± 0.29	1.97 ± 0.83	2.01 ± 0.37	2.10 ± 0.46	2.40 ± 0.90
Lymphocytes	(% WBC)	32.00 ± 2.30	30.47 ± 4.13	25.54 ± 2.89	35.18 ± 5.44	36.87 ± 7.66	28.45 ± 11.99
	($\times 10^4$ µl)	1.25 ± 0.35	0.98 ± 0.20	1.33 ± 0.52	1.21 ± 0.38	1.30 ± 0.34	1.20 ± 0.40
Monocytes	(% WBC)	1.14 ± 0.59	1.39 ± 0.74	1.51 ± 0.40	2.08 ± 1.69	1.65 ± 0.62	2.08 ± 1.33
	($\times 10^4$ µl)	0.04 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.05	0.06 ± 0.02	0.08 ± 0.05
Neutrophils	(% WBC)	1.64 ± 0.98	2.05 ± 1.08	2.35 ± 1.35	2.58 ± 1.64	2.30 ± 1.08	2.70 ± 1.17
	($\times 10^4$ µl)	0.06 ± 0.03	0.06 ± 0.03	0.07 ± 0.03	0.09 ± 0.06	0.08 ± 0.05	0.08 ± 0.01

4 week		CTRL	STRIATA	PHAEO	NANNO	GRAC	ULVA
Thrombocytes	(% WBC)	68.03 ± 9.45	62.57 ± 13.06	67.22 ± 9.35	61.56 ± 8.92	52.89 ± 10.27	65.45 ± 9.07
	($\times 10^4$ µl)	2.79 ± 0.65	2.41 ± 0.65	2.46 ± 0.60	2.12 ± 0.68	2.00 ± 0.67	2.15 ± 0.53
Lymphocytes	(% WBC)	28.81 ± 8.50	33.91 ± 12.22	29.78 ± 9.01	34.11 ± 8.42	43.11 ± 11.43	29.30 ± 8.09
	($\times 10^4$ µl)	1.15 ± 0.25	1.10 ± 0.37	1.11± 0.50	1.10 ± 0.16	1.59 ± 0.39	0.93 ± 0.22
Monocytes	(% WBC)	0.80 ± 0.62	2.02 ± 1.10	2.00 ± 1.19	2.25 ± 1.14	2.00 ± 1.15	1.62 ± 0.93
	($\times 10^4$ µl)	0.04 ± 0.03	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.03	0.05 ± 0.03
Neutrophils	(% WBC)	1.24 ± 0.48	1.50 ± 0.93	1.00 ± 0.65	2.08 ± 1.17	1.40 ± 0.80	0.84 ± 0.97
	($\times 10^4$ µl)	0.05 ± 0.02	0.06 ± 0.04	0.04 ± 0.02	0.07 ± 0.04	0.07 ± 0.04	0.03 ± 0.03

3. Humoral immune parameters

Results from humoral immune parameters of all sampling points are displayed in Figure 11-14 and none showed statistical differences among experimental diets.

Considering the IgM parameter (Figure 11), despite not being statistically significant among dietary treatments, it was observed a general tendency to increase during the experimental trial. All other parameters show a varying pattern among dietary treatments and no general tendency was perceivable between sampling points.

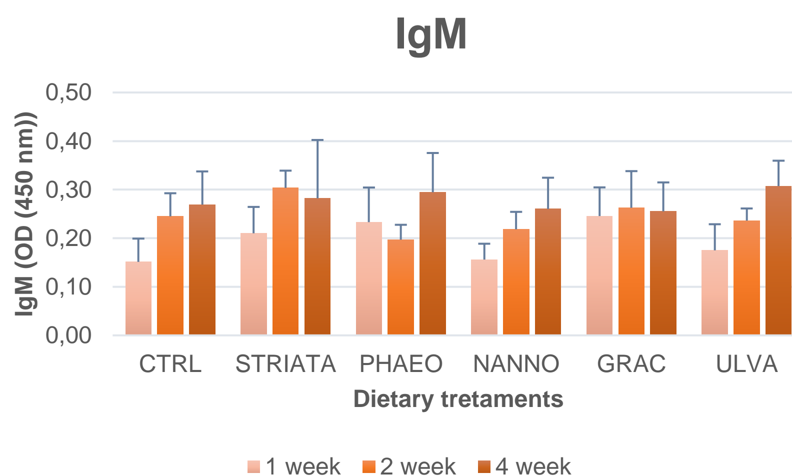


Figure 11- IgM in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

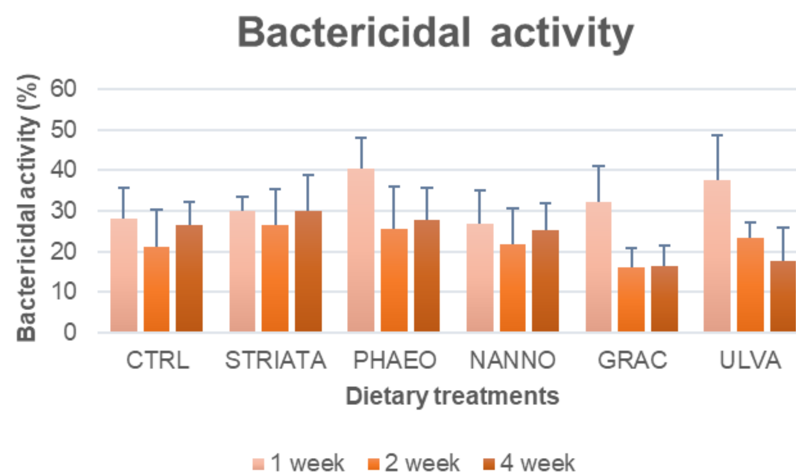


Figure 12- Bactericidal activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

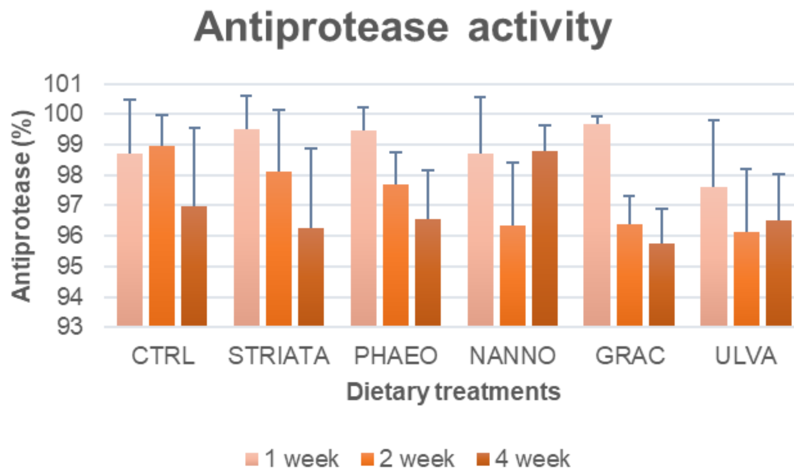


Figure 13- Antiprotease activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

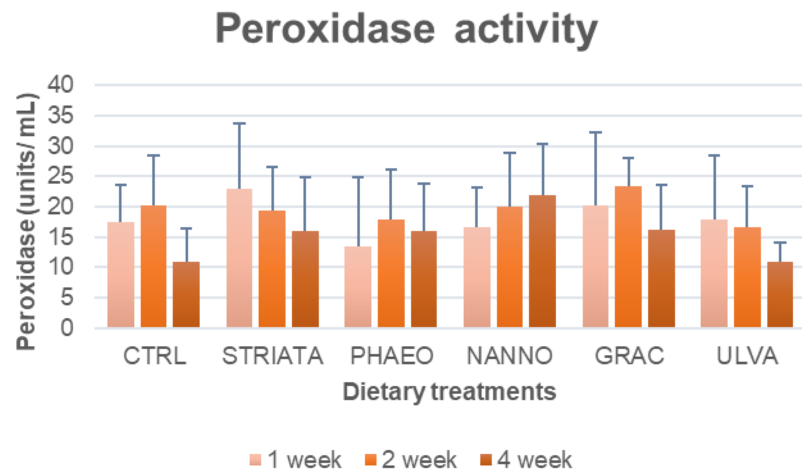


Figure 14- Peroxidase activity in plasma of gilthead seabream fed experimental diets during 1, 2 and 4 weeks. Values are expressed as mean ± SD (n= 6)

Discussion

The bioactive compounds of algae species are widely recognized in the literature and have been identified with potential immunostimulant activity. Nevertheless, to the best of our knowledge, there is scarce information regarding the specific role of algae biomasses in fish immunity, even more in gilthead seabream, a species of great commercial value. The use of biomasses is a more practical approach, since mechanical or chemical procedures would increase production costs and these algae products would reach the aquafeeds market with more constraints. Therefore, in the present work, fish were fed diets containing 2% of different algal biomasses (*Tetraselmis striata*, *Phaeodactylum* sp., *Nannochloropsis* sp., *Gracilaria* sp. and *Ulva rigida*) during 4 weeks and samples were taken in the 1st, 2nd and 4th week of trial.

In general, results showed that STRIATA, PHAEO, NANNO, GRAC and ULVA dietary treatments had no significant effects neither in the tested humoral immune parameters nor on growth performance when fed for 1, 2 and 4 weeks. The same occurred for the overall haematological profile.

However, findings from this study suggested a significant variation in the number of peripheral lymphocytes after 1 week of trial in fish fed GRAC and PHAEO diets. In fish fed GRAC diet, the number of these cells increased whereas in PHAEO diet the opposite occurred. This tendency was not verified in the number of WBC (except for PHAEO diet, although not statistically significant) which could mean that the alteration had little impact on the total number of WBC.

The suggestion that GRAC diet can stimulate the proliferation of lymphocytes in a short period of time could strengthen the general immunity of fish since these cells are responsible for the production of antibodies, coordinate the immune response of other cells and act directly against non-self or damaged cells (Castro & Tafalla, 2015; Ellis, 2001). Hence, fish diets with *Gracilaria* sp. inclusion could improve the immune competence to cope with pathogens as it was recently recognized by Peixoto et al. (2019) when fed European seabass with 5% *Gracilaria* sp. aqueous extract. Accordingly, findings from the present study were similar to those of Mendonça et al. (2019) which discovered that an inclusion up to 5% of dry *Gracilaria domingensis* increased the production of T lymphocytes for mullet juveniles after 60 days of feeding.

The absence of effects in the haematological parameters was in agreement for instance with Guerreiro et al. (2017) for an inclusion of 5% *Ulva lactuca* in gilthead seabream.

Regarding the humoral parameters, although not statistically significant, there are some observations that could be further interpreted. That is, among the experimental times, feeding fish for a short period of time (one or two weeks) with algal biomasses could be the preferable feeding duration to obtain an immunostimulatory effect. This is directly related to the significant findings observed in the number of peripheral lymphocytes, but also because of values obtained for bactericidal activity. The later data appeared to have higher levels for the first sampling point (1 week), for instance in fish fed PHAEO and ULVA dietary treatments. Although this observation was not statistically significant, it could be speculated that some humoral components of the immune system seem to be triggered in a short-term basis. Nevertheless, further studies are definitely needed to infer the veracity of these observations in fish fed algae from the genus Phaeodactylum and Ulva.

In the present study, the IgM levels showed a general tendency to increase over time in fish fed the experimental weeks. This effect seems to be related to the normal fish development since it was observed in seabream from all dietary treatments. In contrast, Cerezuela et al. (2012a) observed an increase in IgM levels when gilthead seabream were fed with diets containing *Tetraselmis chuii* and *Phaeodactylum tricornutum* for 2 weeks. Still, in another comparable study, the same authors found no significant variation in IgM levels when fed the same alga species plus *Nannochloropsis gaditana* (Cerezuela et al., 2012b). Since data available for this parameter is scarce and not consistent our findings may contribute to the expand of knowledge about the tested algae effects on IgM of gilthead seabream

In short, despite some variations in the measured immune parameters, it is important to keep in mind that in the present study fish were not stimulated by a triggering agent (e.g. antigen) or a pathogen challenge, which might explain the lack of response obtained. Therefore, the presence of a pathogen would trigger an immune response by the activation of effector cells (e. g phagocytic cells and natural antibodies) that may release a broad-spectrum of humoral antimicrobial agents as is the case of lysozyme, antiproteases, peroxidases and changes in the bactericidal activity of plasma (Ellis, 2001).

In relation to the growth performance, results showed no significant differences in the final body weight, weight gain, relative growth rate (RGR), feed conversion ratio (FCR), survival and feed intake among diets in the 4th week of trial. Similar results have been described for the same fish species, although for higher concentrations of algae inclusion (Guerreiro et al., 2017; Queiroz et al., 2014). Likewise, it was observed that supplementation of *Gracilaria bursapastoris* and *Ulva rigida* up to 10 % levels had no impact

on growth performance, nor on nutrient utilization for European seabass (Valente et al., 2006).

Although the tested algae biomasses did not improve the general performance of fish, our results could be interesting in terms of dietary supplementation. This is, in addition to the bioactive compounds of algae species, they also have a great content of lipids and proteins (Valente et al., 2006). Typically, fish feed formulations require high levels of these nutrients that come from increasingly scarce and unsustainable sources such as fish oils and meals. Thus, there have been efforts in fish nutrition to evaluate the potential of other alternatives mainly derived from plants (Caruso, 2015; Valente et al., 2006). Considering that our results showed that feeding diets with 2 % inclusion of algae biomasses for 4 weeks, did not compromise the growth performance and feed utilization of gilthead seabream, this could give rise to new studies that investigate the potential of the tested algae species as novel ingredients and alternatives for fish feeds.

Conclusion and future perspectives

Aquaculture is an activity that is increasing worldwide and with possibilities to cope with the nutritional demands of the growing population. Nonetheless, and because it is a recent industry, much of the development goes through scientific research as a way for evolution in knowledge and innovation.

In this study, it was evaluated the potential of five algae species whose bioactive properties and beneficial role for the immune system were already demonstrated in the literature. Results showed an increase in the circulating lymphocytes number after feeding gilthead seabream with GRAC dietary treatment for 1 week. This may suggest that the inclusion of *Gracilaria* sp. in fish diets could enable some degree of stimulation in these immune cells that are crucial for the general immunity of fish. Also, it was verified that the 2 % inclusion of the tested algae biomasses did not compromise fish growth performance which could indicate suitability to be included in fish feed formulations.

Further studies are still needed to elucidate in detail the potential of the tested algae biomasses both in immunological and nutritional fields. For instance, it should be interesting to analyze the effects of the chosen algae at immunohistochemistry and transcriptomic levels in gut tissue.

In conclusion, this study provides preliminary and considerable data, contributing to the improvement of knowledge, especially with regard to the use of algae in fish immunonutrition.

References

Adrien, A., Bonnet, A., Dufour, D., Baudouin, S., Maugard, T., & Bridiau, N. (2019). Anticoagulant activity of sulfated Ulvan isolated from the green macroalga *Ulva rigida*. *Marine Drugs, 17*(5). doi:10.3390/md17050291

Afonso, A., Lousada, S., Silva, J., Ellis, A. E., & Silva, M. T. (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. A light and electron microscopic cytochemical study. *Diseases of Aquatic Organisms, 34*(1), 27-37. doi:10.3354/dao034027

Ahmed, N., & Thompson, S. (2019). The blue dimensions of aquaculture: A global synthesis. *Science of the Total Environment, 652*, 851-861. doi:10.1016/j.scitotenv.2018.10.163

Ahmed, O., & Ahmed, R. (2014). Anti-proliferative and apoptotic efficacies of ulvan polysaccharides against different types of carcinoma cells in vitro and in vivo. *Journal of Cancer Science and Therapy, 6*, 202-208. doi:10.4172/1948-5956.1000272

Akbary, P., & Aminikhoei, Z. (2017). Effect of water-soluble polysaccharide extract from the green alga *Ulva rigida* on growth performance, antioxidant enzyme activity, and immune stimulation of grey mullet *Mugil cephalus*. *Journal of Applied Phycology, 30*(5), 1-9. doi:10.1007/s10811-017-1299-8

Ali, T. E. S., S Martínez-Llorens, Moñino, A. V., Cerdá, M. J., & Tomás-Vidal, A. (2016). Effects of weekly feeding frequency and previous ration restriction on the compensatory growth and body composition of Nile tilapia fingerlings. *The Egyptian Journal of Aquatic Research, 42*(3), 357- 363. doi:10.1016/j.ejar.2016.06.004

Almeida, C. L. F. d., Falcão, H. d. S., Lima, G. R. d. M., Montenegro, C. d. A., Lira, N. S., Athayde-Filho, P. F. d., . . . Batista, L. M. (2011). Bioactivities from marine algae of the genus Gracilaria. *International Journal of Molecular Sciences, 12*(7), 4550-4573. doi:10.3390/ijms12074550

Appenheimer, M. M., & Evans, S. S. (2018). Temperature and adaptive immunity. In A. A. Romanovsky (Ed.), *Handbook of Clinical Neurology* (Vol. 156, pp. 397-415): Elsevier.

APROMAR. (2019). *La Acuicultura en España*.

Araújo, M., Rema, P., Pinto, I. S., Cunha, L., Peixoto, M., Pires, M., . . . Valente, L. (2016). Dietary inclusion of IMTA-cultivated *Gracilaria vermiculophylla* in rainbow trout (*Oncorhynchus mykiss*) diets: effects on growth, intestinal morphology, tissue

pigmentation, and immunological response. *Journal of Applied Phycology, 28*, 679-689. doi:10.1007/s10811-015-0591-8

Austin, B., Baudet, E., & S, S. M. (1992). Inhibition of bacteria fish pathogens by *Tetraselmis suecica*. *Journal of Fish Diseases, 15*, 55-61. doi:10.1111/j.1365-2761.1992.tb00636.x

Austin, B., & Day, J. G. (1990). Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. *Aquaculture (Amsterdam, Netherlands), 90*(3), 389-392. doi:10.1016/0044-8486(90)90261-K

Bansemir, A., Blume, M., Schröder, S., & Lindequist, U. (2006). Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. *Aquaculture (Amsterdam, Netherlands), 252*(1), 79-84. doi:10.1016/j.aquaculture.2005.11.051

Basurco, B., Lovatelli, A., & García, B. (2011). Current status of sparidae aquaculture. In Whiley- Blackwell (Ed.), *Sparidae: Biology and aquaculture of gilthead sea bream and other species* (pp. 1- 50).

Bonilla-Ahumada, F. d. J., Khandual, S., & Lugo-Cervantes, E. d. C. (2018). Microencapsulation of algal biomass (*Tetraselmis chuii*) by spray-drying using different encapsulation materials for better preservation of beta-carotene and antioxidant compounds. *Algal Research, 36*, 229-238. doi:10.1016/j.algal.2018.10.006

Borowitzka, M. A. (2018). Biology of Microalgae. In I. A. Levine & J. Fleurence (Eds.), *Microalgae in Health and Disease Prevention* (pp. 23-72): Academic Press.

Borrego, J. J., Labella, A. M., Castro, D., Ortiz-Delgado, J. B., & Sarasquete, C. (2017). Updated of the pathologies affecting cultured gilthead seabream, *Sparus aurata*. *Annals of Aquaculture and Research, 4*(2), 1- 14.

Calder, P. C. (2003). Immunonutrition. *BMJ (Clinical research ed.), 327*(7407), 117-118. doi:10.1136/bmj.327.7407.117

Carballo-Cárdenas, E. C., Tuan, P. M., Janssen, M., & Wijffels, R. H. (2003). Vitamin E (α-tocopherol) production by the marine microalgae *Dunaliella tertiolecta* and *Tetraselmis suecica* in batch cultivation. *Biomolecular Engineering, 20*(4), 139-147. doi:10.1016/S1389-0344(03)00040-6

Caruso, G. (2015). Use of plant products as candidate fish meal substitutes: An emerging issue in aquaculture productions. *Fisheries and Aquaculture Journal*. doi:10.4172/2150-3508.1000e123

Carvalho, L. G., & Pereira, L. (2014). Review of marine algae as source of bioactive metabolites. In L. Pereira & J. M. Neto (Eds.), *Marine Algae Biodiversity, Taxonomy, Environmental Assessment and Biotechnology* (pp. 195-227): CRC Press.

Castro, R., Piazzon, M. C., Zarra, I., Leiro, J., Noya, M., & Lamas, J. (2006). Stimulation of turbot phagocytes by *Ulva rigida* C. Agardh polysaccharides. *Aquaculture (Amsterdam, Netherlands), 254*(1), 9-20. doi:doi.org/10.1016/j.aquaculture.2005.10.012

Castro, R., & Tafalla, C. (2015). Overview of fish immunity. In B. H. Beck & E. Peatman (Eds.), *Mucosal Health in Aquaculture* (pp. 3-54). San Diego: Academic Press.

Cerezuela, R., Guardiola, F. A., Cuesta, A., & Esteban, M. Á. (2016). Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: Effects on skin mucosal immunity. *Fish & Shellfish Immunology, 49*, 100-109. doi:doi.org/10.1016/j.fsi.2015.12.028

Cerezuela, R., Guardiola, F. A., González, P., Meseguer, J., & Esteban, M. Á. (2012a). Effects of dietary *Bacillus subtilis*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata* L.). *Fish & Shellfish Immunology, 33*(2), 342-349. doi:10.1016/j.fsi.2012.05.004

Cerezuela, R., Guardiola, F. A., Meseguer, J., & Esteban, M. A. (2012b). Enrichment of gilthead seabream (*Sparus aurata* L.) diet with microalgae: effects on the immune system. *Fish Physiology and Biochemistry, 38*(6), 1729-1739. doi:10.1007/s10695-012-9670-9

Charoonnart, P., Purton, S., & Saksmerprome, V. (2018). Applications of microalgal biotechnology for disease control in aquaculture. *Biology, 7*(2). doi:10.3390/biology7020024

Colloca, F., & Cerasi, S. (2019). FAO fisheries and aquaculture department. In *Cultured aquatic species information programme. Sparus aurata. Cultured aquatic species information programme*.

Custódio, L., Justo, T., Silvestre, L., Barradas, A., Duarte, C. V., Pereira, H., . . . Varela, J. (2012). Microalgae of different phyla display antioxidant, metal chelating and acetylcholinesterase inhibitory activities. *Food Chemistry, 131*(1), 134-140. doi:10.1016/j.foodchem.2011.08.047

Dawood, M. A. O., Koshio, S., & Esteban, M. Á. (2017). Beneficial roles of feed additives as immunostimulants in aquaculture: A review. *Reviews in Aquaculture, 0*, 1-25. doi:10.1111/raq.12209

Desbois, A., Spragg, A. M., & Smith, V. (2008). A fatty acid from the diatom *Phaeodactylum tricornutum* is antibacterial against diverse bacteria including multi-resistant *Staphylococcus aureus* (MRSA). *Marine Biotechnology (New York, N.Y.), 11*(1), 45-52. doi:10.1007/s10126-008-9118-5

Docapesca. (2017). Ficha de produto dourada (gilthead seabream) *Sparus aurata*. from Docapesca portos e lotas S.A.

Duraiarasan, S., Vijay, M., Razack, S., Subramaniyan, T., Ammavasai, S., Shellomith, K., & Tamilselvam, K. (2014). A green synthesis of antimicrobial compounds from marine microalgae *Nannochloropsis oculata*. *Journal of Coastal Life Medicine, 2*. doi:10.12980/JCLM.2.2014APJTB-2014-0138

Durmaz, Y. (2007). Vitamin E (α-tocopherol) production by the marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae) in nitrogen limitation. *Aquaculture (Amsterdam, Netherlands), 272*, 717-722. doi:10.1016/j.aquaculture.2007.07.213

Ebrahimzadeh, M. A., Khalili, M., & Dehpou, A. A. (2018). Antioxidant activity of ethyl acetate and methanolic extracts of two marine algae, *Nannochloropsis oculata* and *Gracilaria gracilis* - an in vitro assay. *Brazilian Journal of Pharmaceutical Sciences, 54*(1). doi:10.1590/s2175-97902018000117280

El-Kassas, H. Y., & El-Sheekh, M. M. (2016). Induction of the synthesis of bioactive compounds of the marine alga *Tetraselmis tetrathele* (West) Butcher grown under salinity stress. *The Egyptian Journal of Aquatic Research, 42*(4), 385-391. doi:10.1016/j.ejar.2016.10.006

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology, 25*(8), 827-839. doi:10.1016/S0145-305X(01)00038-6

Emre, Y., Ergün, S., Kurtoglu, A., Güroy, B., & Güroy, D. (2013). Effects of Ulva meal on growth performance of Gilthead seabream (*Sparus aurata*) at different levels of dietary lipid. *Turkish Journal of Fisheries and Aquatic Sciences, 13*, 841-846. doi:10.4194/1303-2712-v13_5_08

EUMOFA. (2017). *The EU fish market*.

EUMOFA. (2019). *The EU fish market- 2019 edition*. Luxembourg.

European Commission. (2017). *Agriculture, forestry and fishery statistics 2017 edition*. Luxembourg.

European Commission. (2019). *Agriculture, forestry and fishery statistics 2019 edition*. Luxembourg.

Eurostat. (2020, 23/07/2020). Production from aquaculture excluding hatcheries and nurseries (from 2008 onwards) [fish_aq2a]. Retrieved from https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do

Fabregas, J., Otero, A., Domínguez, A., & Patiño, M. (2001). *Growth rate of the microalga Tetraselmis suecica changes the biochemical composition of artemia species* (Vol. 3).

FAO. (2018). *The state of world fisheries and aquaculture 2018. Meeting the sustainable development goals*. Rome.

FAO. (2020). *The state of world fisheries and aquaculture 2020. Sustainability in action*. Rome.

Francavilla, M., Franchi, M., Monteleone, M., & Caroppo, C. (2013). The red seaweed *Gracilaria gracilis* as a multi products source. *Marine Drugs, 11*, 3754-3776. doi:10.3390/md11103754

Fu, W., Nelson, D. R., Yi, Z., Xu, M., Khraiwes, B., Jijakli, K., . . . Salehi-Ashtiani, K. (2017). Bioactive compounds from microalgae: Current development and prospects. In R. A. ur (Ed.), *Studies in natural products chemistry* (Vol. 54, pp. 199-225): Elsevier.

Ganesan, K., Kumar, K., & Rao, P. V. S. (2011). Comparative assessment of antioxidant activity in three edible species of green seaweed, Enteromorpha from Okha, Northwest coast of India. *Innovative Food Science & Emerging Technologies, 12*(1), 73-78. doi:10.1016/j.ifset.2010.11.005

German-Báez, L. J., Flores, M. V., Félix-Medina, J. V., Norzagaray, C., Ballardo, D. S., Moreno, C. R., . . . Valdez, A. (2017). Chemical composition and physicochemical properties of *Phaeodactylum tricornutum* microalgal residual biomass. *Food Science and Technology International, 23*(8), 681-689. doi:10.1177/1082013217717611

Guedes, A., & Malcata, F. X. (2012). Nutritional value and uses of microalgae in aquaculture. In Z. Muchlisin (Ed.), *Aquaculture (Amsterdam, Netherlands)* (Vol. 10, pp. 59- 78): InTech.

Guerreiro, I., Magalhães, R., Couto, A., Machado, M., Oliva-Teles, A., & Peres, H. (2017). *Evaluation of two seaweeds species, Ulva lactuca and Chondrus crispus, as aquafeed ingredients in gilthead seabream Sparus aurata*. Paper presented at the Aquaculture Europe 2017, Dubrovnik, Croatia.

Hardy, R., & Barrows, F. (2003). Diet formulation and manufacture. In J. Halver & R. Hardy (Eds.), *Fish Nutrition* (pp. 588).

Holdt, S., & Kraan, S. (2011). *Bioactive compounds in seaweed: Functional food applications and legislation* (Vol. 23).

Instituto Nacional de Estatística. (2020). *Estatísticas da pesca 2019 edition*

Lisboa.

Irianto, A., & Austin, B. (2002). Probiotics in aquaculture. *Journal of Fish Diseases, 25*(11), 633-642. doi:10.1046/j.1365-2761.2002.00422.x

Janeway, C. A. J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). *Immunobiology: The immune system in health and disease*. New York.

Jiao, L., Jiang, P., Zhang, L., & Wu, M. (2010). Antitumor and immunomodulating activity of polysaccharides from *Enteromorpha intestinalis*. *Biotechnology and Bioprocess Engineering, 15*(3), 421-428. doi:10.1007/s12257-008-0269-z

Jin, D. Q., Lim, C. S., Sung, J. Y., Choi, H. G., Ha, I., & Han, J. S. (2006). *Ulva conglobata*, a marine algae, has neuroprotective and anti-inflammatory effects in murine hippocampal and microglial cells. *Neuroscience Letters, 402*(1-2), 154-158. doi:10.1016/j.neulet.2006.03.068

Kızılkaya, I. T., Unal, D., Sukatar, A., & Cadirci, B. (2006). *Antimicrobial activities of the extracts of marine algae from the coast of Urla (İzmir, Turkey)* (Vol. 30).

Kokou, F., Makridis, P., Kentouri, M., & Divanach, P. (2012). Antibacterial activity in microalgae cultures. *43*(10), 1520-1527. doi:10.1111/j.1365-2109.2011.02955.x

Kwak, H. W., Kang, M. J., Bae, J. H., Hur, S. B., Kim, I.-S., Park, Y. H., & Lee, K. H. (2014). Fabrication of *Phaeodactylum tricornutum* extract-loaded gelatin nanofibrous mats exhibiting antimicrobial activity. *International Journal of Biological Macromolecules, 63*, 198-204. doi:10.1016/j.ijbiomac.2013.11.004

Leiro, J. M., Castro, R., Arranz, J. A., & Lamas, J. (2007). Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. Agardh. *International Immunopharmacology, 7*(7), 879-888. doi:10.1016/j.intimp.2007.02.007

Lerat, Y., Cornish, M., & Critchley, A. (2018). Applications of algal biomass in global food and feed markets: From traditional usage to the potential for functional products. In S. L. Barre & S. S. Bates (Eds.), *Blue Biotechnology: Production and Use of Marine Molecules, Volume 1* (pp. 143-189): Wiley-VCH Verlag GmbH & Co. KGaA.

Lieschke, G. J., & Trede, N. S. (2009). Fish immunology. *Current Biology, 19*(16), 678- 682. doi:10.1016/j.cub.2009.06.068

Lopes, A. S. d. C. (2016). *From consumption to production– development of fisheries and aquaculture in Portugal.* (Master), Faculdade de Ciências e Tecnologia (FCT),

Lubián, L. M., Montero, O., Moreno-Garrido, I., Huertas, I. E., Sobrino, C., Valle, M. G.-d., & Parés, G. (2000). *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments. *Journal of Applied Phycology, 12*(3), 249-255. doi:10.1023/A:1008170915932

Machado, M., Azeredo, R., Diaz-Rosales, P., Afonso, A., Peres, H., Oliva-Teles, A., & Costas, B. (2015). Dietary tryptophan and methionine as modulators of European seabass (*Dicentrarchus labrax*) immune status and inflammatory response. *Fish & Shellfish Immunology, 42*(2), 353-362. doi:10.1016/j.fsi.2014.11.024

Machado, M., Azeredo, R., Fontinha, F., Fernández-Boo, S., Conceição, L. E. C., Dias, J., & Costas, B. (2018). Dietary methionine improves the European seabass

(*Dicentrarchus labrax*) immune status, inflammatory response, and disease resistance. *Frontiers in Immunology, 9*(2672). doi:10.3389/fimmu.2018.02672

Maftuch, Kurniawati, I., Adam, A., & Zamzami, I. a. (2016). Antibacterial effect of *Gracilaria verrucosa* bioactive on fish pathogenic bacteria. *The Egyptian Journal of Aquatic Research, 42*(4), 405-410. doi:10.1016/j.ejar.2016.10.005

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & Shellfish Immunology, 20*(2), 137-151. doi:10.1016/j.fsi.2004.09.006

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Goncalves, J. F. M., Rocha, C. M. R., . . . Perez-Sanchez, J. (2017). Dietary supplementation of heat-treated Gracilaria and Ulva seaweeds enhanced acute hypoxia tolerance in gilthead sea bream (*Sparus aurata*). *Biology Open, 6*(6), 897-908. doi:10.1242/bio.024299

Makridis, P., Costa, R., & Dinis, M. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched Artemia metanauplii. *Aquaculture (Amsterdam, Netherlands), 255*(1-4), 76-81. doi:10.1016/j.aquaculture.2005.12.010

Maruyama, I., Nakamura, T., Matsubayashi, T., Ando, Y., & Maeda, T. (1986). Identification of the alga known as marine chlorella as a member of the eustigmatophyceae. *Japanese Journal of Phycology, 34*(4), 319-325.

Mazumder, S., Ghosal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B., & Ray, B. (2002). Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (Gracilariaceae, Rhodophyta). *International Journal of Biological Macromolecules, 31*(1-3), 87-95.

Mendonça, A. J. C. d., Rosas, V. T., Monserrat, J. M., Romano, L. A., & Tesser, M. B. (2019). The inclusion of algae *Gracilaria domingensis* in the diet of mullet juveniles (Mugil liza) improves the immune response. *Journal of Applied Aquaculture, 31*(3), 210-223. doi:10.1080/10454438.2018.1539693

Messina, M., Bulfon, C., Beraldo, P., Tibaldi, E., & Cardinaletti, G. (2019). Intestinal morpho-physiology and innate immune status of European sea bass (*Dicentrarchus labrax*) in response to diets including a blend of two marine microalgae, *Tisochrysis lutea* and *Tetraselmis suecica*. *Aquaculture (Amsterdam, Netherlands), 500*, 660-669. doi:10.1016/j.aquaculture.2018.09.054

Millao, S., & Uquiche, E. (2016). Antioxidant activity of supercritical extracts from *Nannochloropsis gaditana*: Correlation with its content of carotenoids and tocopherols. *The Journal of Supercritical Fluids, 111*, 143-150. doi:10.1016/j.supflu.2016.02.002

Montero, D., Mathlouthi, F., Tort, L., Afonso, J. M., Torrecillas, S., Fernández-Vaquero, A., . . . Izquierdo, M. S. (2010). Replacement of dietary fish oil by vegetable oils affects

humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*. *Fish & Shellfish Immunology, 29*(6), 1073-1081. doi:10.1016/j.fsi.2010.08.024

Morelli, A., Puppi, D., & Chiellini, F. (2017). Perspectives on biomedical applications of ulvan. In J. Venkatesan, S. Anil, & S.-K. Kim (Eds.), *Seaweed Polysaccharides* (pp. 305-330): Elsevier.

Murugan, K., & Iyer, V. (2012). *Antioxidant and antiproliferative activities of marine algae, Gracilaria edulis and Enteromorpha lingulata, from Chennai coast* (Vol. 8).

Nauroth, J. M., Liu, Y. C., Elswyk, M. V., Bell, R., Hall, E. B., Chung, G., & Arterburn, L. M. (2010). Docosahexaenoic acid (DHA) and docosapentaenoic acid (DPAn-6) algal oils reduce inflammatory mediators in human peripheral mononuclear cells in vitro and paw edema in vivo. *Lipids, 45*(5), 375-384. doi:10.1007/s11745-010-3406-3

Norris, R. E., Hori, T., & Chihara, M. (1980). Revision of the genus Tetraselmis (Class Prasinophyceae). *The botanical magazine, 93*, 317-339. doi:10.1007/BF02488737

Okai, Y., & Higashi-Okai, K. (1997). Potent anti-inflammatory activity of pheophytin a derived from edible green alga, *Enteromorpha prolifera* (Sujiao-nori). *International Journal of Immunopharmacology, 19*(6), 355-358. doi:10.1016/S0192-0561(97)00070-2

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of Fish Diseases, 35*(2), 83-108. doi:10.1111/j.1365-2761.2011.01333.x

Paiva, L., Lima, E., Neto, A. I., Marcone, M., & Baptista, J. (2017). Nutritional and functional bioactivity value of selected azorean macroalgae: *Ulva compressa*, *Ulva rigida*, *Gelidium microdon*, and *Pterocladiella capillacea*. *Journal of Food Science, 82*(7), 1757-1764. doi:10.1111/1750-3841.13778

Panagiota, P., & Malandrakis, E. (2018). Aquatic environment and fish welfare in aquaculture. *Reference Module in Earth Systems and Environmental Sciences*. doi:10.1016/B978-0-12-409548-9.10959-5

Patra, S., & Muthuraman, M. S. (2013). *Gracilaria edulis* extract induces apoptosis and inhibits tumor in Ehrlich ascites tumor cells in vivo. *BMC Complementary and Alternative Medicine, 13*, 331-331. doi:10.1186/1472-6882-13-331

Peixoto, M. J., Ferraz, R., Magnoni, L. J., Pereira, R., Gonçalves, J. F., Calduch-Giner, J., . . . Ozório, R. O. A. (2019). Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (*Dicentrarchus labrax*) subjected to bacterial infection. *Scientific Reports, 9*(1), 16134. doi:10.1038/s41598-019-52693-6

Pérez-López, P., González-García, S., Ulloa, R. G., Sineiro, J., Feijoo, G., & Moreira, M. T. (2014). Life cycle assessment of the production of bioactive compounds

from *Tetraselmis suecica* at pilot scale. *Journal of Cleaner Production, 64*, 323-331. doi:10.1016/j.jclepro.2013.07.028

Prestegard, S. K., Erga, S. R., Steinrücken, P., Mjøs, S. A., Knutsen, G., & Rohloff, J. (2015). Specific metabolites in a *Phaeodactylum tricornutum* strain isolated from western norwegian fjord water. *Marine Drugs, 14*(1), 9-9. doi:10.3390/md14010009

Prestegard, S. K., Oftedal, L., Coyne, R. T., Nygaard, G., Skjaerven, K. H., Knutsen, G., . . . Herfindal, L. (2009). Marine benthic diatoms contain compounds able to induce leukemia cell death and modulate blood platelet activity. *Marine Drugs, 7*(4), 605-623. doi:10.3390/md7040605

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology, 65*(6), 635-648. doi:10.1007/s00253-004-1647-x

Qi, H., Zhang, Q., Zhao, T., Chen, R., Zhang, H., Niu, X., & Li, Z. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules, 37*(4), 195-199. doi:10.1016/j.ijbiomac.2005.10.008

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology and Immunopathology, 58*(3), 239-248. doi:10.1016/S0165-2427(97)00048-2

Queiroz, A., Pereira, R., Domingues, A., Peixoto, M., Gonçalves, J., & Ozorio, R. (2014). *Effect of seaweed supplementation on growth performance, immune and oxidative stress responses in gilthead seabream (Sparus aurata)*. Paper presented at the International Meeting on Marine Research. Conference Abstract retrieved from

Ramalho, A., & Dinis, M. (2010). Portuguese aquaculture: status and future perspectives. *Aquaculture Europe Magazine*.

Raposo, M. F. d. J., Morais, R. M. S. C. d., & Morais, A. M. M. B. d. (2013). Health applications of bioactive compounds from marine microalgae. *Life Sciences, 93*(15), 479-486. doi:10.1016/j.lfs.2013.08.002

Ratajczak, W., Niedźwiedzka-Rystwej, P., Tokarz-Deptuła, B., & Deptuła, W. (2018). Immunological memory cells. *Central-European Journal of Immunology, 43*(2), 194-203. doi:10.5114/ceji.2018.77390

Ribeiro, A. R., Gonçalves, A., Barbeiro, M., Bandarra, N., Nunes, M., Carvalho, M., . . . Dias, J. (2017). *Phaeodactylum tricornutum* in finishing diets for gilthead seabream: effects on skin pigmentation, sensory properties and nutritional value. *Journal of Applied Phycology, 29*. doi:10.1007/s10811-017-1125-3

Rindi, F., Soler-Vila, A., & Guiry, M. (2012). Taxonomy of marine macroalgae used as sources of bioactive compounds. In *Marine Bioactive Compounds* (pp. 1-53).

Rizwan, M., Mujtaba, G., Memon, S. A., Lee, K., & Rashid, N. (2018). Exploring the potential of microalgae for new biotechnology applications and beyond: A review. *Renewable and Sustainable Energy Reviews, 92*, 394-404. doi:10.1016/j.rser.2018.04.034

Samarakoon, K. W., Ko, J.-Y., Lee, J.-H., Kwon, O. N., Kim, S.-W., & Jeon, Y.-J. (2014). Apoptotic anticancer activity of a novel fatty alcohol ester isolated from cultured marine diatom, *Phaeodactylum tricornutum*. *Journal of Functional Foods, 6*, 231-240. doi:10.1016/j.jff.2013.10.011

Sanjeewa, K. K. A., Fernando, I. P. S., Samarakoon, K. W., Lakmal, H. H. C., Kim, E.-A., Kwon, O. N., . . . Jeon, Y.-J. (2016). Anti-inflammatory and anti-cancer activities of sterol rich fraction of cultured marine microalga *Nannochloropsis oculata*. *Algae, 31*(3), 277-287. doi:10.4490/algae.2016.31.6.29

Sansone, C., Galasso, C., Orefice, I., Nuzzo, G., Luongo, E., Cutignano, A., . . . Ianora, A. (2017). The green microalga *Tetraselmis suecica* reduces oxidative stress and induces repairing mechanisms in human cells. *Scientific Reports, 7*, 41215. doi:10.1038/srep41215

Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F. (2019). Microalgae metabolites: A rich source for food and medicine. *Saudi Journal of Biological Sciences, 26*(4), 709-722. doi:10.1016/j.sjbs.2017.11.003

Saurabh, S., & Sahoo, P. K. (2008). Lysozyme: An important defence molecule of fish innate immune system. *Aquaculture Research, 39*, 223-239. doi:10.1111/j.1365-2109.2007.01883.x

Secombes, C. J., & Fletcher, T. C. (1992). The role of phagocytes in the protective mechanisms of fish. *Annual Review of Fish Diseases, 2*, 53-71. doi:10.1016/0959-8030(92)90056-4

Shields, R., & Lupatsch, I. (2012). Algae for aquaculture and animal feeds. In C. Posten & C. Walter (Eds.), *Microalgal biotechnology: Integration and economy* (Vol. 21): De Gruyter.

Silva, D., Valente, L., Pinto, I. S., Pereira, R., Pires, M., Travassos, F. S., & Rema, P. (2015). Evaluation of IMTA-produced seaweeds (Gracilaria, Porphyra, and Ulva) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology. *Journal of Applied Phycology, 27*(4), 1671-1680. doi:10.1007/s10811-014-0453-9

Sola, L., Moretti, A., Crosetti, D., Karaiskou, N., Magoulas, A., Rossi, A. R., . . . Tsigenopoulos, C. S. (2007). Genetic effects of domestication, culture and breeding of fish and shellfish, and their impacts on wild populations. Gilthead seabream- *Sparus aurata*. In T. Svåsand, D. Crosetti, E. García-Vázquez, & E. Verspoor (Eds.),

Genetic impact of aquaculture activities on native populations. Genimpact final scientific report (pp. 47- 54).

Souza, B. W., Cerqueira, M. A., Martins, J. T., Quintas, M. A., Ferreira, A. C., Teixeira, J. A., & Vicente, A. A. (2011). Antioxidant potential of two red seaweeds from the Brazilian coasts. *Journal of Agricultural Food Chemistry, 59*(10), 5589-5594. doi:10.1021/jf200999n

Talero, E., Garcia-Maurino, S., Avila-Roman, J., Rodriguez-Luna, A., Alcaide, A., & Motilva, V. (2015). Bioactive compounds isolated from microalgae in chronic inflammation and cancer. *Marine Drugs, 13*(10), 6152-6209. doi:10.3390/md13106152

Thompson, K. D. (2017). Immunology: Improvement of innate and adaptive immunity. In G. Jeney (Ed.), *Fish Diseases* (pp. 1-17): Academic Press.

Tort, L. (2011). Stress and immune modulation in fish. *Developmental and Comparative Immunology, 35*(12), 1366-1375. doi:10.1016/j.dci.2011.07.002

Tort, L., Balasch, J., & Mackenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. *Inmunologia, 22*(3), 277-286.

Tredici, M. R., Biondi, N., Ponis, E., Rodolfi, L., & Chini Zittelli, G. (2009). Advances in microalgal culture for aquaculture feed and other uses. In G. Burnell & G. Allan (Eds.), *New Technologies in Aquaculture* (pp. 610-676): Woodhead Publishing.

Uribe, C., Folch, H., Enríquez, R., & Moran, G. (2011). Innate and adaptive immunity in teleost fish: A review. *Veterinarni Medicina, 56*(10), 486-503. doi:10.17221/3294-VETMED

Valente, L. M. P., Gouveia, A., Rema, P., Matos, J., Gomes, E. F., & Pinto, I. S. (2006). Evaluation of three seaweeds *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture (Amsterdam, Netherlands), 252*(1), 85-91. doi:10.1016/j.aquaculture.2005.11.052

Walport, M. J. (2001a). Complement. First of two parts. *New England Journal of Medicine, 344*(14), 1058-1066. doi:10.1056/nejm200104053441406

Walport, M. J. (2001b). Complement. Second of two parts. *New England Journal of Medicine, 344*(15), 1140-1144. doi:10.1056/nejm200104123441506

Wan, A. H. L., Davies, S. J., Soler-Vila, A., Fitzgerald, R., & Johnson, M. P. (2018). Macroalgae as a sustainable aquafeed ingredient. *Reviews in Aquaculture, 0*(0). doi:10.1111/raq.12241

Wang, S., Said, I. H., Thorstenson, C., Thomsen, C., Ullrich, M. S., Kuhnert, N., & Thomsen, L. (2018). Pilot-scale production of antibacterial substances by the marine diatom *Phaeodactylum tricornutum* Bohlin. *Algal Research, 32*, 113-120. doi:10.1016/j.algal.2018.03.014

Whyte, S. (2007). The innate immune response of finfish - A review of current knowledge. *Fish & Shellfish Immunology, 23*(6), 1127-1151. doi:10.1016/j.fsi.2007.06.005

Yildiz, G., Celikler, S., Vatan, O., & Dere, S. (2011). Determination of the anti-oxidative capacity and bioactive compounds in green seaweed *Ulva rigida* C. Agardh. *International Journal of Food Properties, 2011*(6). doi:10.1080/10942912.2010.517341

Yoshizawa, Y., Tsunehiro, J., Nomura, K., Itoh, M., Fukui, F., Ametani, A., & Kaminogawa, S. (1996). In vivo macrophage-stimulation activity of the enzyme-degraded water-soluble polysaccharide fraction from a marine alga (*Gracilaria verrucosa*). *Bioscience Biotechnology and Biochemistry, 60*(10), 1667-1671.