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Seaweeds as functional aquafeed ingredients: Modulation of nutrient metabolism and stress responsiveness in aquaculture species.

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Seaweeds as functional aquafeed ingredients: Modulation of nutrient metabolism and stress responsiveness in aquaculture species.

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TABLE OF CONTENTS

Author Statement.....vi

Funding information viii

Acknowledgments viiii

Abstract.....xiv

Resumo xvi

Chapter I – Introduction 3

I.1 Aquaculture 3

I.1.2 Fish and shellfish aquaculture 4

I.1.3 Seaweed aquaculture 7

I.2 Bioactives as dietary supplements 12

I.3 Fish and seaweed species 15

References 23

Objectives 31

Chapter II 33

Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*) 33

Chapter III 61

Diets supplemented with seaweed affect metabolic rate, innate immune and antioxidant responses, but not individual growth rate in seabass (*Dicentrarchus labrax*)..... 61

Chapter IV 85

Effects of dietary *Gracilaria* sp. and *Alaria* sp. supplementation on growth performance, metabolic rates and health in meagre (*Argyrosomus regius*) subjected to pathogen infection 85

Chapter V 117

Effects of dietary supplementation of *Gracilaria* sp. extracts on fillet quality, oxidative stress and immune responses in European seabass (*Dicentrarchus labrax*). 117

Chapter VI 143

Oxidative stress and immune responses genes have significant altered expression in European seabass (*Dicentrarchus labrax*) fed *Gracilaria* sp. supplemented diet. 143

Chapter VII - Discussion 185

General appreciation 185

References 194

INDEX FIGURES

Figure 1 – Mural painting of an *Assyrian vivarium* at the Palais des Papes, Avignon, France (https://www.alimentarium.org/en/knowledge/history-aquaculture). 3

Figure 2 – World fisheries and aquaculture production in 2014 (FAO 2006). 4

Figure 3 – Constraints associated with aquaculture production. 5

Figure 4 – *Sargassum* collection for use as fertilizer by Portuguese farmers in the north of the country. (In: https://www.geocaching.com/geocache)..... 8

..... 9

Figure 5 – Seabass cooked with seaweeds “Robalo com algas” a typical dish from Viana do Castelo, Portugal (In: https://www.tripadvisor.pt/Restaurant_Review-g4314675-d4309996-Reviews-Restaurante_Mariana-Afife_Viana_do_Castelo_District_Northern_Portugal.html). 9

Figure 6 – Example ALGAPLUS commercialized products (left side) and their use in culinary by chefs (on the right). 9

Figure 7 – Integrated multi-trophic aquaculture (IMTA) system (Holdt and Edwards 2014). 12

Figure 8 – Illustration and producing countries of European seabass (*Dicentrarchus labrax*) (FAO 2014a)..... 16

Figure 9 – Global production data regarding European seabass (*Dicentrarchus labrax*) (FAO 2014a). 17

Figure 10 – Illustration and producing countries of meagre (*Argyrosomus regius*) (FAO 2005)..... 17

Figure 11 – Global production data regarding meagre (Argyrosomus regius) (FAO 2005). 18

Figure 12 – Photograph and sketch of *Alaria* sp. (In: 19

Figure 13 – Photograph and sketch of *Gracilaria* sp. (In: http://data.nhm.ac.uk/)..... 20

Figure 14 – Photograph and sketch of *Fucus* sp. (In: http://data.nhm.ac.uk/). 21

Figure 15 – Photograph and sketch of *Ulva* sp. (In: http://data.nhm.ac.uk/). 22

TABLES INDEX

Table 1 – Production of farmed aquatic plants in the world (FAO 2016). 7

Table 2 – Pigments, main products and cell wall composition of the main groups of seaweeds. 10

Table 3 – Sources of functional materials of marine origin and their correspondent activity. 13

Table 4 – Summary of the seaweeds species and the parameters analyzed in seabass and meagre. 186

AUTHOR STATEMENT

This thesis was organized in seven chapters, which include a general introduction, five scientific papers and a general discussion.

The five articles that were integrated within the thesis comprise four articles as first author published in peer reviewed international journals and one article as first author submitted and pending approval.

The work included in this thesis was totally or partially executed by the candidate, in close cooperation/co-authorships with supervisors and other researchers. The articles included herein will not appear in other thesis or dissertations.

During the PhD work, the candidate actively participated in other research projects, which entailed additional publications not included in this thesis but included in the attached curriculum.

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"MEN OFTEN BECOME WHAT THEY BELIEVE THEMSELVES TO BE. IF I BELIEVE I CANNOT DO SOMETHING, IT MAKES ME INCAPABLE OF DOING IT. BUT WHEN I BELIEVE I CAN, THEN I ACQUIRE THE ABILITY".

MAHATMA GANDHI

ABSTRACT

Intensive aquaculture practices subject fish to a diversity of stressors, which are responsible for immunosuppression, leading to impaired growth and higher susceptibility to bacterial infections. Consequently, most of expenses associated with aquaculture practices involve feeds and infectious diseases management. The use of antibiotics in aquaculture entails a serious environmental impact and results in increased bacterial resistance. Therefore, a great effort is being made to develop sustainable strategies allowing improving fish welfare through environmental-friendly techniques, thus avoiding therapeutic procedures. One of the most promising strategies is the application of food additives with immunostimulant properties by creating tailor-made diets for each species or specific situation.

Seaweeds are important nutrient sources, hereafter being in the spotlight as novel feed ingredients. Moreover, the industrial processing of seaweed biomass, for food or pharmaceutical industries, generates non-hazardous and rich by-products evaluated as having important immunostimulant and antioxidant properties. Indeed, several molecules with interesting applications for aquafeeds are expected at high concentrations in the seaweed fractions not used for bioplastic production. These fractions may be upcycled as a natural replacement for other ingredients and/or additive in optimized aquafeeds.

For an efficient exploration of seaweeds as supplement in aquafeeds, it is mandatory to determine the overall impact in fish production and welfare. With that purpose, this thesis focused on two fish species: seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*). These species have great importance in the Mediterranean aquaculture industry and are susceptible to waterborne pathogens. Additionally, these species do not naturally feed on seaweeds, aggravating the need for research assuring the safety of dietary seaweed supplementation. In this context, I selected four seaweed species, with different nutritional and mineral compositions: *Ulva* sp., *Fucus* sp., *Gracilaria* sp. and *Alaria* sp., covering a large spectrum of possible applications. The general aim of this thesis was to understand the effects of seaweed application as functional feed ingredient for aquaculture species.

Initially growth performance, digestive capacity and stress responsiveness in European seabass (*Dicentrarchus labrax*) fed practical diets supplemented with *Gracilaria* sp., *Ulva* sp., or *Fucus* sp., were evaluated. Overall, the results indicate that the use of dietary seaweed supplementation improves immune and antioxidant responses in seabass without compromising growth performance.

Subsequently, the effects of seaweed dietary supplementation on European seabass (*Dicentrarchus labrax*) performance including aerobic metabolism, digestive enzymes activity, innate immune status, oxidative damage and growth rates were also investigated. Collectively, the results suggest that dietary seaweed supplementation may alter seabass metabolic rates, innate immune and antioxidant responses without compromising growth parameters.

Additionally, the effects of dietary seaweed supplementation with *Gracilaria* sp. and *Alaria* sp., on basal physiology and health biomarkers in meagre (*Argyrosomus regius*) when challenged with *Photobacterium damselae* subsp. *piscicida* (Phdp) were evaluated. The results of suggest that dietary seaweed supplementation modulates metabolic rates and biomarker responses in meagre, which may confer advantages in coping with biotic stressors.

Furthermore, the effects of two different fractions derived from the methanolic extraction of *Gracilaria* sp. supplemented in European seabass (*Dicentrarchus labrax*) diets were evaluated. The results indicate that dietary supplementation of *Gracilaria* sp. methanolic extracts may have minor influence on the innate immune system and skin color in seabass.

The evaluation of the effect of seaweed dietary supplementation on antioxidant capacity, immunity and disease resistance against *Photobacterium damselae* subsp. *piscicida* (Phdp) infection in European seabass (*Dicentrarchus labrax*) was also performed. In summary, supplementation with 5% *Gracilaria* sp. aqueous extract in seabass diets increases immune and antioxidant capacities of this species when challenged with *P. damselae*.

In overview, this thesis reiterates the use of seaweeds as supplements in aquafeeds for carnivore fish, without compromising growth or altering feed consumption or efficiency rates, point of great concern for aquaculture production. Meanwhile, dietary seaweed supplementation can elicit metabolic shifts in energetic parameters that could be valuable for pond culture. Most importantly, seaweed supplementation positively modulates fish immune system and antioxidant capacity ultimately improving disease resistance.

RESUMO

Em aquacultura, os sistemas de cultivo intensivos sujeitam os peixes a variados fatores de stress, que causam imunossupressão, levando à diminuição do crescimento e a uma maior suscetibilidade a infeções bacterianas. Consequentemente, a maioria dos gastos associados à aquacultura estão relacionados com as rações e o controlo de doenças infeciosas. A utilização de antibióticos na aquacultura implica um sério risco ambiental e culmina em aumento da resistência bacteriana. Nesse sentido, sérios esforços estão direcionados para o desenvolvimento de estratégias sustentáveis que permitam melhorar o bem-estar dos peixes através de técnicas amigas do ambiente, evitando assim o recurso a procedimentos terapêuticos. Uma das estratégias mais promissoras consiste na aplicação de aditivos alimentares com propriedades imunoestimulantes, através da criação de rações especificas para cada espécie e situação especifica.

As macroalgas são fontes nutricionais importantes estando por esse motivo em relevo na procura por ingredientes inovadores para rações. Além disso, o processamento industrial da biomassa de macroalgas, para as indústrias alimentares e farmacêutica, origina valiosos subprodutos não perigosos e avaliados como detentores de importantes características imunoestimulantes e antioxidantes. De facto, várias moléculas com interessante aplicabilidade para a produção de rações poderão encontrar-se em maior concentração nas porções não utilizadas para a produção de bioplásticos. Estas frações poderão ser utilizadas como um substituinte natural de outros ingredientes e/ou como aditivo em rações otimizadas.

Para uma aplicação eficiente das macroalgas em dietas para aquacultura, é obrigatório determinar o impacto global sobre os parâmetros de produção e o bem-estar dos peixes. Com esse propósito, esta tese focou-se em duas espécies de peixes: o robalo (*Dicentrarchus labrax*) e a corvina (*Argyrosomus regius*). Estas espécies são relevantes para a indústria da aquacultura Mediterrânica e são suscetíveis a agentes patogénicos aquáticos. Acresce que estas espécies não se alimentam naturalmente de macroalgas, o que aumenta a necessidade de investigação no sentido de assegurar a biossegurança do seu uso como aditivo alimentar. Neste contexto, selecionei quatro espécies de macroalgas, com diferentes composições nutricionais e minerais: *Ulva* sp., *Fucus* sp., *Gracilaria* sp. e *Alaria* sp., cobrindo deste modo um espectro abrangente de possíveis aplicações. O objetivo geral desta tese era entender os efeitos da aplicação de macroalgas como ingrediente funcional nas dietas de espécies de aquacultura.

Inicialmente foram avaliados a performance de crescimento, a capacidade digestiva e a resposta ao stresse em robalo Europeu (*Dicentrarchus labrax*), quando alimentado com dietas funcionais suplementadas com *Gracilaria* sp., *Ulva* sp., ou *Fucus* sp. De um modo geral, os resultados indicam que a aplicação de macroalgas como suplemento nas dietas de robalo melhoram as respostas imunitária e antioxidante, sem que seja comprometida a performance de crescimento.

Subsequentemente, foram investigados em robalo europeu (*Dicentrarchus labrax*) os efeitos da suplementação de macroalgas na sua performance, incluindo o metabolismo aeróbico, atividade das enzimas digestivas, perfil imunitário inato, stresse oxidativo e taxas de crescimento. Globalmente, os resultados sugerem que a suplementação alimentar de macroalgas poderá alterar as taxas metabólicas de robalo, as respostas imunitárias e antioxidantes, sem prejudicar os parâmetros de crescimento.

Adicionalmente, foram avaliados os efeitos da suplementação de *Gracilaria* sp. e *Alaria* sp. na fisiologia basal e em biomarcadores de saúde da corvina (*Argyrosomus regius*) quando desafiada com *Photobacterium damselae* subsp. *piscicida* (Phdp). Os resultados sugerem que a suplementação dietética com macroalgas modula as taxas metabólicas e outros biomarcadores em corvina, o que poderá ser vantajoso em situações de stress biótico.

Ademais, foram avaliados os efeitos de duas frações diferentes resultantes da extração metanólica de *Gracilaria* sp. quando suplementadas a dietas de robalo (*Dicentrarchus labrax*). Os resultados indicam que a suplementação de extratos metanólicos de *Gracilaria* sp. poderá ter uma ligeira influência sobre o sistema imunitário inato e a coloração da pele do robalo.

Foi também realizada a avaliação da suplementação dietética com macroalgas na capacidade antioxidante, imunidade e resistência à infeção por *Photobacterium damselae* subsp. *piscicida* (Phdp) do robalo europeu (*Dicentrarchus labrax*). Sumariamente, a suplementação com 5% de extrato aquoso de *Gracilaria* sp. nas dietas de robalo aumentam as capacidades imunes e antioxidantes deste peixe quando desafiado com *P. damselae*.

Em suma, esta tese reitera o uso de macroalgas como suplemento alimentar em dietas para aquacultura de peixes carnívoros, sem que haja repercussões no crescimento ou alterações no consumo ou taxas de eficiência, ponto de elevada importância para a produção em aquacultura. Entretanto, a suplementação dietética com macroalgas desencadeia alterações metabólicas nos parâmetros energéticos com interesse económico para o setor da aquacultura. Essencialmente, a suplementação alimentar com macroalgas modela positivamente o sistema imunitário e antioxidante dos peixes, resultando em maior resistência a doenças.

CHAPTER I

INTRODUCTION

CHAPTER I – INTRODUCTION

I.1 AQUACULTURE

Food and Agriculture Organization (FAO) currently defines aquaculture as the culture or farming in water such as the aquatic equivalent of agriculture or farming on land. Meaning "agriculture includes farming both animals (animal husbandry) and plants (agronomy, horticulture and forestry). Similarly, aquaculture covers the farming of both animals (including crustaceans, finfish and mollusks) and plants (including seaweeds and freshwater macrophytes). While agriculture is predominantly based on use of freshwater, aquaculture occurs in both inland (freshwater) and coastal (brackishwater, seawater) areas". Aquaculture provides approximately 50% of fish and shellfish that the world consumes annually, the remaining is harvested from naturally existing populations — principally from marine fisheries, which are at their maximum sustainable yields, in decline, or have completely collapsed (FAO 2006; Georgiadis *et al.* 2001). This farming implies anthropogenic involvement in the rearing processes, which aims to enhance production; for instance, regular stocking, feeding, protection from predators, protection against pathogens, among others (FAO 2017). Reports of aquaculture practices date back to 6000 B.C. by Australian indigenous. In Europe, aquaculture first began in Ancient Rome. The Romans love for sea fish and oysters resulted in the creation of oyster farms and the use of Assyrian vivarium (Figure 1). These *vivarium* were built inside wealthier homes and consisted of a 'swimming pool' where fish and crustaceans caught in lagoons were kept alive until people chose the fish they wished to eat.

Figure 1 – Mural painting of an ***Assyrian vivarium*** at the Palais des Papes, Avignon, France (https://www.alimentarium.org/en/knowledge/history-aquaculture).

I.1.2 FISH AND SHELLFISH AQUACULTURE

Fish and shellfish aquaculture remains the fastest growing animal food producing sector worldwide (Srivastava and Pandey 2015) with food fish supplies increasing annually (FAO 2014c). The increasing growth of global population bestows grave pressure over aquatic food products. The yield of capture fisheries stabilized, or in some regions decreased, since most of fishing areas has reached their maximum potential (Figure 2). Sustaining fish supplies from capture fisheries alone will therefore not be able to meet the growing global demand for aquatic food. Hence, aquaculture is considered an opportunity to bridge the supply and demand gap of aquatic food in most regions of the world (Subasinghe *et al.* 2009).

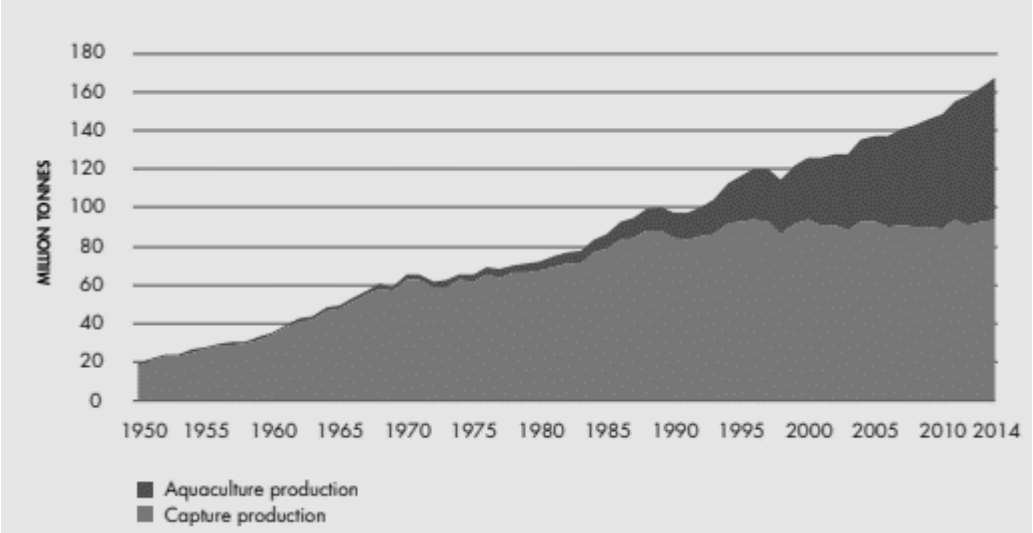

Figure 2 – World fisheries and aquaculture production in 2014 **(FAO 2006).**

Nevertheless, aquaculture production entails many constraints (Figure 3), some common to the production of all food animals, and others exclusive of aquaculture procedures. In the Conference on Aquaculture in the Third Millennium, held in Thailand in February 2000, FAO identified worldwide limitations to the industry and highlighted the main knowledge gaps to be addressed in order to successfully develop the aquaculture sector and achieve profitable fish farms (FAO 2001):

(a) Water: demands for intake (human and animal) and concerns about its quality including environmental aspects;

(b) The ability to comply with policies and regulations, including production aspects, such as cages/ponds characteristics, feed quality, control of bacterial pathogen outbreaks, etc.;

(c) Improvements in technology and marketing to include health management, genetics, culture systems and product quality.

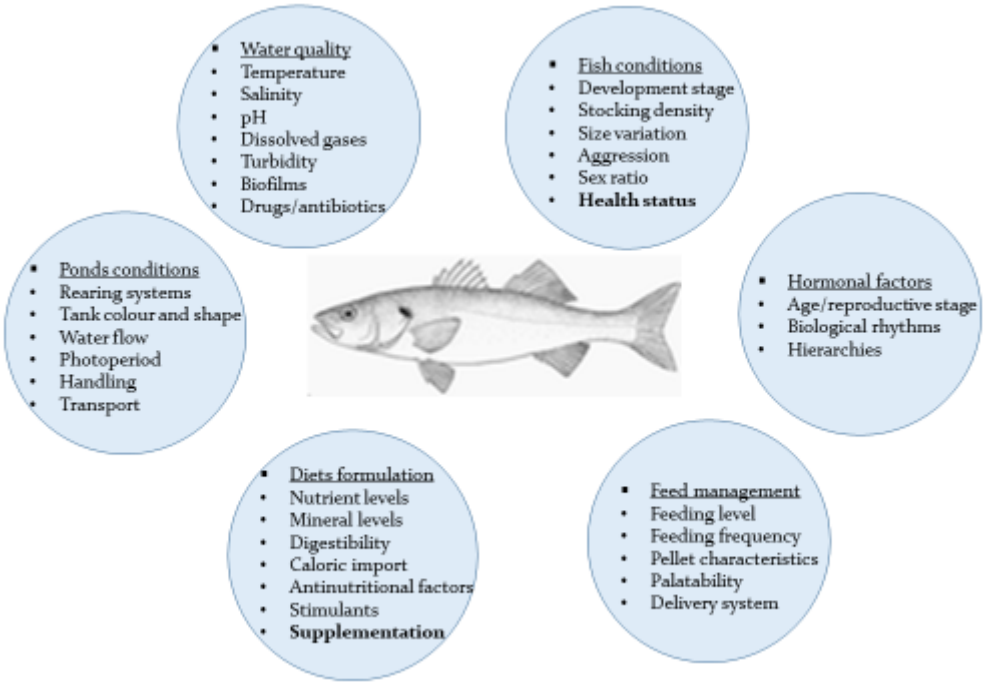

Figure 3 – Constraints associated with aquaculture production.

Water is a matrix containing: dissolved gases, organic matter, and inorganic substances such as minerals, in addition to support microorganisms, plant and animal life forms and providing a medium for chemical exchange among these populations. Therefore, water quality affects growth and survival of all living beings. In turn, several ecological parameters and management practices also influence water quality. The stocking density of fish or crustaceans in ponds usually exacerbates problems with water quality and sediment deterioration. Wastes consisting of faeces and unconsumed feed accumulate in the bottom, resulting in degraded organic matter increasing metabolite toxicity and decreasing O_2 quantity and availability. Hence, strict regulation needs to account for fish density, feeding rations and chemical products used as medication against infectious agents. Moreover, the intrinsic water characteristics, such as temperature and salinity, greatly influence production yield as well as influence bacterial ecosystems (Verschuere *et al.* 2000).

To address concerns about feed or the influence of feed rations on fish physiology, a good knowledge on nutrient requirements is crucial to optimize growth performance. In fact, feed rations affect a wide range of biochemical parameters in fish, which in turn modulate their physiological response to environmental stimuli (Chatzifotis *et al.* 2011) and should therefore be regulated and controlled. In intensive aquaculture more than 50% of the total production costs are associated with feed production, where protein is the most expensive constituent

(Cheng *et al.* 2010). Fishmeal is an ideal source of protein in feeds for most fish (Naylor *et al.* 2000) and has been the main protein in the feeds for cultured marine fish during the last decades (Tacon and Metian 2008). Essentially, fishmeal prices determine feed costs and the general profitability of fish farming. Consequently, the increase of aquaculture, particularly the intensification of carnivorous fish species production, like salmon or seabass, heightened the need of producing high performance aquafeeds in large scale. This rise in feed needs generates a great demand in fish by-products, in particular fishmeal and fish oil (Bostock *et al.* 2010; Dallaire *et al.* 2007). In addition to high performance aquafeeds, some species, such as tuna, further require the use of small pelagic fish, introducing an added pressure over aquatic ecosystems (Bostock *et al.* 2010). Fishmeal and fish oil are also used in livestock diet production, therefore the issues regarding overexploitation are not restricted to the aquaculture sector. Nonetheless, the use of substitute ingredients is easier in livestock industry than aquaculture, which means that aquaculture takes the majority share of this resource (Bostock *et al.* 2010). According to Tacon and Metian (2009), wild-caught feedstock of fish meal and oil ranged from 1 million and 6 million tonnes per year respectively, for the past 20 years. Furthermore, in 2008, aquaculture practices consumed approximately 90% of the fish oil and 71% of the fishmeal available worldwide. This trend will remain a menacing problem if alternative sources are not developed. Nevertheless, the production of fishmeal from whole fish is gradually declining, with an increasing portion of fishmeal originated from fishery by-products. Likewise, fish oil causes a growing demand for fishery by-products, hence its increasing price (FAO 2014c). This collective pressure on fish populations forage, together with the restrictions on the use of several animal derived proteins in fish feed formulations, fuel the research for feed ingredients alternatives, targeting more sustainable protein sources. In this sense, proteins derived from plants have received most of the attention (Food *et al.* 2001). Many aquaculture producers have turned to plant-based feeds (terrestrial plants and seaweeds) due to their higher abundance and consequent low prices. Thus, seaweeds are currently under scrutiny as farmed fish feed ingredients. A huge advantage to creating a feed based on seaweed is that it would likely be very cost-effective. Overall, growth of farmed fish and the global profitability of aquaculture enterprises depend on feed quality and feed cost. In addition, the use of poor quality feeds increases the production period, affects fish quality and increases susceptibility of fish to diseases.

The emergence of diseases that affect farmed fish is majorly associated with water quality deterioration, oscillations of water physical characteristics or feed conveying foreign microorganisms, often resulting in bacterial outbreaks that conduct to fish death (Pulkkinen *et al.* 2010). Traditionally, the emergence of such pathogens and the resulting pathologies lead to the application of antibiotics and/or, attempting to prevent them, vaccination. However,

health management strategies and the development of environmentally friendly technologies are constantly pushing for the use of alternatives with lower impact on aquatic ecosystems.

I.1.3 SEAWEED AQUACULTURE

Seaweed aquaculture is a growing industry with approximately 20% of total marine aquaculture production by weight (Table 1). World production of farmed seaweeds more than doubled from 2000 to 2012, reaching a revenue value of US $6.7 billion in 2013 (FAO 2018). Worldwide, 291 seaweed species are used for food, hydrocolloid or medicine production, paper, fertilizer, and animal feeds (Lindsey White and Wilson 2015). In 2012 close to 21 million tonnes of seaweeds, almost exclusively originated from aquaculture, were used.

Table 1 – Production of farmed aquatic plants in the world (FAO 2016).

	2005	**2010**	**2013**	**2014**
(Thousand tonnes)				
Kappaphycus alvarezii and *Euchemia* spp.	2444	5629	10394	10992
Laminaria japonica	4371	5147	5942	7655
Gracilaria spp.	936	1696	3463	3752
Undaria pinnatifida	2440	1537	2079	2359
Porphyra spp.	1287	1637	1861	1806
Sargassum fusiforme	86	78	152	175
Spirulina spp.	48	97	82	86
Other aquatic plants	1892	3172	2895	482
Total	13504	18993	26868	27307

Archaeological studies in Chile revealed cooked and partially eaten seaweeds at a 14,000-year-old site. These findings suggest that seaweeds have been part of the human diet in the Western Hemisphere for a very long time (Dillehay *et al.* 2008). In the past centuries, seaweeds have been prominent in Asian cuisine' in agreement, most of seaweed production occurs in Asia. In 2014, about 9 million tonnes of farmed seaweeds were used for direct human consumption, mostly in East Asia (FAO 2014c). Farmed *wakame* (*Undaria pinnatifida*) and *Porphyra* sp. seaweeds are almost entirely destined for direct human consumption, either as crude ingredient or as supplement. In addition to direct human consumption, in China, several non-identified seaweed species and *Gracilaria* spp. are produced and used as feed for abalone (*Haliotis* sp.) and sea cucumber (*Holothuroidea* sp.) culture. A small portion (less than 20 %) of *Undaria pinnatifida* is used for iodine and algin extraction. The total harvest from the Indian coast is about 100,000 tonnes, out of which only 35 to 40 % of the seaweed raw material is used by small-scale industrial units, for the extraction of phycocolloids (Dhargalkar and Pereira 2005). China production of *Laminaria* was about 4.8 million tonnes, *Gracilaria* 1.9 million

tonnes, *Undaria* 1.7 million tonnes, and *Porphyra* 1.1 million tonnes. Indonesia produces 5.7 million tonnes of *Eucheuma*. The Philippines produce about 1.7 million tonnes of *Eucheuma* (Lindsey White and Wilson 2015). In Europe, seaweeds (mostly brown) are used to produce food additives. Their application as human or animal food relates to their mineral content or functional properties (Fleurence 1999). However, worldwide, people unknowingly use seaweeds on daily basis in the form of processed foods, such as meat and fruit, but also as cosmetics or toothpaste (Dhargalkar and Pereira 2005). For that reason studies in nutrition and health using seaweeds, constitute a novel and promising research area (Denis *et al.* 2010; Mohamed *et al.* 2012) that may bring, in long term, significant benefits for the aquaculture industry.

In Portugal, the main application of seaweed farming has been as fertilizer. Historically, the collection of beach casted seaweed was recorded as far as the 14th century (Figure 4).

Figure 4 – *Sargassum* collection for use as fertilizer by Portuguese farmers in the north of the country. (In: https://www.geocaching.com/geocache).

Nowadays due to the training of young people and fishermen, integration of aquaculture with other activities and creation of more aquaculture-related research and demonstrations seaweeds are recognized as valuable for feed, energy and biomedical purposes. Nonetheless, seaweeds are established in Portuguese feeding habits, with some regions of the country using seaweeds in traditional culinary dishes, such as in “Robalo com macroalgas” (Figure 5) from the northern coastal area of the country, or as an addition to the modern trend of eating sushi, which is being progressively explored by local chefs and small companies.

Figure 5 – Seabass cooked with seaweeds “Robalo com algas” a typical dish from Viana do Castelo, Portugal (In: https://www.tripadvisor.pt/Restaurant_Review-g4314675-d4309996-Reviews-Restaurante_Mariana-Afife_Viana_do_Castelo_District_Northern_Portugal.html).

Seaweeds are therefore increasingly introduced in the everyday life of the Portuguese, through the efforts of specialists and entrepreneurs that are developing modern products using seaweeds as main or flavoring ingredients. An example is the company ALGAPLUS and their seaweed-based products, as well as their partnership with renowned chefs (Figure 6).

Figure 6 – Example ALGAPLUS commercialized products (left side) and their use in culinary by chefs (on the right).

Currently in Portugal, several policies focus in developing the marine economy and harnessing marine resources for industrial development. The country geographical location encompasses a wide coastal area with approximately 1793 km, with temperature and light conditions suitable for a wide variety of seaweed development. According to Ardré (1970), in Portugal the

presence of 60 different species of Chlorophyta, 98 Phaeophyta and 246 species of Rhodophyta has been recognized. Seaweed are divided in three main groups based on their chemical composition, specially their photosynthetic pigments.

Table 2 – Pigments, main products and cell wall composition of the main groups of seaweeds.

	Phaeophyta	**Rhodophyta**	**Chlorophyta**
Pigments	Chlorophyll a and c; fucoxanthin; β-carotene; lutein	Chlorophyll a and d; phycoerythrin; phycocyanin; a and b-carotene	Chlorophyll a and b; β-carotene; lutein
Products	Laminaran; mannitol	Floridean starch	Starch
Cell Wall	Cellulose; alginate	Cellulose; carrageenan; agar	Cellulose

Chlorophyta or green algae, are the less rewarding in natural products and only a limited number of secondary metabolites are known from this phylum (Blunt *et al.* 2013). The main chemical characteristic of green algae is the presence of 1,4-diaceoxybutadiene dienolate ester (Amsler 2008). Phaeophyta or brown algae are primarily composed of poly-phloroglucinol phenolic compounds, such as phlorotannins; however, over 1140 secondary metabolites have been associated with brown seaweeds (Amsler 2008). Rhodophyta or red algae present a secondary metabolite chemistry richer comparatively to other macroalgae, both in variety and abundance, and are impressive producers of halogenated compounds (Amsler 2008). The protein content varies within these groups, being generally established as 3-15 % (dry weight) for brown seaweeds and 10-47 % (dry weight) for red and green seaweeds (Fleurence 1999). Also the levels of seaweed proteins is determined by seasonality, with higher protein contents registered in the winter period (Cardoso *et al.* 2014). They are an exceptional source of minerals such as Ca, P, Na, K, but also vitamins A, B, B12, C, D and E, riboflavin, niacin, pantothenic acid and folic acid (Dhargalkar and Pereira 2005).

Energetic balance through lipid metabolism is also a possible metabolic target for dietary seaweed supplementation. Seaweed intake was shown to decrease cholesterol levels in hypercholesterolemic rats (Amano *et al.* 2005; Bocanegra *et al.* 2006). Also in rat models, caulerpenyne, isolated from Chlorophyta, inhibited lipase activity (Bitou *et al.* 1999). Furthermore, the administration of a *Laminaria* polysaccharide was shown to alter intestinal mucosal composition, pH and short chain fatty acid production, functioning as a prebiotic in humans (Devillé *et al.* 2007). In black seabream (*Spondyliosoma cantharus*) changes in

accumulation and mobilization of lipids were reported upon seaweed supplementation (Nakagawa *et al.* 1987); seaweed extracts were also found to activate lipolysis in sweetfish (*Plecoglossus altivelis*) *in vitro*, by stimulating lipolytic hormones and/or influencing the adipose tissue structure (Nematipour *et al.* 1990). In addition, the solvent used in bioactives extraction from seaweed may lead to fractions with diverse chemical compositions, therefore differently affecting fish health. Lipids are important for flesh quality although their excessive accumulation leads to accelerated carcass deterioration (Nakagawa 1997). Considering that cultured fish exhibit higher lipid visceral deposition than their wild counterparts, producers in the aquaculture sector need to reduce the effects of excessive lipid deposition, to ensure fillet quality, shelf-life span and therefore manage possible correspondent economic losses. Seaweeds are therefore being studied as possible modulators of lipid metabolism in cultured species (Nelson *et al.* 2002). Even though seaweed supplementation seems to influence lipid synthesis and mobilization, decreasing lipid deposition, the mechanisms behind this reduction are not well understood. A plausible explanation is the synergistic effect between vitamins, antioxidants and minerals from seaweeds, leading to a more efficient lipid mobilization (Nakagawa 1997).

The main setbacks in aquaculture industry are related to disease outbursts that consequently cause severe losses in production numbers. In general, antibiotics are used to control bacterial diseases, which eventually lead to antibiotic resistance (de la Banda *et al.* 2012). Therefore, alternative strategies are constantly being explored and a great effort is being made to improve fish welfare through environmental-friendly techniques such as marine by-products application as food additives. Simultaneously, the increasing concern with animal welfare has expanded the search for alternative techniques in aquaculture. Additionally, concerns with the public perception of farmed fish which influences product acceptability, as well as the effect over production efficiency and quality, are driving the exploration of new concepts and methodologies in the industry (Ashley 2007). As an example, extensive sea areas may be cultivated to produce crops that require no freshwater or fertilizers, while providing a variety of valuable ecosystem services. Furthermore, ecologic engineering tools which encompass different species in a combined ecosystem such as integrated multi-trophic aquaculture (IMTA) system are cutting-edge technologies. This approach has been proven to solve sea pollution problems associated with fish culture (Troell 2009). IMTA systems incorporate species that are usually commercially valuable, such as seabass and mussels, but are also environmentally sustainable, based on the concept that the wastes consisting of uneaten feed, feces and metabolic excretion of one species are a useful input for growth of another species, such as seaweeds, working in a natural self-cleansing mechanism (Figure 7) (Largo *et al.* 2016). Seaweeds contribute to the system by assimilating the fish-excreted ammonia, phosphate and

CO_2. These are then converted to potentially valuable biomass ensuring that the effluents can recirculate back to the fish ponds or be discharged without endangering the environment (Abreu *et al.* 2011). The endpoint is an integrated culturing of fed species, such as finfish, inorganic extractive species such as seaweeds, and organic extractive species such as suspension and deposit-feeders (Troell 2009).

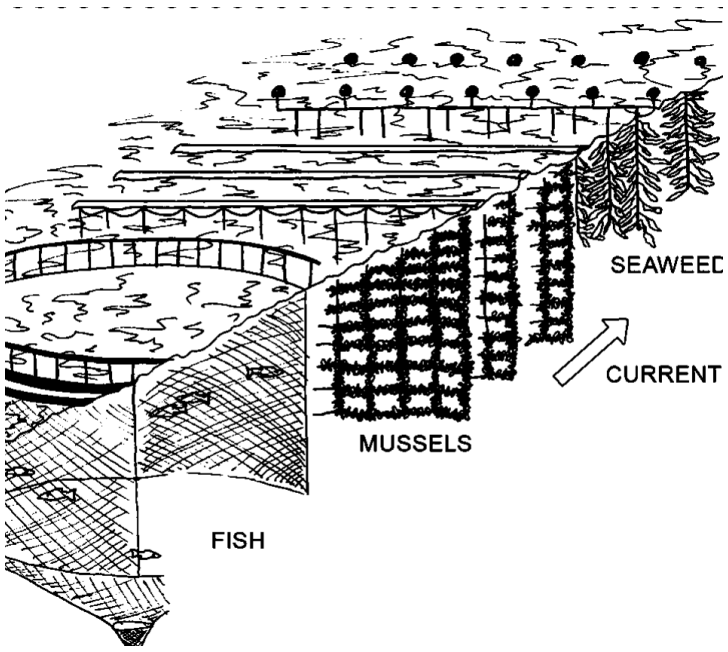

Figure 7 – Integrated multi-trophic aquaculture (IMTA) system (Holdt and Edwards 2014)**.**

I.2 BIOACTIVES AS DIETARY SUPPLEMENTS

During the course of time, humans have explored Nature to provide for their basic needs. Terrestrial plants are in the ontogeny of medicinal systems, with the earliest records dating from around 2600 B.C., documenting the uses of approximately 1000 plant-derived substances (Cragg and Newman 2013). However, more than 70% of our planet surface is constituted by oceans which are recognized as a source of matchless natural products that are mainly accumulated in living organisms (Pandey 2012). Hence, the extensive list of marine organisms being recognized as valuable sources of functional materials, including polyunsaturated fatty acids (PUFA), polysaccharides, natural pigments, essential minerals, vitamins, enzymes and bioactive peptides (Table 3) (Pangestuti and Kim 2011).

Table 3 – Sources of functional materials of marine origin and their correspondent activity.

Source	Activity
Marine Microbes	Enzymes, fungicides, biosensors, antifouling agents, antibiotics, toxins.
Marine Organisms	Immune modulators, U.V. protecting compounds, peptides, biosensors, antifouling agents.
Marine Biowastes	Fish diets, nutraceuticals, tissue-engineering, biochemicals.

Among marine organisms, seaweeds are identified as an under-exploited aquatic plant resource, although they have long been used in diets as well as traditional therapies in Eastern hemisphere. Oceans flora has been intensively studied as a valuable source of bioactive molecules such as marine microorganisms, including certain bacteria, fungi and algae, that produce secondary metabolites which may have some degree of bioactivity: either against another microorganism or acting against certain physiological states of a diseased body (Bhatnagar and Kim 2010). A secondary metabolite is considered a compound that is not directly involved in the development and/or maintenance of the organism and that is usually produced for an ecological interaction, being, therefore, species specific (Amsler 2008). Numerous marine-based compounds have been linked to exogenous biological activities, including prevention and treatment of pathogenesis (D'Orazio *et al.* 2012). Since several species of seaweed produce or contain secondary metabolites with antiviral, antibacterial, anti-inflammatory and antioxidant capacities.

Furthermore, marine resources can be applied as nutraceuticals expanding their applicability in the food and dietary supplements industries. Thus, the use of seaweed as nutraceutical in aquafeeds may confer fish a certain resistance to disease (Gupta and Abu-Ghannam 2011; Mohamed *et al.* 2012). Intensive aquaculture practices subject animals to a variety of stresses such as high density, sub-optimal water conditions and handling. These stressors are responsible for immunosuppression, leading to impaired growth and higher susceptibility to bacterial infections. Thus, currently efforts are being made to produce balanced diets capable of fulfilling the nutritional requirements for fish growth (Peres and Oliva-Teles 2006) while enhancing immune status (Torrecillas *et al.* 2007) and antioxidant capacities of fish (Mourente *et al.* 2005a; Mourente *et al.* 2005b; Tovar-Ramírez *et al.* 2010).

For instance, algal lectin, phlorotannins, sesquiterpenes, bromoditerpenes, and halogenated furanones have been found to affect positive and negative Gram bacteria (Arvinda Swamy 2011). Bioactive peptides isolated from fish protein hydrolysates, as well as algal fucans, galactans and alginates, possess anticoagulant, anticancer and hypocholesterolemic activities

(Kim and Wijesekara 2010; Pihlanto-Leppälä 2000). The action of immunostimulants on both specific and non-specific mechanisms mainly includes the modulation of phagocytosis, antibacterial activity, non-specific cytotoxicity and antibody production (Raa 1996; Robertsen 1999), thus positively affecting the overall immunocompetency and disease resistance of the host. With respect to immunity enhancement and improvement of resistance against infection, polysaccharides, obtained from fungi and seaweeds, are currently used in animal feed supplementation (Sakai 1999). β-glucans from seaweeds appear to stimulate the immune system through the rapid release of reactive oxygen species (ROS) and signaling proteins (Castro *et al.* 2006; Diaz-Rosales *et al.* 2008). The mechanism of action possibly involves an array of cell surface receptors, lectins, scavenger receptors and integrins, which bind β-glucans, triggering an inflammatory response: leukocyte activation followed by cytokines and chemokines production and release (Brown and Gordon 2005). Another group of seaweed-derived immunostimulants includes carrageenan and sodium alginate/alginic acid, which enhance non-specific immunity, increase expression levels of immune-related genes and improve resistance against bacterial challenge (Fujiki and Yano 1997). Seaweeds are also being studied as inhibitors of the excessive production of pro-inflammatory mediators, such as nitric oxide (NO), interleukins (IL-6 and IL-1) and tumor necrosis factor-R (TNF-R) (Dang *et al.* 2008). Fucoxanthin is a carotenoid present in the chloroplasts of brown seaweeds that has been proved to act as a cytokine that selectively induces apoptosis in many tumor cells and is an attractive candidate for antitumor therapies (Jin *et al.* 2018). Phenolic compounds and alginates extracted from brown edible seaweeds were proven to act as potent α-amylase inhibitors, thus potentially retarding glucose liberation from starches and consequently assuaging postprandial hyperglycemia (Zaharudin *et al.* 2018). Polysaccharides from red seaweed *Gracilaria folifera* have interesting functional properties as antioxidant and prebiotic (Vidhya Hindu *et al.* 2017).

ROS scavengers and free radicals main function is to prevent cellular oxidation: mostly consisting in peroxidation of the lipid layers in cell walls, oxidative stress in mitochondria and DNA denaturation in the nucleus. Therefore, under normal conditions, these molecules are eliminated by the animals' antioxidant defense system, which includes enzymes and non-enzymatic factors. Substances such as vitamins C and E, glutathione and carotenoids, together with several enzymes capable of reducing ROS or oxidized products are therefore responsible for maintaining the redox equilibrium. However, under stress or pathological conditions, the equilibrium between the generation and elimination of ROS becomes unbalanced, leading to oxidative stress and cell damage (Je *et al.* 2009). The final products of free radicals action are aldehydes, which inhibit the action of glucose-6-phosphate adenylate cyclase, a membrane enzyme, ultimately causing tissue damage (Borza *et al.* 2013). There is

growing evidence in vertebrates that dietary supplements of natural antioxidant and plant products may mitigate the negative effects of excessive oxidative stress (Gatlin *et al.* 2007; Landete 2013). Such application is relevant during oxidative stress, when a variety of antioxidant systems are usually switched-on to protect against oxidative damage, including up-regulation of the expression levels and activity of key enzymes such as glutathione peroxidase (GPX), and catalase (CAT). In fact, seaweeds were found to contain compounds with a scavenging effect, decreasing ROS formation in animal tissues (Fleurence 1999; MacArtain *et al.* 2007). For instance, phenolic compounds from seaweeds can act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system (Cox *et al.* 2010). Prebiotic effect of *Laminaria* polysaccharides has been shown in the gut metabolism through its effects on mucosal composition, intestinal pH and short chain fatty acids production (Devillé *et al.* 2007).

Despite the possible advantages in using marine bioactives as dietary nutraceuticals, there is little information available of their modulatory effects in marine fish. Regardless, seaweeds have been tested as an alternative protein source in aquafeeds with contradictory growth results (Davies *et al.* 1997; El-Tawil 2010; Mustafa *et al.* 1995; Soler-Vila *et al.* 2009; Valente *et al.* 2006) and relevant efforts have focused on their role as dietary supplement to modulate the overall immune status of farmed fish (Araújo *et al.* 2015; Bansemir *et al.* 2006; Gabrielsen and Austreng 1998).

I.3 Fish and Seaweed Species

European seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*) are species of great importance in the European aquaculture sector. Seabass, together with seabream are the most intensively farmed marine fish in southern Europe. Meagre, on the other hand, has a growing relevance for species diversification in the sector, as Mediterranean aquaculture is mainly established on gilthead seabream and european seabass (Chatzifotis *et al.* 2017).

Dicentrarchus labrax

This perciform fish from the family Moronidae is a carnivore euryhaline (3 – 35 ‰) and eurythermal (5 – 28 °C) species that lives in estuaries, lagoons and estuarine habitats. European seabass is found in the eastern Atlantic Ocean, from the British Isles and southern Norway to Senegal, including the Macaronesia Islands (Madeira, the Canary Islans and Cape Verde). It is present throughout the Mediterranean Sea and in Black Sea coasts (Pollard 2015)

(Figure 8). Depending on water temperature their eggs hatch in 4 to 9 days post-fertilization and the planktonic larvae stage varies from 2 to 3 months (Naciri *et al.* 1999). Larvae can inhabit coastal waters, estuaries or lagoons where they can grow for 2 years. At adulthood, seabass migrates to offshore spawning sites every year (Naciri *et al.* 1999). In their natural environment seabass breed once per year, however in captivity, through photoperiod manipulation it is possible to breed them up to 3 times per year (Kousoulaki *et al.* 2015).

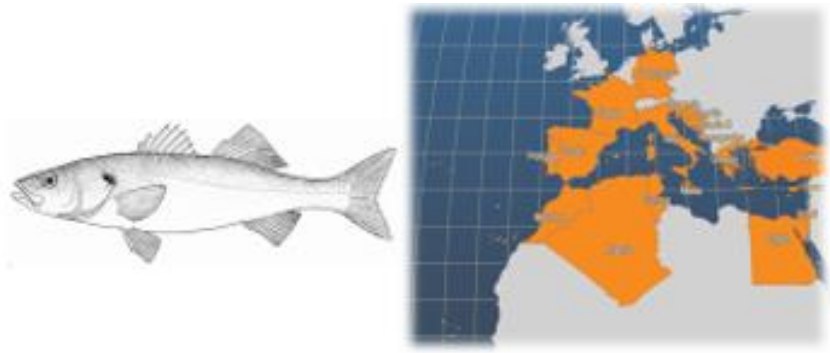

Figure 8 – Illustration and producing countries of European seabass (*Dicentrarchus labrax*) (FAO 2014b).

Seabass aquaculture, albeit one of the most developed (Figure 9), still encounters numerous challenges that affect production efficiency, namely growth performance, feeding efficiency and mortality. These challenges are often linked with feed management issues and biomass losses associated with environmental factors, in addition to disease outbreaks. Research has focused in increasing productivity by improving diet formulations and quality, including formulations that induce higher resistance against pathogens (Kousoulaki *et al.* 2015). The improvement of feed management practices by adjustment of feed delivery to fish appetite fluctuations, or by fine-tuning meals size and frequencies, results in improved feed conversion ratio (FCR) and overall higher growth rates (Kousoulaki *et al.* 2015). Another factor that influences production efficiency is seabass nutritional status, which when precarious, weakens immune-competence and renders seabass more susceptible to disease. These diseases can include *photobacteriosis*, a worldwide septicemic disease caused by *Photobacterium damselae* (Essam *et al.* 2016).

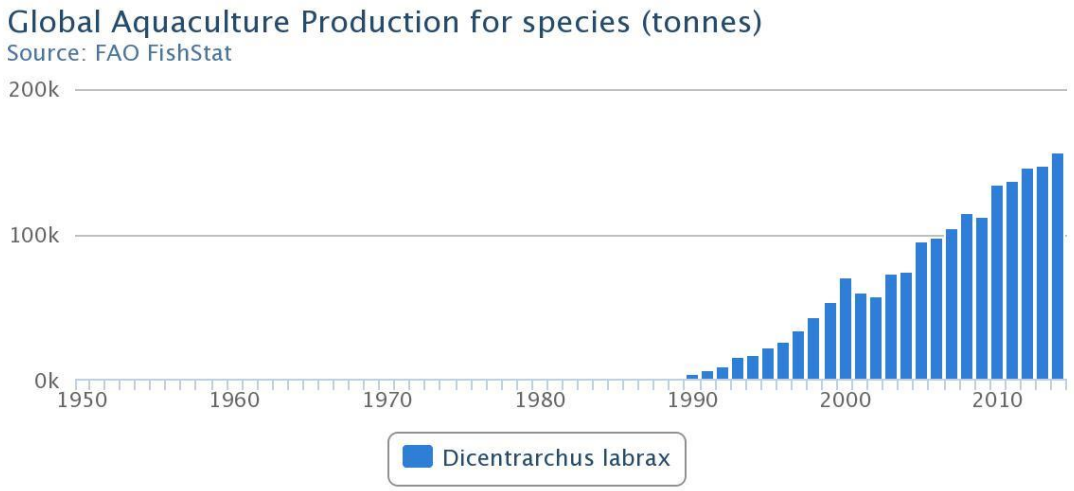

Figure 9 – Global production data regarding European seabass (*Dicentrarchus labrax*) (FAO 2014b).

Argyrosomus regius

Meagre is a perciform fish belonging to the Sciaenidae family that can grow up to 200 cm of total body length and 50 kg of weight in the wild. This species can be found distributed in subtropical waters including estuaries in the Mediterranean Sea, the Atlantic Coast of Europe and North of Africa (Figure 10) (Mylonas *et al.* 2013). The spawning occurs nearby estuaries and/or salt marshes where both very large adults and small-sized juveniles can be observed in specific time windows (González-Quirós *et al.* 2011). Meagre is a euryhaline fish able to adapt to diverse environments, making land-based production possible (Hernández *et al.* 2009).

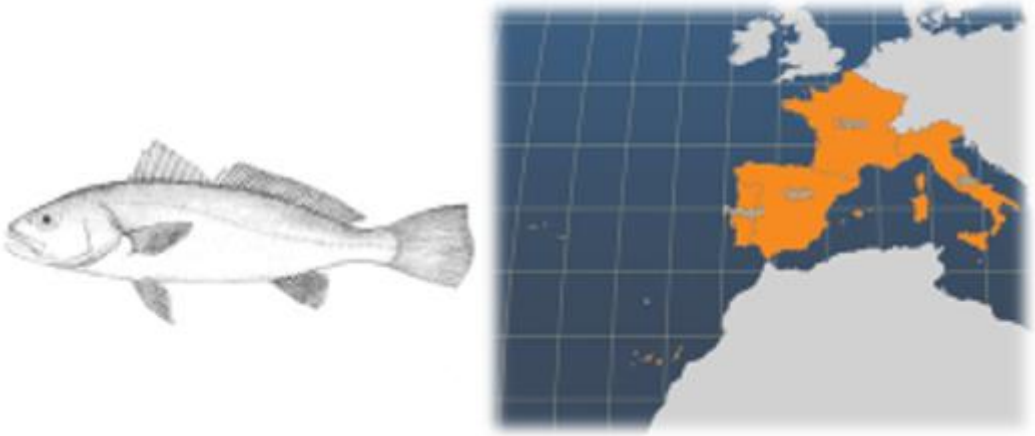

Figure 10 – Illustration and producing countries of meagre (*Argyrosomus regius*) (FAO 2014a).

Meagre aquaculture has been rapidly increasing in the last years (Figure 11), although their reproduction in captivity has presented various limitations and demanding further studies (Mylonas *et al.* 2013). This species thrives in tank captivity exhibiting high growth rates with

good FCR (Hernández *et al.* 2009). One of meagre's most interesting traits, in the context of aquaculture is related with the low amount of fat they accumulate viscerally and within muscle, which is lower than most species including seabass (Hernández *et al.* 2009).

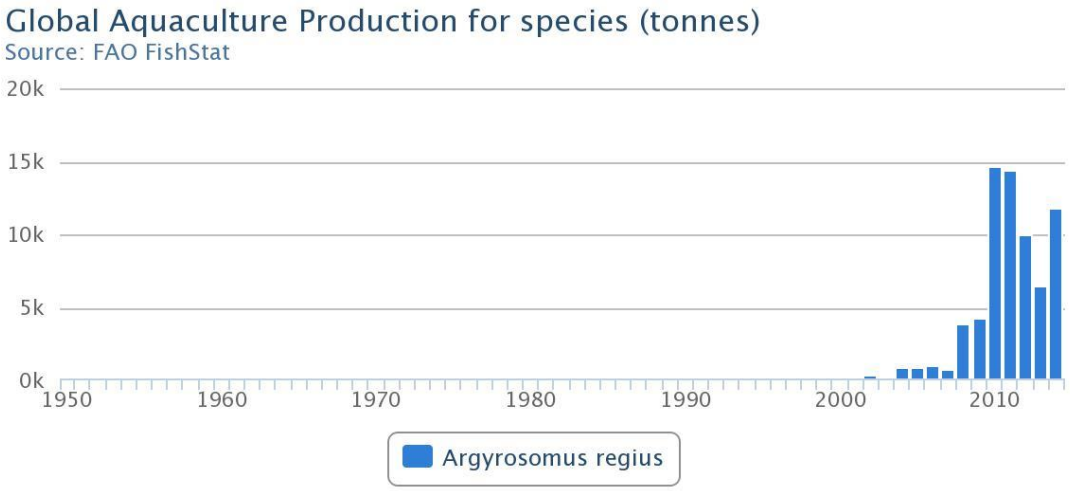

Figure 11 – Global production data regarding meagre (Argyrosomus regius) (FAO 2014a).

Several studies addressed the modulation of aquaculture fish species immune system by dietary changes, including partial replacement of fish oil (Deng *et al.* 2013), or replacement of the yeast supplement using bacteria (Panigrahi *et al.* 2004) or microalgae (Reyes-Becerril *et al.* 2013). These studies propose that dietary changes produce significant innate immune response alterations in marine and freshwater fish, reinforcing the value of immunomodulators with potential benefits for the farming sector. The addition of small quantities of seaweed and/or their extracts in the diet of marine and freshwater fish may increase their resistance to infectious diseases and improve fish welfare.

Alaria sp., *Gracilaria* sp., *Fucus* sp. and *Ulva* sp. are four seaweed species representatives of the three seaweed Phyla (Phaeophyta, Rhodophyta and Chlorophyta) and widely distributed in the Portuguese coast.

Alaria sp.

This perennial seaweed species belongs to the Phaeophyta class and it grows in exposed rocky outcrops often forming a band at low water and in shallow subtidal areas, though it can also be found in rock pools on the lower shore. *Alaria* spp. color can be yellowish, olive-green, olive-brown, yellow-brown or a rich brown (Figure 12). This seaweed has been found to grow fronds up to 5 meters long and 25 centimeters wide, with a seasonal growth rate reaching 20

- 25 centimeters per month. Although usually they are described to have 1 or 2 meters due to the strength of wave battering.

Figure 12 – Photograph and sketch of *Alaria* sp. (In: **http://data.nhm.ac.uk/**).

Alaria sp. has been used for human consumption, being commercialized as whole, flaked, milled, or powdered. It is also highly demanded in alginate production (alginic acid content around 42%), as well as in the cosmetic industry.

This seaweed is rich in calcium, vitamins A, B2, B6, B12, K, iodine and bromine rendering this species important as a nutritional supplement. The high vitamin content in *Alaria* is important in releasing energy from carbohydrates, altering the energetic metabolism. Its vitamins may also play a role in the creation of antibodies in the immune system, help maintain normal nerve function and support the chemical reactions of proteins. However, more recently, *Alaria* was claimed to help cell reproduction, red cell production, stimulate immunity and assist with the formation of some hormones (MACOI 2018).

Gracilaria sp.

Gracilaria sp. is a red seaweed from the phylum Rhodophytae that can be found as loose-lying thalli or attached to small stones and shells (Figure 13). It is a branched cartilaginous seaweed with cylindrical “leaves” up to 50 cm long (MACOI 2018).

Figure 13 – Photograph and sketch of *Gracilaria* sp. (In: http://data.nhm.ac.uk/).

Gracilaria species are important for both industrial and biotechnological purposes due to their phycocolloids content, which is the main source of agar α-(1,4)-3,6-anhydro-l-galactose and β-(1,3)-d-galactose. These seaweeds also produce important bioactive metabolites with antibiotic activity such as acrylic acid and eicosanoids, the precursors of prostaglandins. These relevant molecules, which result from polyunsaturated fatty acid (PUFA) oxidation that originate from arachidonic acid and Eicosapentaenoic acids, are essential for inflammation processes. Lipids are abundant in this genus being mainly prostaglandins, steroids, such as cholesterol and clionasterol (de Almeida *et al.* 2011).

Fucus sp.

This seaweed species is usually around 40 centimeters in length, attached to rocky substrates. Its color ranges from olive green to olive brown, or reddish brown to almost black (Figure 14). *Fucus* is characterized by the small nearly spherical gas–filled vesicles (bladders) which look like bubble wrap and serve to keep the seaweed floating enhancing its light exposure and the ability to photosynthesize. Naturally, it grows gregariously, forming dense mats of long ribbons (MACOI 2018).

Figure 14 – Photograph and sketch of *Fucus* sp. (In: http://data.nhm.ac.uk/).

Fucus sp. has been widely applied and explored by the cosmetic industry. The primary chemical constitution of *Fucus* include mucilage, algin, mannitol, beta-carotene, zeaxanthin, iodine and iodine salts, bromine, potassium, volatile oils and many other minerals, as well as polysaccharides. The main use of *Fucus* in medicine is as an iodine source, essential for the thyroid gland used in the treatment of hypothyroidism and goiter. Its ability as a thyroid stimulant may also help counter obesity by increasing metabolic rate and diminishing hypodermic lipids deposits. Despite the low quantities of iodine present in seaweeds its effects are reinforced through synergetic relationships with carbohydrates, amino acids and mineral salts.

Fucus sp. has also a high sulphate content, ranging from 46.2 to 55.0 %, from which fucoidan can be extracted. This sulphated polysaccharide has been described to have high thrombin inhibitory effects, with broad applications in the medical and pharmacological fields (MACOI 2018).

Ulva sp.

Ulva sp. commonly called sea lettuce, is usually a vivid green, however it can range from light yellowish green to darker green (Figure 15). This seaweed may have a diameter up to 30 centimeters, although usually smaller. It can be found in sheltered bays or in protected and semi-protected areas with limited wave action: in tide pools, rock pools, cobble, boulders, and bedrock in mid- to lower levels of the intertidal zone. *Ulva* tolerates brackish conditions and is present on suitable substrates in estuaries. *Ulva* sp. can be collected year round, but is mostly abundant in summer and fall (MACOI 2018).

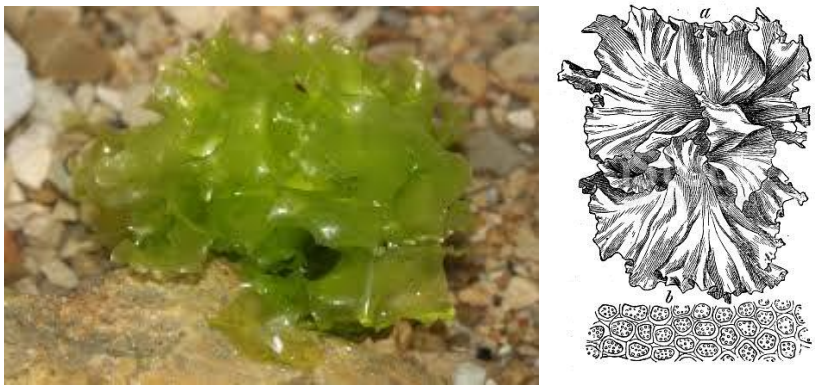

Figure 15 – Photograph and sketch of *Ulva* sp. (In: http://data.nhm.ac.uk/).

Ulva sp. is sold both in fresh and dried forms and is used for human consumption and as key ingredient in many cosmetic and personal care items. It is also a common component in gardening and fertilizer products. *Ulva* sp. is described has having a high concentration of amino acids, such as proline, glycine and lysine, which stimulate the cells in the connective tissues to synthesize collagen. This effect has important repercussions in health but also in the cosmetic industry since it can improve the skin's elasticity in scars and healing tissues but also reducing lines and wrinkles (MACOI 2018).

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OBJECTIVES

OBJECTIVES

This thesis presents the general hypothesis that seaweed supplementation, or their extracts, benefits the overall health of farmed fish, which translates into increased profits. The overall objectives are:

1) Evaluation of combinatorial factors such as dosage, duration, type of seaweed and rearing conditions to validate the effectiveness of dietary seaweed supplementation;
2) Quantification of the effects of seaweeds on antioxidant and immune molecular mechanism at the gene-expression level.

The combination of biochemical and molecular tools aims to provide a better understanding of the physiological foundations of the complex relationship between fish health, growth and environment, and enable a better use of seaweeds in modern aquaculture practices

More specifically, the current PhD study aimed to assess the possible effects of dietary supplementation on fish growth and health under various biotic and abiotic conditions, using European seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*) as animal models. In fact, despite the possible advantages of seaweed use as dietary nutraceuticals, the available information on their modulatory effects in aquaculture fish species is scarce.

In this work I tested four seaweed species representatives of all three seaweed Phyla (Phaeophyta, Rhodophyta and Chlorophyta): *Alaria* sp., *Gracilaria* sp., *Fucus* sp. and *Ulva* sp.. Apart from *Alaria* sp., all species are widely distributed in the Portuguese coast. Both crude and selected bioactive extracts were supplemented to aquafeeds and fish immune responses and physiological base-line conditions, as well as energetic metabolism, were evaluated.

CHAPTER II

Aquaculture Reports 3 (2016) 189–197

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Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (*Dicentrarchus labrax*)

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CHAPTER II

ROLE OF DIETARY SEAWEED SUPPLEMENTATION ON GROWTH PERFORMANCE, DIGESTIVE CAPACITY AND IMMUNE AND STRESS RESPONSIVENESS IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*)

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Abstract

This work aimed to appraise growth performance, digestive capacity and stress responsiveness in European seabass (*Dicentrarchus labrax*) when fed seaweed supplemented diets. A *Ctrl* diet (without supplementation) was tested against 6 practical diets supplemented either with *Gracilaria* spp., *Ulva* spp., or *Fucus* spp., at 2.5 or 7.5 % levels, plus an additional diet with a blend of the three seaweeds, each supplemented at 2.5 % (*Mix*). Seabass juveniles (24.0 ± 6.3 g) were fed the experimental diets for 84 days. Dietary seaweed supplementation had no effect on growth rate (DGI: 1.0-1.1), voluntary feed intake (11.3-12.6 g. kg $^{-1}$ ABW^{-1}. day^{-1}), feed conversion ratio (FCR: 1.2-1.4) and protein efficiency ratio (PER: 1.5-1.8). Lipase activity was significantly higher in fish fed *Mix* diet when compared to *Ctrl*. Glutathione peroxidase (GPx) was affected by seaweeds presence regardless its level of supplementation ($P<0.05$). The innate immune system was significantly altered by dietary seaweed supplementation. Fish fed the 7.5 % seaweed supplemented diets had a significant decrease in the ACH50 level, when compared to fish fed 2.5 % seaweed supplemented diets. Moreover, a combined effect of seaweed and supplementation level significantly affected lysozyme (LYS) activity ($P<0.05$). Fish fed *Ul2.5* diet had an increase in LYS when compared to fish fed *Ctrl* and *Ul7.5* diets. Overall, our results indicate that the use of dietary seaweed supplementation improves immune and antioxidant responses in European in seabass without compromising growth performance.

Keywords: Digestive enzymes, Growth, Innate immunity, Oxidative stress, Seaweeds.

Introduction

Seaweeds are considered a vast source of biologically active substances, especially rich in essential nutrients for human and animal nutrition (Jiménez-Escrig *et al.* 2011). Under *in vitro* conditions, relevant antimicrobial and anti-viral activities (Hemmingson *et al.* 2006; Cox *et al.* 2010; Narasimhan *et al.* 2013), and efficient antioxidant capacity (Leonard *et al.* 2011; Narasimhan *et al.* 2013), have been widely demonstrated in extracts obtained from representative species of Chlorophyta, Rhodophyta and Phaeophyta phyla. *In vivo* studies carried out in swine, demonstrated that the dietary supplementation with *Laminaria* sp. (*Phaeophyta)* extracts, containing laminarin and fucoidan (polysaccharides), improved gastrointestinal health and growth performance of starter and weaned pigs (Reilly *et al.* 2008; Leonard *et al.* 2011). In ruminants subjected to pre-slaughter stress conditions, the dietary supplementation with brown seaweed (*Ascophyllum nodosum)* increased the antioxidant status by lowering lipid peroxidation (LPO), increasing glutathione peroxidase activity, and reducing stress indicators (Kannan *et al.* 2007; Archer 2005). In crustaceans, extracts and sulphated galactans from red seaweeds (*Gracilaria fisheri* and *Asparagopsis* spp.) administered to black tiger shrimp *Penaeous monodon* also caused a significant immune-stimulant effect and an increased protection against the pathogen *Vibrio* spp*.* and white spot syndrome virus (Kanjana *et al.* 2011; Wongprasert *et al.* 2014; Rudtanatip *et al.* 2015; Manilal *et al.* 2012). Additional research efforts are undoubtedly required to carry out *in vivo* studies, which are scarce to date, that accurately evaluate the potential of seaweed application as a nutritional tool (Makkar *et al.* 2015), especially useful to industries with high economic impact.

The utilization of immunostimulant functional ingredients, such as meals, extracts and isolated compounds, may unfold be particularly appropriate in aquaculture. This successful food production activity is facing a major challenge supplying quality fish food to an exponentially increasing world population (FAO 2014).

Notwithstanding, the dietary seaweed incorporation in aquafeeds for finfish has shown contradictory growth results (El-Tawil 2010; Mustafa *et al.* 1995; Davies *et al.* 1997; Valente *et al.* 2006; Soler-Vila *et al.* 2009). *In vitro* studies have reported an increased respiratory burst and immune system stimulation, through rapid release of reactive oxygen species (ROS) and signaling proteins, by *Ulva rigida* and *Chondrus crispus* extracts and β-glucans in turbot (*Scophthalmus maximus*) and Atlantic salmon (*Salmo salar* L.) phagocytes (Dalmo and Seljelid 1995; Castro *et al.* 2004). Besides immunocompetency, a positive correlation has been reported between the phenolic content and antioxidant capacity of seaweeds (Devi *et al.* 2011), using strategies as ROS

scavenging activity or lipid peroxidation inhibition (Heo *et al.* 2005). Orange-spotted grouper (*Epinephelus coicoides*), previously inoculated with sodium alginate from *Macrocystis pyritera* and carrageenan from *Chondrus crispus* showed an increase in respiratory burst, superoxide dismutase (SOD) and phagocytic activities after exposition to *Vibrio alginolyticus* (Cheng *et al.* 2007). Recent results observed in Nile tilapia (*Oreochromis mossambicus*) have demonstrated that the administration of extracts and products from red (*Gracilaria folifera*) and brown (*Padina gymnospora* and *Sargassum cinereum*) seaweeds may be effective as therapeutic and prophylactic treatments against *Pseudomonas* spp. infection (Thanigaivel *et al.* 2015a; Thanigaivel *et al.* 2015b). Practical studies assessing if dietary seaweed incorporation contributes to enhance the basal health status of cultivated fish are, therefore, highly recommended, since stocking and harvesting routine practices such as crowding, size sorting and transport, constitute usual stressors in fish farming.

The objective of this study was to examine the effect of dietary seaweed supplementation on growth performance, digestive capacity and stress responsiveness, using seabass (*Dicentrarchus labrax*) as experimental model. Specifically, we tested the hypothesis that seaweeds could be used to enhance immune and antioxidant defences in fish, without growth impairments.

Material and Methods

The current study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

Seaweeds and experimental diets

Seaweed from 3 distinct phyla, *Gracilaria* spp. (Rhodophyta), *Ulva* spp. (Chlorophyta) and *Fucus* spp. (Phaeophyta) were cultivated in an integrated multi-trophic aquaculture (IMTA) system and supplied by ALGAPLUS (Portugal). The 3 seaweeds were supplemented separately to the experimental diets at two levels, 2.5% and 7.5 % dry matter basis (DM). Two additional diets were formulated, one control diet without seaweed supplementation (*Ctrl*) and the other (*Mix*) with all three seaweeds, each at 2.5 % level of supplementation, dry matter basis (DM). Proximate and mineral compositions of the seaweeds are depicted in Table 1.

Table 1 Proximate and mineral composition of the used seaweed meals.

	Limit Detection (LD)	*Gracilaria* spp.		*Fucus* spp.		*Ulva* spp.	
Mineral comp. (mg. kg^{-1} dry tissue)		Mean	SD	Mean	SD	Mean	SD
Lead (Pb)	0.004	1.1	0.1	0.8	0.1	2.8	0.1
Mercury (Hg)	0.005	< LD		< LD		< LD	
Cadmium (Cd)	0.031	0.2	0.0	0.1	0.0	0.1	0.0
Tin (Sn)	0.005	0.4	0.0	0.9	0.2	0.5	0.0
Arsenic (As)	0.074	< LD		< LD		< LD	
Iron (Fe)	1.021	511.0	31.0	560.0	19.0	4080.0	246.0
Zinc (Zn)	1.908	43.0	1.0	109.0	2.0	47.0	1.0
Copper (Cu)	0.206	1.5	0.1	2.8	0.2	5.0	0.1
Selenium (Se)	0.832	6.5	0.3	8.8	0.5	9.8	0.3
Mineral comp. (%)							
Potassium (k)	0.034	13.1	0.5	11.3	0.5	9.3	2.0
Sodium (Na)	0.025	11.7	1.6	3.3	0.1	19.9	1.1
Magnesium (Mg)	0.001	0.3	0.0	0.4	0.0	2.0	0.2
Calcium (Ca)	0.002	0.2	0.0	0.9	0.1	0.4	0.0
Phosphorus (P)	0.006	0.3	0.1	0.2	0.0	0.4	0.1
Proximate composition (%DM)							
Dry matter		93.4	0.0	87.0	0.0	85.7	0.0
Crude protein		25.9	0.2	17.2	0.2	23.2	0.2
Ash		34.3	0.2	20.7	0.1	34.8	0.3
Crude fat		1.1	0.7	3.4	0.1	1.5	0.1
Gross energy (kJ. g^{-1} DM)		12.8	0.2	15.1	0.0	12.1	0.1

The 8 experimental diets were formulated to be isoenergetic (22 kJ. g^{-1} DM), isoproteic (47 % DM) and isolipidic (18 % DM) (Table 2). All diets were formulated with the same practical ingredients. Marine-derived ingredients (fishmeal and fish oil) represented 36.5% DM of the diet, whereas dietary protein and fat levels were chosen in accordance with recommendations for juvenile seabass (Webster and Lim 2002; FAO 2005-2015). All diets were supplemented with crystalline indispensable amino acids (L-Lysine and DL-Methionine) to meet this species requirement. The dietary macronutrient balance after seaweed incorporation was achieved by adjusting the soy protein concentrate and wheat meal contents. All ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 2.0 mm pellet size, SPAROS, Portugal). Diets were finally dried at 45 ºC for 12 h and stored at 4ºC until used.

Table 2 Feed formulation and proximate composition of the experimental diets.

	Dietary treatments							
	Ctrl	Gr2.5	Gr7.5	Ul2.5	Ul7.5	Fu2.5	F7.5	Mix 7.5
Fish ingredients (%DM)								
Fishmeal Standard	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Fishmeal SOLOR	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	11.3	10.0	11.5	10.4	11.5	11.0	10.3
Wheat gluten	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn gluten	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Soybean meal 48	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Rapeseed meal	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Wheat meal	9.0	7.0	3.3	6.8	2.9	6.8	2.3	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Fish oil - COPPENS	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Soybean oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Rapeseed oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vit & Min Premix PV01	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder (Kieselghur)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
MCP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Lysine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
DL-Methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gracilaria sp.		2.5	7.5					2.5
Ulva sp.				2.5	7.5			2.5
Fucus sp.						2.5	7.5	2.5
Proximate composition (%DM)								
Dry matter	94.7	96.5	95.9	94.3	94.2	94.5	95.0	94.8
Ash	8.6	9.4	10.8	9.5	11.1	8.9	10.0	10.6
Crude protein	47.8	47.4	47.8	47.8	47.7	47.7	47.9	47.9
Crude fat	19.1	18.1	19.6	19.5	19.1	18.1	18.4	19.3
Gross energy (kJ. g^{-1} DM)	22.7	22.8	22.4	22.7	22.3	22.9	22.7	22.4

Fish and rearing conditions

The growth trial was conducted at the Aquatic Engineering laboratory of ICBAS (Porto, Portugal). Seabass juveniles were supplied by a commercial fish farm (MARESA, Spain). After arrival at the experimental unit, fish were kept under quarantine conditions for a two-week period. Thereafter, fish were individually weighed (initial body weight: 24.0 ± 6.3 g) and distributed to the 24 units of 80 L tanks (3 tanks/treatment; 17 fish/tank) connected to a closed recirculation seawater system. Total ammonium (≤ 0.5 mg. L^{-1}), nitrate (≤ 3 mg. L^{-1}) and pH levels (≈8) were measured during the entire trial to ensure levels within the recommended ranges for marine species. A photoperiod was set for 12 h light:12 h dark and the flow rate in each tank set for 90 L/h. Fish were hand-fed to apparent satiety twice a day (10.00 and 16.00 h) for 84 days.

At the end of the feeding trial, fish were fasted for 24 h, prior to sampling. Fish were anesthetized with 100 mg. L^{-1} of MS-222 (Sigma-Aldrich Co. LLC, Bellfonte, USA) and

individually weighed (g). Thereafter, fish were sacrificed by decapitation and body tissues were collected. Liver and viscera were weighted from 4 fish per tank to calculate the hepatosomatic (HIS) and viscerosomatic (VSI) indexes. Other 4 fish from each tank were used for whole body composition analyses. Additionally, 6 fish were sampled from each tank for the analyses to be carried out in the intestine, blood and liver. Plasma was obtained by blood centrifugation (5 min, 10000 rpm). All samples were stored at -80°C until subsequent analysis.

Analytical methods

Carcasses from 4 fish from each tank were milled, pooled and the moisture content determined (105 ºC for 24 h). Samples were subsequently lyophilized before further analyses. Both feed and whole-body analyses were carried out in duplicate following the methodology described by AOAC (2006). Ash was analyzed by combustion (500 ºC during 6 h) in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany) and crude protein (N×6.25) using a Leco N analyser (Model FP-528, Leco Corporation, St. Joseph, USA). Crude lipid content was determined by petroleum ether extraction (40-60 ºC) using a Soxtec™ 2055 Fat Extraction System (Foss, Hilleroed, Denmark) while gross energy was quantified in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Staufen, Germany).

Whole intestines were homogeneized for digestive enzymes activity quantification according to the method described by Rungruangsak-Torrissen (2007). Lipase activity was determined according to Winkler and Stuckmann (1979). Trypsin and chymotrypsin were assessed through the production of nitroaniline (Rungruangsak-Torrissen and Sundby 2000). α-Amilase activity was examined by formation of the disacharide maltose (Bernfeld 1951). In adittion, the ratios amilase to trypsin (A/T) and trypsin to chymotripsin (T/C) where calculated to infer about preferable energy source and growth rates, respectively. According to Hidalgo *et al.* (1999), an elevated A/T ratio can translate into higher capacity to digest carbohydrates, sparing protein for growth. The ratio T/C has been associated with feed convertion efficiency and specific growth rate, and can indicate if fish are in a fast or slow growth phase (Rungruangsak-Torrissen *et al.* 2006). The protein content (Lowry *et al.* 1951) of the homogenate was analized to establish results in terms of enzimatic units per protein unit.

Innate immune response was evaluated in plasma by determination of alternative complement (ACH50) and lysozyme activities, as well as peroxidase content. The ACH50 activity followed the protocol described by Sunyer and Tort (1995) and was

defined as the reciprocal of the serum dilution capable of inducing 50% lysis of rabbit erythrocytes. Lysozyme activity was evaluated by turbidimetric assay (Ellis 1990) of the *Micrococus lysodeikicus* lysis and later standardization by hen egg white lysozyme (Sigma, Portugal) reaction curve. Peroxidase levels were examined as the chemical reduction of 3,3', 5,5' – tetramethylbenzidine hydrochloride (Sigma, Portugal), according to Quade and Roth (1997).

Livers were homogeneized (phosphate buffer, 0.1 M pH 7.4) as a previous step to measure indicators related to oxidative status. The protein content was determined according to Bradford (1976) and used to standardize antioxidant enzymes activities. Lipid peroxidation was determined by quantifying the presence of thiobarbituric acid reactive substances (Ohkawa *et al.* 1979). Catalase (EC 1.11.1.6.) activity was studied based on Clairborne (1985), with hydrogen peroxyde (30%) as substrate. Glutathione s-transferase (GST) (EC 2.5.1.18) was determined by absorbance at 340 nm, using 1-chloro-2,4-dinitrobenzene as substrate, consistent with the methods described in Habig *et al.* (1974). Glutathione peroxidase (Gpx) (EC 1.11.1.9.) and Glutathione reductase (GR) (EC 1.8.1.7) were evaluated based on NADPH (Sigma, Portugal) oxidation at 340 nm (Cribb *et al.* 1989; Mohandas *et al.* 1984). Total and oxidized Glutathione were evaluated at 412 nm by the formation of 5-thio-2-nitrobenzoic acid, as detailed in Baker *et al.* (1990).

Growth performance calculations

Daily growth index (DGI) was calculated as: $100 \times [(FBW)^{1/3} – (IBW)^{1/3}] \times$ days, where IBW and FBW are the initial and the final average body weights (g). Feed conversion ratio (FCR) was calculated as dry feed intake, FI (g) × weight gain (g) while protein efficiency ratio (PER) considered: weight gain (g) × crude protein ingested (g). The hepatosomatic and viscerosomatic index (HSI and VSI) were calculated as: HSI = 100 × [liver weight (g)/fish weight (g)]; VSI = 100 × [viscera weight (g) /fish weight (g)]. Voluntary feed intake (VFI) (g. kg ABW^{-1} day^{-1}) was calculated as: VFI = 100 × [feed intake (g) / ABW (kg) / trial duration (days)], where ABW (average body weight) is (IBW + FBW) / 2.

Statistical analyses

All data were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test). The analysis of variance was performed applying two-way ANOVA test, with seaweed (SW) (*Gr*, *Ul*, *Fu* and *Mix*) and concentration (C) (2.5 and 7.5 %) as

independent factors, with an unsupplemented diet as control treatment. Significant differences were considered when $P<0.05$. The statistic software package used was SPSS Statistics 21 (IBM Corp., Armonk, NY, USA).

Results

Growth performance, feed intake and whole-body composition

Fish mortality was lower than 1% in all experimental treatments. All dietary treatments exhibited 2.2 fold increase in their initial body weights (24 ± 6.3g) after 12 weeks of feeding trial. No significant differences were observed on growth (DGI range of 1.0-1.1), VFI (11.3-12.6 g. kg ABW^{-1}. day^{-1}), FCR (1.2-1.4) and PER (1.5-1.8), among dietary treatments (Table 3). Both HIS, VFI and body composition did not vary among the dietary treatments ($P>0.05$) (Table 4). Especially relevant was the fact that whole body composition, evaluated in terms of macronutrients and energy, also showed no significant differences between dietary treatments (Table 4).

Table 3 Growth performance, feed utilization and feed intake of sea bass fed the experimental period for 84 days. Corresponding P values to seaweed (S) and concentration (C) factors, as same as interaction S x C, are exhibited for every parameter.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
Growth	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Initial Body Weight (g)	23.5	1.2	23.6	1.2	24.5	1.2	24.5	0.9	23.2	1.1	24.3	1.7	24.4	1.2	23.9	1.9	0.502	0.679	0.971
Final Body Weight (g)	51.7	3.5	55.5	3.9	58.2	4.3	54.8	0.8	53.9	5.7	55.3	1.3	51.9	3.6	53.1	6.1	0.554	0.217	0.953
DGI	1.0	0.1	1.1	0.1	1.2	0.1	1.1	0.0	1.1	0.1	1.1	0.1	1.0	0.1	1.1	0.1	0.270	0.215	0.868
FCR	1.4	0.1	1.3	0.1	1.2	0.1	1.3	0.0	1.3	0.2	1.2	0.1	1.4	0.2	1.3	0.1	0.380	0.29	0.841
PER	1.5	0.1	1.7	0.1	1.8	0.1	1.7	0.0	1.6	0.2	1.7	0.1	1.5	0.2	1.6	0.2	0.319	0.290	0.869
Intake (g. kg ABW^{-1}. day $^{-1}$)																			
DM	12.6	0.4	12.0	0.6	11.5	0.4	11.6	0.3	12.3	0.8	11.3	0.6	11.7	0.7	11.7	0.8	0.213	0.771	0.696

N = 3 tanks. Absence of letters indicates non-significant differences between treatments (P≥0.05) for every studied factor and subsequent interaction
DGI, Daily growth index = 100 x ((Final body weight)$^{1/3}$ - (Initial body weight)$^{1/3}$) / days
FCR, Feed conversion ratio = dry feed intake / weight gain
PER, Protein efficiency ratio = weight gain / crude protein intake
ABW, Average body weight
DM, Dry matter

Table 4 Somatic index and whole-body composition of sea bass fed the experimental diets for 84 days. Corresponding P values to seaweed (S) and concentration (C) factors, as same as interaction S x C, are exhibited for every parameter.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
Somatic index (%)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
HSI	1.2	0.1	1.5	0.2	1.5	0.0	1.4	0.1	1.5	0.1	1.4	0.1	1.4	0.1	1.5	0.3	0.675	0.437	0.651
VSI	7.9	0.4	8.2	0.4	8.4	0.7	8.6	0.6	8.6	0.3	8.1	0.6	8.2	0.4	8.0	1.1	0.852	0.730	0.567
Whole body composition (%WW)																			
DM	31.4	0.3	32.3	3.6	31.3	0.5	32.0	1.1	31.2	0.7	31.8	1.9	31.9	1.4	31.8	3.2	0.987	0.791	0.777
Ash	4.8	0.1	4.1	0.4	4.4	0.8	4.3	0.6	4.3	0.7	4.9	0.7	4.7	0.2	4.5	0.5	0.645	0.212	0.943
Protein	18.0	0.3	18.0	1.6	17.7	0.7	17.9	0.6	16.9	1.0	17.8	1.1	18.1	1.0	17.7	0.6	0.780	0.531	0.405
Fat	9.7	0.5	10.6	2.1	9.4	0.6	10.3	0.4	9.7	0.9	9.2	1.4	9.1	0.5	9.8	2.7	0.755	0.269	0.794
Energy (kJ. g^{-1} WW)	7.8	0.3	8.1	1.2	7.6	0.1	8.1	0.2	7.5	0.1	7.7	0.8	7.6	0.5	7.7	1.7	0.957	0.360	0.654

N = 3 tanks. Absence of letters indicates non-significant differences between treatments (P≥0.05) for every studied factor and subsequent interaction
HSI, Hepatosomatic index
VSI, Viscerosomatic index
WW, Wet weight

Digestive enzyme activities

Amylase (1.0-1.6 U. mg protein^{-1}), chymotrypsin (4.1-6.5 U. mg protein^{-1}), trypsin (10.0-14.8 U. mg protein^{-1}) activities and the ratios amylase/trypsin (111.0-221.6) and trypsin/chymotrypsin (2.0-3.5) are reported in Table 5. Digestive enzyme activities were not significantly among the dietary treatments ($P>0.05$). Fish fed *Ulva* spp. had lower amylase activity (Fig. 1) among all treatments, significantly different from fish fed *Mix* diet ($P<0.05$). The interaction between seaweed type and incorporation level was not observed ($P>0.05$).

Table 5 Digestive enzyme activities in intestines of sea bass fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Amylase (U. mg protein^{-1})	1.2	0.1	1.5	0.3	1.4	0.4	1.6	0.0	1.4	0.3	1.0	0.1	1.5	0.1	1.3	0.2	0.179	0.100	0.416
Chymotrypsin (U. mg protein^{-1})	4.4	2.1	4.1	1.4	4.2	0.6	4.2	1.4	5.5	2.0	5.6	0.6	6.5	3.2	4.2	1.6	0.569	0.065	0.880
Trypsin (U. mg protein^{-1})	14.8	4.5	12.5	6.3	10.0	5.5	13.5	7.2	11.4	6.3	12.8	5.8	10.0	1.3	11.3	11.2	0.999	0.853	0.698
A/T	119.7	50.5	156.5	92.9	195.6	52.4	221.6	116.4	172.3	106.0	111.0	68.4	199.0	25.0	150.6	75.2	0.625	0.424	0.550
T/C	3.5	1.7	3.0	1.0	2.4	1.2	2.9	0.6	2.2	1.0	2.5	0.6	2.0	0.6	2.8	1.6	0.890	0.317	0.707

N = 6 intestines/treatment. Values presented as Mean ± Standard Deviation

Absence of letters indicates non-significant differences between treatments (P≥0.05) for every studied factor and subsequent interaction

A, Amylase

T, Trypsin

C, Chymotripsin

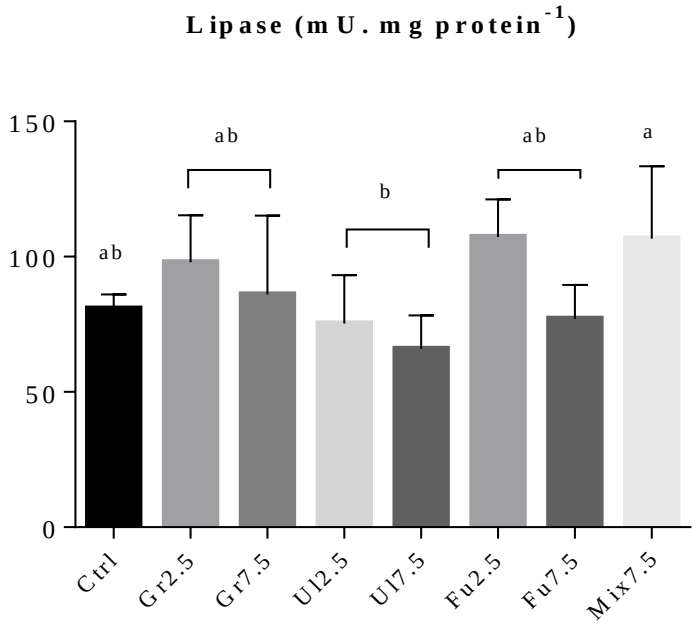


Fig. 1 Lipase activity measured in intestines of sea bass fed the experimental diets for 84 days. Different letters indicate significant differences for the presence of seaweed, despite the level of supplementation.

Antioxidant status indicators

Seabass juveniles fed the experimental diets exhibited similar (P>0.05) levels of hepatic catalase (CAT) and lipid peroxidation (LPO) as presented in Table 6.

Glutathione s-transferase (GST), total glutathione (GT), glutathione reductase (GR) and oxidized glutathione (GSSG) are shown in Table 7. Both seaweed type and supplementation level revealed no differences for GST, GT, GR and GSSG (P>0.05).

In contrast, glutathione peroxidase (GPx) activity (Fig. 2) was significantly higher in fish fed *Gracilaria* spp., *Ulva* spp. and *Mix*, when compared to the other dietary treatments (P<0.05).

Table 6 Catalase activity and lipid peroxidation determined in the liver of sea bass fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Catalase (µmol,min^{-1}.mg protein^{-1})	89.5	14.2	86.8	7.8	89.8	6.2	90.2	6.2	91.5	4.9	98.5	3.9	81.1	17.7	86.7	7.8	0.471	0.761	0.278
LPO (nmol TBARS. g tissue^{-1})	41.5	28.5	43.2	10.6	59.3	20.3	52.6	8.0	22.7	2.6	36.8	26.6	44.1	28.2	32.7	18.2	0.528	0.089	0.809

N = 6 livers/treatment. Values presented as Mean ± Standard Deviation
Absence of letters indicates non-significant differences between treatments (P≥0.05) for every studied factor and subsequent interaction

Table 7 Antioxidant enzyme activities (nmol. min^{-1}. mg protein^{-1}) measured in liver of fish fed the experimental diets for 84 days.

	Dietary treatments																P value		
	Ctrl		Gr2.5		Ul2.5		Fu2.5		Gr7.5		Ul7.5		Fu7.5		Mix7.5		S	C	S x C
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
GST	44.5	13.4	50.1	5.9	51.9	1.8	51.3	8.2	52.0	11.6	60.8	13.3	53.5	17.7	54.2	7.9	0.855	0.416	0.826
GT	8.3	2.4	15.8	3.7	13.2	3.5	12.5	3.9	8.3	1.6	16.5	4.6	9.8	2.4	13.6	7.4	0.381	0.244	0.097
GR	56.1	3.3	56.0	6.4	31.9	9.6	56.2	10.0	52.5	5.0	50.1	14.9	45.0	16.2	45.0	10.4	0.176	0.812	0.066
GSSG	4.8	0.7	5.3	0.8	3.2	0.3	5.1	1.5	3.4	0.5	5.0	0.5	3.1	0.3	6.1	3.9	0.240	0.353	0.092

N = 6 livers/treatment. Values presented as Mean ± Standard Deviation
Absence of letters indicates non-significant differences between treatments (P≥0.05) for every studied factor and subsequent interaction

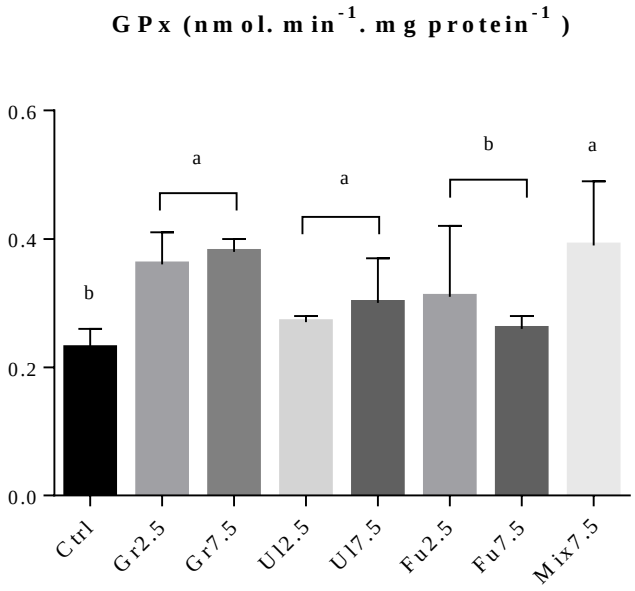
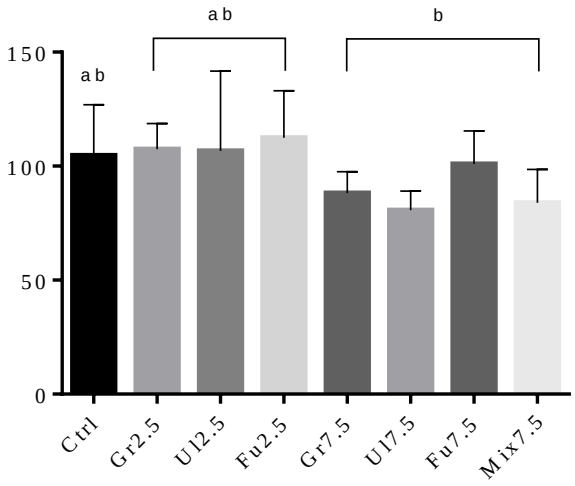


Fig. 2 Glutathione peroxidase (GPx) activity measured in liver of sea bass fed the experimental diets for 84 days. Different letters indicate significant differences for the presence of seaweed, despite the level of supplementation.

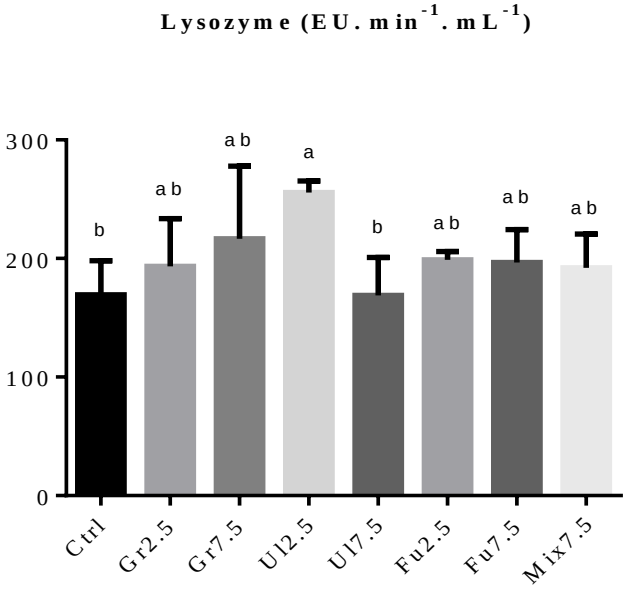
Innate immune parameters

Fish fed dietary seaweed supplementation at 7.5 % DM incorporation level showed a decrease in alternative complement pathway (ACH50) when compared to the other dietary treatments (Fig. 3a). A combined effect caused by both seaweed type and level of supplementation had a significant effect on lysozyme activity in the case of diets containing *Ulva* spp. (Fig. 3b). Fish fed *Ul2.5* diet showed a significantly higher lysozyme activity (255.6 EU. min^{-1}. mL^{1}) in comparison with those fed the *Ctrl* (169.4 EU. min^{-1}. mL^{-1}) and *Ul7.5* diets (168.9 EU. min^{-1}. mL^{-1}). Plasma peroxidase level (Fig. 3c) showed no significant differences (P >0.05) between dietary treatments.

a



b



c

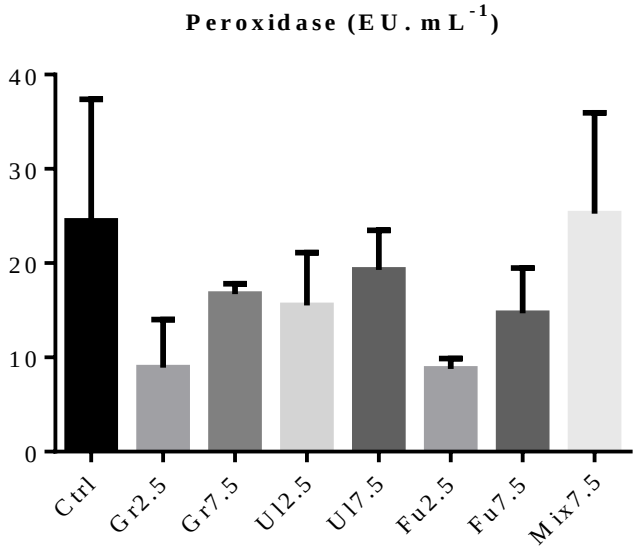


Fig.3 Alternative complement pathway (a), lysozyme activity (b) and peroxidase level (c) determined in plasma of sea bass fed the experimental diets for 84 days. In Fig. 3 (a), different letters are an indication of statistical differences between levels of supplementation. In Fig. 3 (b), significant differences were found for the interaction of factors presence of the seaweed and level of supplementation.

Discussion

In the present work, the dietary supplementation of *Gracilaria* spp., *Ulva* spp., and *Fucus* spp., at 2.5 and 7.5 % levels, in diets for European seabass had no effect on final body weight, DGI, FCR and PER (Table 3). The results from growth performance and feed utilization are in agreement with previous data reported for seabass juveniles fed to satiety with diets containing similar protein (47 %) and lipid (16-18 %) levels (Guroy 2006; Bonaldo *et al.* 2010). Previous studies showed that dietary supplementation of *Gracilaria* spp*.* and *Ulva* spp*.* were an adequate protein source at 10 % inclusion level (Valente *et al.* 2006). Similarly, Queiroz *et al.* (2014) and Linares *et al.* (2014) working with gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), respectively, did not observe changes on growth performance when fish were fed diets supplemented with seaweed. Marinho *et al.* (2013) established a 10 % maximal level of *Ulva* spp. supplementation for Nile tilapia (*Oreochromis niloticus*). In contrast, Silva *et al.* (2015) and Valente *et al.* (2015) working with rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), established a 5 % inclusion of *Gracilaria* spp*.* as the recommended dietary supplementatin level.

Amylase, trypsin and chymotrypsin (Table 5) revealed no influence from the dietary treatments. Despite the high variability reported for alkaline enzymes activity, our results are within the reported range for marine species (Hidalgo *et al.* 1999; Frouël *et al.* 2008). Lipase activity (Fig. 1) however, showed a decrease in the *Ul2.5* and *Ul7.5* treatments. In literature, lipid source and level of inclusion in diets are the most reported modulators of lipase activity (Infante and Cahu 1999). The observed *Ulva* spp. negative effect over lipase activity found in this work is in accordance with the reported lipase inhibiting proprieties found for Chlorophyta seaweeds (Bitou *et al.* 1999). Moreover, Francis *et al.* (2001) reported the presence of antinutrients in seaweeds, particularly lectins (polyphenolic functional group) that interfer with digestion and feed utilization processes. Nevertheless, this eventual antinutritional effect associated to *Ulva* spp., is not so severe that causes growth impairment, and is apparently compensated by the presence of *Gracilaria* spp*.* and *Fucus* spp*.* in *Mix* diet. Albeit, Valente *et al.* (2006) found no negative consequences on apparent digestibility coefficient with the inclusion of *Gracilaria* spp*.* and *Ulva* spp*.* up to 10 % DM. To our knowledge, *Dicentrarchus labrax* and seaweed supplemented diets have not been previously studied in the digestive enzymes perspective.

Glutathione peroxidase activity (GPx) revealed significant differences between treatments. Results showed *Gr2.5*, *Gr7.5* and *Mix* diet to have higher GPx activity than all other treatments. However, the similarity between *Gr2.5* and the *Gr7.5* diets, shows

a lack of dose-response effect. Moreover, the higher GPx activity level in *Mix* diet supports the theory that a 2.5 % *Gracilaria* spp. inclusion level is sufficient to promote GPx activity. Despite the extensive work on seaweed antioxidant properties (Vadlapudi and Kaladhar 2012), experiments tackling the potential effects of dietary seaweed supplementation on the oxidative stress condition in fish are scarce. However, red seaweeds as *Gracilaria* spp. contain high levels of antioxidants and represent a good scourse of selenium (Devi *et al.* 2011). Therefore, it is plausible that *Gracilaria* spp. influence over GPx activity was due to a selenium increment. This result has been reported in other studies with *Dicentrarchus labrax* (Martínez-Páramo *et al.* 2014). The deficiency of selenium is also known to jeopardise GPx activity (Heisinger and Dawson 1983), since GPx molecule requires selenium not for maintenance purposes, but for actively protecting cells (Rotruck *et al.* 1973).

Cell detoxification from the nocive action caused by reactive oxygen species, ROS, is the primary function of the antioxidant systems (Urso and Clarkson 2003). An accumulation of these molecules may naturally arise from celular metabolism via mitochondrial respiratory chain (Fulle *et al.* 2004). This accumulation leads to an oxidative imbalance (Ben Ameur *et al.* 2012) that may result in cell membrane lipid peroxidation (LPO) (Lesser 2006; Ran *et al.* 2007). In the present study, the similarities found for LPO between dietary treatments (Table 6) and the remaining detoxifying enzymes (Table 7), however, further support the evidences that dietary seaweed supplementation causes no nutritional stress to European seabass.

In the present work, the alternative complement pathway results (Fig. 3a) were modulated by the level of seaweed supplementation. A decrease in this parameter was detected when seabass were fed diets with 7.5 % DM supplementation level. ACH50 modulating factors are well established, and include stress conditions, temperature, nutritional deficiencies and additives (Boshra *et al.* 2006; Montero *et al.* 1998). Furthermore, ACH50 activity is interpreted by numerous authors as a sign of a more promp innate immune system, improving the resistance to pathogens (Chiu *et al.* 2008; Biller-Takahashi *et al.* 2012). However, in our study the 7.5 % supplementation appears to jeopardise seabass immune condition. These results are similar to Araújo *et al.* (2015) who reported a decrease in ACH50 in rainbow trout fed 10 % *Gracilaria* spp. supplemented diet. The reason may rely in the antioxidant import carried by seaweed inclusion. Ortuno *et al.* (2000) showed that an excessive inclusion of Vitamine E reduced ACH50 activity in gilthead seabream (*Sparus aurata*), suggesting also that an unbalaced dose of antioxidants may be the cause for ACH50 decrease. As mentioned before, seaweed represent a good source of antioxidants, and the 7.5 % DM may represent an unbalacing point for the correct dose of antioxidants in seabass diet. The role of the

complement system in fish immunity is associated with chemotaxis, opsonisation and pathogen destruction functions (Holland and Lambris 2002). Several compounds such as glucans, tocopherol and ascorbic acid are recognized as potential complement activity enhancers (Bagni *et al.* 2000). Previous works have stated the presence of such compounds in seaweeds (Leiro *et al.* 2007; Bobadilla *et al.* 2013; Kanimozhi *et al.* 2013). However, scarce work has still been done regarding dietary seaweed supplementation influence over teleost innate immune system.

Lysozyme activity levels, demonstrated an increase in this parameter related to the presence of seaweed. Seabass fed the *Ul2.5* diet showed an enhanced lysozyme activity when compared to the *Ctrl* and the *Ul7.5* diet, suggesting a dose-dependent response in this immune parameter. The remaining seaweed supplemented diets showed lysozyme results similar to *Ctrl* diet. Lysozyme levels can vary considerably between different fish species and in most cases is positively correlated with disease resistance (Fevolden *et al.* 1994). Similarly to ACH50, lysozyme has vast literature regarding both its importance as broad-spectrum enzyme with strong action against Gram-negative bacteria (Yano 1996) and its modulation factors, both physical (Valero *et al.* 2014) and nutritional (Cecchini *et al.* 2000). Valente *et al.* (2015) showed *Ulva* spp. dietary inclusion at 5 and 10 % DM levels to be indifferent in Nile tilapia (*Oreochromis niloticus*). Nevertheless, a lysozyme increase was found for olive flounder (*Paralichthys olivaceus*) fed increasing levels of kelp (*Ecklonia cava*) (Kim and Lee 2008), and flathead grey mullet (*Mugil cephalus*) fed *Sargassum* spp. supplemented diets and tested against *Pseudomonas fluorescence* (Kanimozhi *et al.* 2013). The actual immunostimulants compounds in seaweed supplements are unknown. However, some studies suggest that polysaccharides present in seaweeds may activate the non-specific immune responses in both teleosts and shrimps (Kim and Lee 2008). This is in accordance with known lysozyme molecular triggers, as pathogenic microorganisms often possess polysaccharides, and other characteristic components that are not normally on the surface of multicellular organisms (Uribe *et al.* 2011). The combined results of these immunologic indicators point to an immunostimulating effect of the seaweeds.

Therefore, results in the present work demonstrate that dietary seaweed supplementation (up to 7.5 %) have no detrimental effects on the growth performance and feed utilization of European seabass juveniles, supporting the subsequent utilization of dietary seaweed supplementation for antioxidant and immunostimulant purposes.

Conclusions

Seaweed supplementation (*Gracilaria* spp., *Ulva* spp., and *Fucus* spp.) in practical diets for European seabass have no impact in growth performance, up to 7.5 % supplementation level. A minimal modulation of digestive enzyme activities was found for diets with *Ulva* spp. supplementation. Dietary *Gracilaria* spp. supplementation led to an antioxidant capacity enhancement that, however, should be confirmed testing other Rhodophyta species. A dietary 2.5 % incorporation level improved innate immune system indicators. Overall, our results indicate that seaweed supplementation in aquafeeds may be a valuable tool to increase the immunocompetency of valuable aquaculture fish species, usually subjected to stressful or immunologically challenging situations, without compromising growth performance.

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CHAPTER III

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Diets supplemented with seaweed affect metabolic rate, innate immune, and antioxidant responses, but not individual growth rate in European seabass (*Dicentrarchus labrax*)

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CHAPTER III

DIETS SUPPLEMENTED WITH SEAWEED AFFECT METABOLIC RATE, INNATE IMMUNE AND ANTIOXIDANT RESPONSES, BUT NOT INDIVIDUAL GROWTH RATE IN SEABASS (*DICENTRARCHUS LABRAX*).

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Abstract

This study investigated the effects of seaweed dietary supplementation on measures of fish performance including aerobic metabolism, digestive enzymes activity, innate immune status, oxidative damage and growth rate using European seabass (Dicentrarchus labrax). Fish were fed for 49 days with three different diets: control diet (CTRL), a Gracilaria supplemented diet (GR7.5) and a mixed diet (Mix) composed of Gracilaria, Fucus and Ulva genera representatives. All diets were isoenergetic (22 kJ.g-1 adjusted for dry matter (DM)), isoproteic (47 %DM) and isolipidic (18 %DM) and tested in triplicate groups of 20 fish (initial body weight 25.5 ± 4.1 g). Final results showed similar growth rates and digestive activities between diets. Maximum and standard metabolic rates and aerobic metabolic scope revealed comparable results for the three diets. In contrast, fish fed with GR7.5 exhibited elevated routine metabolic rate (190.7 mg O_2 kg^{-1} h^{-1}). Fish fed the GR7.5 and Mix diets had lower alternative complement pathway (62.5 and 63 $U^{-1}.ml^{-1}$ respectively) than CTRL (84 $U^{-1}.ml^{-1}$). GR7.5 increased lipid peroxidation and cholinesterase levels, as well as glutathione s-transferase activity. Mix diet increased glutathione reductase activity when compared to CTRL. Collectively, our findings suggest that dietary seaweed supplementation may alter seabass metabolic rate, innate immune and antioxidant responses without compromising growth parameters.

Keywords: Digestive enzymes, growth rate, innate immune response, metabolic rate, oxidative stress, seaweeds.

Introduction

Marine derived bioactives have high nutraceutical potential, encompassing anti-inflammatory, antioxidant and pathogen inhibition proprieties (Chandini *et al.* 2008; Kadam and Prabhasankar 2010; Lordan *et al.* 2011; Okuzumi *et al.* 1993; Yan *et al.* 1999). Because performance traits are modulated by specific nutrients (Atherton and Smith 2012), functional diets could affect the physiological, innate immunity and antioxidant capacities of fish. Interestingly, various types of seaweed possess valuable nutritional compounds such as vitamins, minerals, proteins, polysaccharides, steroids and dietary fibers (El-Said and El-Sikaily 2013). In addition, seaweed often contains important carotenoids, saturated and polyunsaturated fatty acids (De Almeida *et al.* 2011) and several phytochemicals advantageous for the control of diseases including hyperlipidemia, thrombosis, tumor and obesity (Plaza *et al.* 2008).

There is an emerging interest in determining the role of dietary seaweed supplementation on antioxidant and immune responses. Understanding the effects of seaweed supplementation is particularly important in aquaculture, because farming protocols often induce stress conditions that affect the immune system responses (Scapigliati *et al.* 2002), including an increase in reactive oxygen species (ROS) production. In the long term, this situation may compromise growth performance (Leal *et al.* 2011), animal welfare and ultimately cause a reduction in revenues. Previous studies suggested that feed supplemented with seaweed may mitigate stress responses and improve vitality, illness resistance (Araújo *et al.* 2015; Mohamed *et al.* 2012; Samad 2013) and flesh quality of fish (Hamauzu and Yamanaka 1997; Valente *et al.* 2015), all parameters with direct commercial interest for the aquaculture sector (Luna-Acosta *et al.* 2011).

The determination of innate immune and oxidative stress responses can be used to monitor fish condition by analyzing lysozyme, peroxidase and complement systems (i.e. non-specific protection systems) that defend fish against bacteria, fungi and parasites (Sunyer and Tort 1995; Tort *et al.* 1996) and by measuring the oxidative stress enzymes activities that are involved in the clearance of reactive oxygen species (Blier 2014).

However, maintaining a functional immune system is energetically and nutritionally costly (Lailvaux and Husak 2014), requiring energy and nutrients that could have been allocated for growth (Sheldon and Verhulst 1996). Indeed, up-regulation of the immune system is associated with elevated energy and protein expenditures (Lochmiller and Deerenberg 2000). Newsholme and Newsholme (1989) compared the metabolic costs of elicited macrophages by infection to a maximally functioning heart, emphasizing the need of macrophages for large amounts of ATP. The fuel for these energetic demands derives from augmented processes such as lipolysis, proteolysis and glycolysis (Lochmiller and Deerenberg 2000). Consequently,

an enhanced immune system could be metabolically costly and result in reduced allocation of energy to somatic growth (Bashir-Tanoli and Tinsley 2014). If confirmed, this mechanism might counter any positive influence of seaweed on fish health and survival.

Two important physiological parameters to understand aerobic energy metabolism in ectothermic animals are standard metabolic rate (SMR) and maximum metabolic rate (MMR), measured as the lower and upper boundaries of oxygen consumption rates (Norin and Malte 2011; Roche *et al.* 2013). Subtracting SMR from MMR provides a limit measure for the total aerobic expanse of simultaneous metabolically demanding processes (Clark *et al.* 2013; Norin and Malte 2011), known as the aerobic metabolic scope (AMS). Many fish show spontaneous bouts of activity, typically fueled by aerobic energy with metabolism fluctuating around the average level, termed the routine metabolic rate (RMR) (Wieser 1985). Previous studies have suggested that metabolic rates are coupled with immune responses in endothermic animals (Downs *et al.* 2013). For example, Ots *et al.* (2001) found that in wintering adult birds even a non-pathogenic immune challenge affects basal metabolism (equivalent to SMR in ectothermic animals). The effects of diet on metabolism, and interactions with immune system variables, are largely unknown in fish, in particular in relation to aquaculture.

The objective of this study was to examine metabolic and immunological effects of diets supplemented with seaweed, using seabass *Dicentrarchus labrax* as a model. Specifically, we tested the hypothesis that dietary seaweed supplementation enhances the innate immune and anti-oxidant responses of fish. Because the immune system is energetically expensive, such enhanced responses could be associated with elevated metabolic rates and therefore decreased growth performance.

Materials and Methods

This experiment was carried out under the guidance of a Laboratory Animal Science certified supervisor (1005/1092, DGV-Portugal, C category, FELASA), according to European Union directives (2010/63/UE).

Experimental diets

Gracilaria spp., *Ulva spp.* and *Fucus spp.* were reared in an integrated multi-trophic aquaculture (IMTA) system (Abreu *et al.* 2011) at Algaplus Lda. (Ílhavo, Portugal). Crude seaweeds were dried and added as supplements (concentrations of 2.5 and 7.5 % adjusted for dry matter content (DM)) to the experimental diets. The experiment comprised three isoenergetic (22 $kJ.g^{-1}$ DM), isoproteic (47 %DM) and isolipidic (18 %DM) diets. Details on formulation and proximate composition are presented in Table 1. All diets were formulated with the same basic ingredients. Dietary protein and fat levels were adjusted in accordance with recommendations for seabass (FAO 2005-2015; Webster and Lim 2002). Dietary macronutrient balance after seaweed incorporation was achieved by reducing contents of soy protein and wheat meal.

All ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 2.0 mm pellet size, SPAROS, Portugal). Diets were finally dried at 45 ºC for 12 h and stored at 4ºC until use.

Table 1 Formulation and proximate composition of the experimental diets. DM means dry matter.

	Dietary treatments		
	CTRL	GR7.5	Mix7.5
Fish ingredients (%DM)			
Fishmeal Standard	10.0	10.0	10.0
Fishmeal SOLOR	20.0	20.0	20.0
Soy protein concentrate (Soycomil)	11.8	10.0	10.3
Wheat gluten	4.0	4.0	4.0
Corn gluten	8.0	8.0	8.0
Soybean meal 48	12.0	12.0	12.0
Rapeseed meal	5.0	5.0	5.0
Wheat meal	9.0	3.3	3.0
Peas gelatinized (Aquatex 8071)	3.2	3.2	3.2
Fish oil - COPPENS	6.5	6.5	6.5
Soybean oil	4.0	4.0	4.0
Rapeseed oil	4.0	4.0	4.0
Vit & Min Premix PV01	1.0	1.0	1.0
Binder (Kieselghur)	0.5	0.5	0.5
Antioxidant powder (Paramega)	0.2	0.2	0.2
MCP	0.5	0.5	0.5
L-Lysine	0.2	0.2	0.2
DL-Methionine	0.1	0.1	0.1
Gracilaria		7.5	2.5
Ulva			2.5
Fucus			2.5
Proximate composition			
Dry matter (%DM)	94.74	95.90	94.83
Ash (%DM)	8.59	10.76	10.55
Crude protein (%DM)	47.80	47.76	47.90
Crude fat (%DM)	19.09	19.57	19.28
Gross energy (kJ g^{-1} DM)	22.70	22.41	22.39

Fish and rearing conditions

A total of 180 seabass (initial body weight: 25.5 ± 4.1 g) were obtained from a fish farm (MARESA) in Spain and transported to University of Porto in Portugal. For the physiological and immunological sampling, 27 random seabass were marked with Passive Integrated Transponder (PIT) tags, and body mass was measured (to nearest 0.1 g) (Boel *et al.* 2014). Next, seabass were acclimated to a basal diet (CTRL) and the new holding facilities for two weeks, and then randomly distributed in 9 tanks of 80 L each (n=3 tanks $treatment^{-1}$). The tanks were connected to a flow-through seawater system providing environmental conditions optimized for seabass (30 ‰ salinity, oxygen content ≥ 80 % air saturation (O_{2sat}), 20 ± 1 ºC, ammonia ≤ 0.5 mg l^{-1} and nitrites ≤ 3 mg l^{-1}).

The experimental diets were randomly assigned to the tanks establishing triplicate groups of fish per treatment that were hand-fed, twice a day (09:30 and 17:30 h) for 49 days until

apparent visual satiety. Food intake was determined by weighing the daily amount of diet distributed to each tank (Araújo *et al.* 2015). Subsequently, the three tagged fish per tank (n=9 fish treatment^{-1}) were sampled for analyses of growth performance in addition to physiological and immunological variables as outlined below.

Respirometry

Three static respirometers (each 0.42 L) and a mixing pump were submerged in a 150 L opaque tank. The tank was in the same room as the dietary treatment tanks and filled with water (30 ‰ salinity) from the same source as used for the dietary groups. The tank was partly covered by polystyrene sheets to isolate experimental fish from outside stimuli. Air stones were used to maintain water O_{2sat} in the tank at a normoxic level (> 95 % O_{2sat}), and water temperature was maintained at 20 ± 0.1 ˚C using a temperature controlling instrument (TMP-REG; Loligo Systems; Tjele, Denmark). Water in the tank was recirculated through a loop consisting of a separate biological filter (TMC-IBERIA, Portugal) and a UV sterilizer (UV-10000; Tetra Pond, Melle, Germany) (Rosewarne *et al.*, in press).

Measurements of MO_2 (mg O_2 kg^{-1} h^{-1}) were carried out every 7 min using intermittent flow respirometry, allowing long term (> 24 h) repeated measurements (Forstner 1983; Steffensen 1989). Each respirometer was fitted with two inlet and outlet ports and two water pumps as described previously (Svendsen *et al.* 2012). Oxygen partial pressure (kPa) was measured inside the respirometers at 1 Hz using galvanic oxygen sensors (Mini DO Probe; Loligo Systems; Tjele, Denmark). Oxygen levels above 80 % O_{2sat} in the respirometers were secured using flush pumps that were activated intermittently and controlled by AutoResp software (Loligo Systems, Tjele, Denmark). Between flushings, the declining oxygen partial pressure (kPa) was recorded to calculate MO_2 using the equation:

$$MO_2 = \frac{K\,V\,\beta}{M} \quad (1)$$

where *K* is the linear rate of decline (kPa h^{-1}) in the oxygen content over time (h) in the respirometer, *V* is the volume of the respirometer (L) corrected for the volume of fish, β is the solubility of oxygen in the water (mg O_2 L^{-1} kPa^{-1}) (β = 0.3683) and M is the body mass of the fish (kg). The coefficient of determination (r^2) associated with each MO_2 measurement was always > 0.98, similar to previous studies (Schurmann and Steffensen 1997; Svendsen *et al.* 2013). Corrections of background respiration (i.e. microbial respiration) followed Rosewarne *et al.* (in press). Measurements of MO_2 were adjusted to a common body mass of 53 g (Claireaux *et al.* 2006; Herskin and Steffensen 1998) using a mass exponent of 0.77.

Seabass were fasted for 48 h to ensure a post absorptive state prior to sampling (Axelsson 2002; Dupont-Prinet *et al.* 2010). Fish were transferred to respirometers to determine MMR

and left undisturbed for 24 h for measurements of RMR, SMR and AMS as described below. For each 24 h period, data collection involved a fish from each of the three dietary groups. Selection of respirometer for each fish was randomized. Promptly after the respirometry trials, seabass were euthanized by an anesthetic overdose of ethylene glycol monophenyl ether (Merck, Germany) for measurements of individual growth parameters as well as innate immune and antioxidant systems.

Maximum metabolic rate (MMR), routine metabolic rate (RMR), standard metabolic rate (SMR) and aerobic metabolic scope (AMS)

MMR was measured using a standard chase protocol (Cutts *et al.* 2002). Briefly, individual seabass were transferred to a circular trough and chased to exhaustion (Dupont-Prinet *et al.* 2010; Roche *et al.* 2013; Svendsen *et al.* 2014). Upon exhaustion, identified by no further response after 5 min of manual stimulation, seabass were transferred to the respirometer where MO_2 measurements started immediately. MMR was the highest of three consecutive MO_2 measurements (Svendsen *et al.* 2012).

Fish were left undisturbed in respirometers after the measurement of MMR. The mean value of MO_2 measurements collected during the last 4 h of the full 24 h respirometry period was used to estimate routine metabolic rate (RMR) (between 09:00 and 13:00 h the day after the fish was introduced in to the respirometer) following Killen *et al.* (2012a).

SMR in individual fish was estimated using two different methods: 1) SMR was estimated as the average of the lowest 10 MO_2 values collected over the 24 h periods (Schurmann and Steffensen 1997; Svendsen *et al.* 2014). This method to estimate SMR was employed because it provides measurements that are repeatable in individual fish (Norin and Malte 2011); 2) SMR was estimated as the lowest 10th percentile of all MO_2 values collected over the 24 h periods (Killen *et al.* 2012b).

Growth performance

Growth response parameters were evaluated by calculating weight gain (2), daily growth index (3), voluntary feed intake (4) and feed conversion ratio (5).

Weight gain (WG) was calculated as:

$$WG = Wf - Wi \quad (2)$$

where Wi is the initial body weight at the start of the feeding trial and Wf is the final body weight at the end of the respirometry trial;

Daily growth index (DGI) was calculated as percentage of body weight increase per day (% BWday^{-1}):

$$DGI = \frac{FBW^{1/3} - IBW^{1/3}}{t} \times 100 \quad (3)$$

where t is the feeding duration in days and FBW and IBW are the final and initial body weight respectively;

Voluntary feed intake (VFI; % BW day^{-1}) was determined as:

$$VFI = \frac{\frac{Total\ feed\ intake}{Average\ body\ weight\ (ABW)}}{t} \times 100 \quad (4)$$

where $ABW = \frac{IBW+FBW}{2}$.

Feed conversion ratio (FCR) was calculated as:

$$FCR = \frac{Total\ feed\ intake}{Weight\ gain} \times 100 \quad (5)$$

Biochemical analysis

Immediately after the respirometry trials, blood was collected and centrifuged and resulting plasma stored for posterior analysis, and liver and intestines were sampled for enzymatic screening. All samples were immediately stored at -80 ˚C.

Fish innate immune status was accessed by plasma contents of lysozyme and peroxidase activity as well as alternative complement pathway response. Lysozyme is a bacteriolityc protein widely used because of its capacity to cleave bacterial peptidoglycans (Guardiola *et al.* 2014). It was measured by a turbidimetric assay based on *Micrococus lysodeikicus* lysis and using hen egg white lysozyme (Sigma, Portugal) as standard (Valero *et al.* 2014).

Peroxidase levels were used as indicator of activation state of circulating leucocytes that are known to increase in response to infection or stress (Cuesta *et al.* 2006). Peroxidase levels were determined by 3,3', 5,5' – tetramethylbenzidine hydrochloride (Sigma, Portugal) reduction (Quade and Roth 1997).

Hemolytic activity of the alternative complement system was assayed via rabbit red blood cells agglutination as described by Sunyer and Tort (1995).

Antioxidant systems are divided by enzymatic, catalase and peroxidases, and non-enzymatic such as lipid peroxidation processes. To quantify oxidative damages, liver was used and its protein content determined as described by Bradford (1976).

Lipid peroxidation was accessed by the presence of its by-product, thiobarbituric acid reactive substances (Ohkawa *et al.* 1979). Catalase (EC 1.11.1.6.) was determined by its action over

peroxide hydrogen according to previous studies (Clairborne 1985). Glutathione s-transferase (EC 2.5.1.18) was evaluated by conjugation of Glutathione (GHS) with 1-chloro-2,4-dinitrobenzene (Habig *et al.* 1974). Glutathione peroxidase (EC 1.11.1.9.) and reductase (EC 1.8.1.7) were studied by oxidation of NADPH based on previous studies (Cribb *et al.* 1989; Mohandas *et al.* 1984). Total glutathione was evaluated by the formation of 5-thio-2-nitrobenzoic acid as detailed by Baker *et al.* (1990). Cholinesterase was measured using Acetylcholine as substrate and accessed according to Ellman *et al.* (1961).

Intestines were homogenized for digestive enzymes extraction (Rungruangsak-Torrissen 2007) and the protein content analysis performed according to previous studies (Lowry *et al.* 1951). Amylase was examined by formation of maltose (Bernfeld 1951). Measurements of nitroaniline production allowed trypsin and chymotrypsin determination (Rungruangsak-Torrissen and Sundby 2000). Lipase assay was carried out using p-nitrophenyl for substrate as described by Winkler and Stuckmann (1979).

Statistical analysis

Statistical analyses followed the methods outlined by Zar (1999). Data were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's test, respectively. Then data were analyzed with one-way analysis of variance (ANOVA) to test for differences between dietary treatments. When this test showed significance, individual means were compared using Tukey's test. Significant differences were considered when $P < 0.05$. When ANOVA criteria were not fulfilled, data were submitted to the nonparametric tests (Kruskal–Wallis test), followed by Mann–Whitney test when needed. All values are reported as means ± S.E. unless noted otherwise. Tests were carried out using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA).

Results

Metabolic rates

Metabolic rates were estimated after correcting MO_2 for background respiration and body mass. No statistical differences were found between dietary treatments for SMR (table 2). Both supplemented diets (GR7.5 and Mix) revealed higher MMR values than the control diet (CTRL), however, differences were not significant ($P > 0.05$) (table 2). AMS was calculated by subtracting SMR from MMR and demonstrated no statistical difference ($P > 0.05$) (table 2) between diets, although mean values were higher for both supplemented diets. This result was consistent for both methods to estimate SMR (method 1 and 2; see above).

Table 2 Standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic metabolic scope (AMS) (mg O_2 kg^{-1} h^{-1}) in seabass at 20 °C fed three different diets

Diet	SMR[1]	SMR[2]	MMR	AMS
CTRL	117.61 ± 4.88	122.62 ± 15.41	420.01 ± 19.29	302.40 ± 0.53
GR7.5	124.98 ± 6.00	134.28 ± 17.56	455.06 ± 21.71	330.08 ± 14.98
Mix	112.76 ± 5.43	120.37 ± 14.01	432.41 ± 22.04	319.65 ± 16.36

* N = 9 for each diet. No statistical differences between diets ($P > 0.05$). Values presented as mean ± S.E.
[1] Determined using the 10 lowest MO_2 values (i.e. Method 1).
[2] Determined using the 10 % lowest MO_2 values (i.e. Method 2).
No differences in AMS were detected ($P > 0.05$) regardless of the method used to calculate SMR.

RMR differed significantly between diets (Fig. 1). GR7.5 revealed higher RMR than CTRL ($P < 0.05$) whereas mix was intermediate. This finding indicated that GR7.5 exhibited higher routine metabolic expenditures than CTRL.

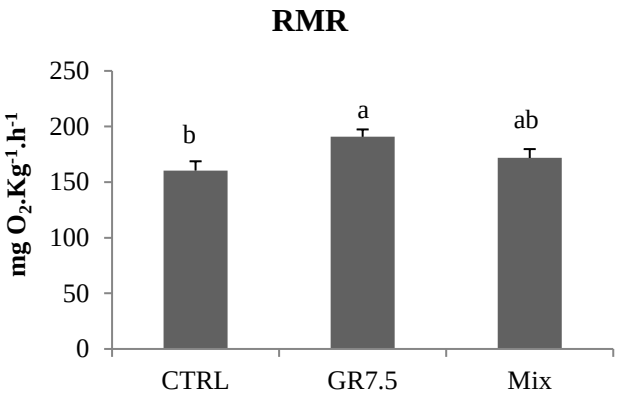

Fig. 1 Routine metabolic rate (RMR; mg O_2 kg^{-1} h^{-1}) in seabass at 20 °C fed three different diets. N = 9 for each diet. Different letters indicate statistical differences ($P < 0.05$) between diets. Values presented as mean ± S.E.

Morphometric and growth data

Growth parameters showed no statistical differences between diets (table 3) indicating no direct effect of seaweed supplementation on fish growth capacity.

Table 3 Growth parameters of seabass reared at 20 ºC and fed three different diets.

Diets	WG (g-1)	FCR	DGI (%BW.day-1)	VFI (g-1.kg-1. day-1)
CTRL	22.56 ± 1.57	1.20 ± 0.08	1.26 ± 0.06	1.24 ± 0.05
GR7.5	22.19 ± 1.64	1.20 ± 0.09	1.31 ± 0.10	1.30 ± 0.04
Mix	19.82 ± 1.66	1.31 ± 0.11	1.12 ± 0.09	1.19 ± 0.04

*N = 9 for each group. No statistical differences between diets ($P > 0.05$). Values presented as mean ± S.E.

Digestive Enzymes data

Digestive enzymes analysis provides an overall indicator of fish nutritive physiology. There was no effect of diets on seabass digestive enzymes (table 4).

Table 4 Digestive enzymes study of seabass reared at 20 ºC and fed three different diets. All data normalized by protein content.

Diets	Amylase (mU-1mg-1protein)	Lipase (mU-1mg-1protein)	Chymotrypsin (mU-1mg-1protein)	Trypsin (mU-1mg-1protein)
CTRL	1786.69 ± 186.35	113.71 ± 18.68	4.00 ± 0.52	16.20 ± 3.74
GR7.5	1987.67 ± 88.94	106.10 ± 9.41	3.80 ± 0.43	22.40 ± 3.25
Mix	1831.03 ± 150.22	107.23 ± 13.30	4.64 ± 0.55	15.81 ± 1.89

*N = 9 for each dietary group. No statistical differences between diets ($P > 0.05$). Values presented as mean ± S.E.

Immune Parameters data

Plasma analysis showed no differences between dietary treatments for lysozyme and peroxidase contents (table 5).

Table 5 Innate immune parameters analyzed in plasma from seabass reared at 20 ºC and fed three different diets

Diets	Peroxidase (U-1.ml-1)	Lysozyme (U-1.ml-1)
CTRL	11.29 ± 0.82	386.81 ± 38.22
GR7.5	12.58 ± 1.97	366.05 ± 37.41
Mix	17.32 ± 3.34	466.67 ± 45.50

*N = 9 for each group. No statistical differences between diets ($P < 0.05$). Values presented as mean ± S.E.

Conversely, when testing for hemolytic capacity of the alternative pathway complement system (ACH50) (Fig. 2), clear differences were found between control diet and supplemented diets (GR7.5 and Mix) ($P < 0.05$). This finding indicated that diet may influence how and how fast pathogens are opsonized and destroyed by complement proteins.

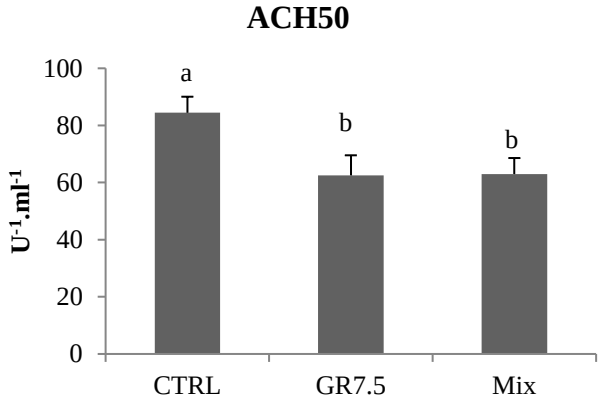

Fig. 2 Plasma Alternative Complement (ACH50) content of seabass reared at 20 ºC and fed three different diets. Values presented as mean ± S.E. Different letters indicate statistical differences ($P < 0.05$) between diets.

Oxidative stress data

Analysis of liver antioxidant systems revealed no significant differences ($P < 0.05$) for catalase (CAT), total glutathione (GT), glutathione peroxidase (GPx) and oxidized glutathione (GSSG) (table 6).

Table 6 Levels of catalase (Cat), total glutathione (GT), glutathione peroxidase (GPx), oxidized glutathione (GSSG), reduced glutathione (GSH) and reduced over oxidized glutathione ratio (GSH/GSSG) in seabass liver at 20 ºC. Fish were fed three different diets.

Diets	Cat	GT	GPx	GSSG	GSH	GSH/GSSG
CTRL	72.62 ± 13.99	15.16 ± 4.72	0.54 ± 0.13	7.19 ± 2.13	7.08 ± 0.55	1.09 ± 0.10
GR7.5	77.09 ± 10.82	17.06 ± 8.55	0.63 ± 0.19	6.19 ± 2.17	10.88 ± 2.25	1.70 ± 0.20
Mix	72.86 ± 8.09	11.75 ± 2.26	0.41 ± 0.15	5.68 ± 2.46	6.55 ± 0.56	1.61 ± 0.40

*N = 9 for each group. No statistical differences between diets ($P < 0.05$). Values presented as mean ± S.E.
Cat - $\mu M^{-1}.min^{-1}.mg^{-1}$protein
GT, GPx, GSSG, GSH - $nM^{-1}.min^{-1}.mg^{-1}$protein

Lipid peroxidation (LPO) revealed higher levels of lipid oxidation in GR7.5 and mix ($P < 0.05$) (Fig. 3a). Cholinesterase enzyme (ChE) followed the LPO pattern and presented differences between control and both supplemented diets, indicating higher circulating esterase content in GR7.5 and mix (Fig. 3b) ($P < 0.01$). Considering cells oxidative state, a higher effort was expected by other antioxidant enzymes, which we found for glutathione s-transferase (GST) in GR7.5 (Fig. 3c) ($P = 0.01$). Also glutathione reductase (GR) showed higher levels for the mix diet (Fig. 3d) ($P < 0.05$). Collectively, these findings indicated that GR inclusion level can be a key factor for the dietary effects on the oxidative stress condition.

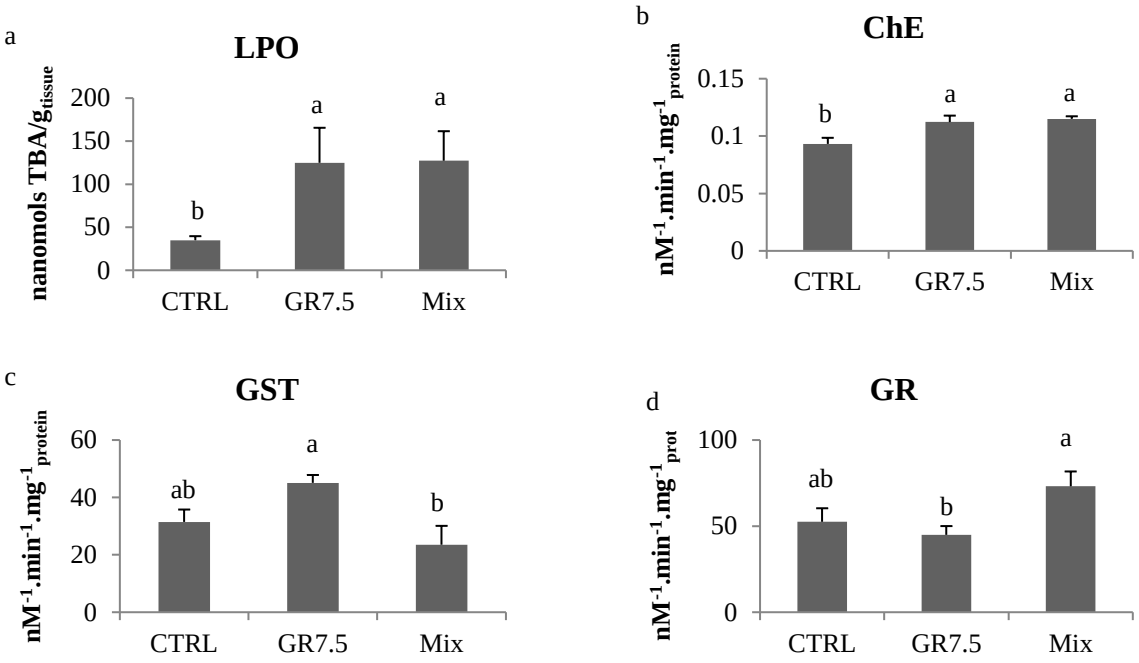

Fig. 3 Liver contents of lipid peroxidation (A), cholinesterase (B), glutathione *s*-transferase (C) and glutathione reductase (D) in seabass reared at 20 ºC. Fish were fed three different diets. Different letters indicate statistical differences ($P < 0.05$) between diets. Values presented as mean ± S.E.

D**iscussion**

This study examined the use of seaweed supplemented diets to improve fish growth, performance and health. Specifically, we tested the hypothesis that enhancing the innate immune and antioxidant responses using seaweed could alter fish metabolism and therefore growth. Our data revealed no effects of seaweed on fish growth and metabolism measured as SMR, MMR and AMS. In contrast, we found evidence of altered innate immune and antioxidant responses in fish fed the seaweed supplemented diets. Collectively, the data suggest that seaweed supplementation can modulate innate immune and antioxidant responses without affecting fish growth and metabolism measured as SMR, MMR and AMS.

SMR and MMR provide the lower and upper boundaries for aerobic energy metabolism. The present study found no influence of diets on SMR and MMR in seabass (table 2). As a direct result, AMS also showed no dietary effect. Conversely, RMR was higher in fish fed the GR7.5 diet suggesting that the routine activity level was directly influenced by ingestion of *Gracilaria*. Shao *et al.* (2013) found a similar result for mice fed *Gracilaria eucheumoides*, where an anti-fatigue effect of this diet was evaluated. Their work indicates altered expression levels in genes responsible for energy metabolism and transport, as well as plasmatic markers of energetic metabolism, when mice were fed increasing levels of *Gracilaria*. Elevated RMR may influence growth (Burton *et al.* 2011; Killen *et al.* 2011) because energy is allocated from storage to activity. This would suggest that seabass fed the GR7.5 diet should be growing less, or feeding more, because they exhibited elevated RMR. However, we found no effects of diets on the growth and feed intake parameters (table 3). These findings support the intriguing possibility that the GR7.5 diet allowed seabass to allocate resources to growth more efficiently and thereby compensated for the energy allocated to routine activity (i.e. RMR). While this hypothesis warrants further study, it is possible that the modulated innate immune and antioxidant responses associated with the GR7.5 diet allowed the fish to allocate resources differently and grow more efficiently.

Detrimental components in diets may influence digestive functions (Hartviksen *et al.* 2014; Krogdahl *et al.* 2010). The present study, however, found no significant differences between diets in terms of digestive enzymes (table 4), which supports two possible conclusions. Firstly, the basic composition of the three diets may have met all the nutrient requirements of seabass (Dias *et al.* 2005). If so, seaweed represent no antinutritional effect over seabass digestive enzymes and we would expect to see no differences between diets. Secondly, the fact that we found no significant differences in digestive enzymes might be related to the non-absorptive state that fish were in, prior to testing, because fish were subjected to a 48 h fasting period (Eroldoğan *et al.* 2008; Hidalgo *et al.* 1999) for the respirometry trials. Thence, the lack of nutritional stimulation of the digestive enzymes production may have inhibited the expression

of the possibly different responses between diets. Further studies are needed to examine the two possible conclusions in detail. Nevertheless, despite the fact that seabass is a piscivorous species (Le Boucher *et al.* 2013) that does not feed on seaweed in the wild, our data support the conclusion that seaweed dietary supplementation has no deleterious effect on this species digestion and growth.

Elevated metabolism may be associated with up regulated specific and non-specific immune mechanisms (Bashir-Tanoli and Tinsley 2014; Burton *et al.* 2011; Skinner *et al.* 2010). According to Råberg *et al.* (2002), variations in metabolic rates may be attributed to differential energetic costs associated with stimulating innate or adaptive immune defenses. Therefore, adaptive immune markers should be analyzed in an attempt to correlate the results found for RMR in GR7.5% diet, since no direct correlation was established with the innate parameters analyzed in this work. However, we found that the plasma alternative complement (ACH50) was elevated in the CTRL group (Fig. 2) while this group did not exhibit elevated metabolism (i.e. SMR, MMR and AMS). Elevated ACH50 in the CTRL group means higher hemolytic capacity (i.e. up regulated immune status) when compared with GR7.5, yet, the CTRL diet caused a low RMR (Fig. 1). Using rainbow trout (Latin name) as model species, previous studies with *Gracilaria* dietary inclusion at 5% and 10% levels showed an increase in ACH50 in the 5% inclusion group, whereas in the 10% inclusion group a decrease was found when compared to 5%, although both appeared higher than control (Araújo *et al.* 2015). These findings suggest that the immunostimulant properties of Gracilaria may decay above 5% inclusion, which seems to be in accordance with the results found in the present work for 7.5% inclusion.

Oxidative stress biomarkers are widely used as a tool to determine toxicity in aquatic ecosystems (Almeida *et al.* 2012; Gravato *et al.* 2006; Rodrigues *et al.* 2013). In the present study, we aimed at identifying if seaweeds, known for their antioxidant abilities (Faten M. 2009; Souza *et al.* 2012; Woo *et al.* 2013), may protect fish from the deleterious effects of reactive oxygen species (ROS). Our data on lipid peroxidation (LPO) (Fig. 3a) when compared to CTRL showed a remarkable increase in seabass fed supplemented diets. These results suggest that seaweed supplementation increases lipid layers degradation. This conclusion is in accordance with a recent study (Woo *et al.* 2013) that revealed a dose-dependent inhibition in lipid accumulation in cells treated with *Gracilaria verrucosa* extracts. Moreover, ROS production increases with physical activity (Liu *et al.* 2000), due to high energy requirements of muscle cells (McClelland 2004). In this sense, the lipid peroxidation increase in fish fed GR7.5 may be directly linked with the observed increase in RMR.

The role of esterase activity in lipid metabolism is not clear. Nonetheless these enzymes are considered capable of hydrolyzing water soluble carboxylic acids (Tocher 2003) and are precursors of lipases that hydrolyze lipids in the digestive tract (Bele 2014). In this study, we

analyzed cholinesterase activity (Fig. 3b), where a pattern similar to LPO (Fig. 3a) was found, with supplemented diets presenting higher levels of this enzyme. The results may suggest that seabass fed CTRL diets suffer an esterase activity inhibition. While similar findings were reported recently (Andrade *et al.* 2013), the biological implications of the inhibition remain unclear.

Analyzing specific antioxidant enzymes, we performed an activity analysis of glutathione s-transferase (GST) (Fig. 3c), an enzyme responsible for removing reactive oxygen intermediates (Tocher 2003). According to previous studies (Leaver *et al.* 1993), this enzyme is also involved with prostaglandins biosynthesis and steroids isomerization, having an essential role in detoxification, as well as metabolism and transport. In our study, we found higher GST activity in seabass fed *Gracilaria* when compared with mix diet (Fig. 3C). CTRL diet showed no differences from the supplemented diets. Considering that mix diet was supplemented with a lower level of *Gracilaria*, although the total level of supplementation was identical, our results could indicate that *Gracilaria* has antioxidant properties.

Glutathione reductase (GR) is the enzyme responsible for recovering the oxidized glutathione (GSSG), to its reduced form, inhibiting the exhaustion of the electron donor, reduced glutathione (GSH) (Srikanth *et al.* 2013; Tocher 2003). Its activity can therefore represent the antioxidant restoration potential. Total glutathione (GT) represents the state of the peptide glutathione, summing both reduced and oxidized forms. Its level is related to antioxidants depletion, considering the incomplete recovery of the GSSG (Eroglu *et al.* 2014; Owen and Butterfield 2010). In our data, no differences were found in GSSG, GSH, GT and GSH/GSSG ratio (table 6). The significant increase in GR observed in the mix diet (Fig. 3d) suggests lower capacity to mold the glutathione metabolism state in this dietary treatment. The differences found for GR (Fig. 3d) might also be related to the tendency for lower GSH concentrations in mix diet (table 6), placing a greater demand in the reduction of GSSG to sustain adequate GSH/GSSG levels. Nevertheless, the fact that data indicated reduced GST activity in mix diet (Fig. 3c) might be a sign of momentary GSH shortage, as this would serve as co-factor for GST activity.

Conclusion

Seaweed supplementation in seabass feeds does not impair growth at 2.5 and 7.5 % supplementation levels. The present results have implications for aquaculture because dietary supplementation may be used to prevent economic losses related to fish disease and stress by improving survival. The use of crude seaweed for experimentation leaves a gap in the mechanistic understanding of the biological effects observed in seabass because the specific chemical compounds responsible for the effects remain unknown. Therefore, further studies would benefit from using specific seaweed extracts to further our understanding of the effects on fish metabolic rate, innate immunity and antioxidant capacity.

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Effects of dietary *Gracilaria* sp. and *Alaria* sp. supplementation on growth performance, metabolic rates and health in meagre (*Argyrosomus regius*) subjected to pathogen infection

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CHAPTER IV

EFFECTS OF DIETARY *GRACILARIA* SP. AND *ALARIA* SP. SUPPLEMENTATION ON GROWTH PERFORMANCE, METABOLIC RATES AND HEALTH IN MEAGRE (*ARGYROSOMUS REGIUS*) SUBJECTED TO PATHOGEN INFECTION

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Abstract

Effects of dietary seaweed supplementation on basal physiology and health biomarkers were assessed in meagre (*Argyrosomus regius*) subjected to bacterial infection, using *Photobacterium damselae* subsp. *piscicida* (*Phdp*) as the etiologic agent. Three test diets were prepared by supplementing a basal control formulation (44 % protein, 16 % lipid, 22 kJg^{-1} energy) with 0% seaweed (control), 5 % *Gracilaria* sp. or 5% *Alaria* sp. During the growth trial, 180 fish (39.70 ± 0.33 g) were daily fed for 69 days with the experimental diets. After the growth trial, 60 fish from each dietary treatment were divided into two groups, infected and non-infected. The infected group was injected intraperitoneally with a saline solution (HBSS) with 2.91 x 10^3 CFU *Phdp*/g fish, whereas the non-infected group was injected with HBSS without *Phdp*. Dietary seaweed supplementation did not affect fish growth performance. Standard and routine metabolic rates, and aerobic metabolic scope did not vary significantly among treatments. Conversely, maximum metabolic rate was significantly higher in fish fed *Alaria* sp. diet when compared to control group. Non-infected fish had higher haematocrit levels than the infected group, regardless of diet. Lactate levels were significantly higher in fish fed *Alaria* sp. diet when compared to control, with no interaction between diet and infection. Lipid peroxidation was significantly higher in fish fed control diet than supplemented diets. Infected groups had lower antioxidant enzymes activities when compared to non-infected. An interaction between infection and diet was found for glutathione peroxidase and reduced glutathione activities.

The current study suggests that dietary seaweed supplementation modulates metabolic rates and biomarker responses in meagre, which may confer advantages in coping with biotic stressors.

Keywords: Aerobic metabolic scope; bacterial infection; immune function; maximum metabolic rate; antioxidants.

Introduction

Meagre (*Argyrosomus regius*) is a teleost fish, belonging to the Sciaenidae family, that can be found along the west coasts of Europe and Africa, as well as in the Mediterranean and the Black Seas (Poli *et al.* 2003). It is a euryhaline and thermohaline species, tolerating wide temperature changes from 2 to 38 °C and salinity variations from 5 to 42 ‰ (Cárdenas 2012; González-Quirós *et al.* 2011). In addition to meagre resilience against abiotic stress factors and consequent high adaptation to captivity, other relevant factors such as high growth rates under optimum temperatures (17-21 ˚C) and its organoleptic characteristics (Poli *et al.* 2003; Quéméner *et al.* 2002; Cárdenas 2012), have increased the scientific interest as potential aquaculture fish model (Monfort *et al.* 2010; Cárdenas 2012; Quéméner *et al.* 2002).

Meagre aquaculture production however, has revealed some constraints, namely highly variable growth rates (Duncan *et al.* 2013) and health issues (Koyuncu *et al.* 2012), which may be related with two reasons. One, the fact that no specific diets have been developed for this specie. Currently, meagre is fed pelleted diets developed for seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) which basal formulation matches the protein and lipid requirements determined for meagre (Chatzifotis *et al.* 2010; Chatzifotis *et al.* 2012); Two: despite being considered a fitness advantage, most fish species don't grow at their physiological maximum (Conover *et al.* 2009), even with no restrictions to feed intake. This submaximal growth has been associated with the elevated energetic costs of high growth rates (Perez and Munch 2015), which constrain resource allocation trade-offs, diverting energy form other physiological traits, such as immune and antioxidant systems (Smith *et al.* 2016). The energetic cost of physiological traits is evaluated trough measurements of metabolic rates, specifically the aerobic metabolic scope (AMS) which indicates the maximum range of aerobic energy available for living and can be determined as the difference between the minimum energy requirement (SMR) and the maximum consumption of aerobic energy (MMR) (Luna-Acosta *et al.* 2011). Metabolic elevations above SMR are typically associated with spontaneous activity and are measured as routine metabolic rate (RMR). Together, SMR, MMR and AMS provide a measure of metabolic phenotype in fish (Metcalfe *et al.* 2016). Fish with high metabolic phenotype may be able to consume more feed per day, which may result in faster growth (Auer *et al.* 2015), however, this increased cellular activity may lead to oxidative damage in tissues and eventually illness and death (Smith *et al.* 2016). Since fish respond to disease throughout several physiological changes, including hematologic alterations (Dethloff *et al.* 1999), the variation of blood biochemistry and metabolic status might be, therefore, indicative of unsuitable environmental conditions or the presence of infection. Bacterial infections have been the most important and frequent disease-related causes of death in marine fish (Cruz *et al.* 2012). *Photobacterium damselae* spp *piscicida* (*Phdp*), is a

well-known pathogen in aquaculture ponds, responsible for diseases outbreaks and important economic losses (Romalde 2002a; Andreoni and Magnani 2014). Pasteurellosis caused by *Phdp* remains the most important septicemia in the Mediterranean grow-out facilities causing very high mortalities (Romalde 2002b).).

The current idea in aquaculture research is to develop an environmental friendly alternative to the excessive use of antibiotics, by using natural products that strengthen the immune and antioxidant system of fisheries. Furthermore, in intensive rearing facilities, fish are continuously exposed to management practices such as confinement, capture, handling and transportation, which are potential stressors (Wendelaar Bonga 1997; Iversen *et al.* 2005; Shabani *et al.* 2016). Hence, the use of functional diets that pursue improved production yield (Wassef *et al.* 2013; Li *et al.* 2008), fish metabolism modulation (Peixoto *et al.* 2015) or enhanced immune (Bansemir *et al.* 2006; Ibrahem *et al.* 2010) and anti-oxidative defences (Xu *et al.* 2011). These improvements are reflected in a higher capacity of fish to resist to both abiotic and biotic stress factors (Pham *et al.* 2006). The incorporation of seaweeds as functional ingredient in fish feeds has been explored in the major aquacultured species. Brown seaweed (Phaeophyceae) and red seaweed (Rhodophyceae) may be used as tools for different applications based on their chemical composition (Makkar *et al.* 2015). Because of their low protein content (3 to 15 %), brown seaweeds, including *Alaria* sp. are mainly used for their bioactive compounds (Makkar *et al.* 2015). In contrast red seaweeds, such as *Gracilaria* sp., tend to have higher protein content (from 10 to 50 %) which is highly valuable for an aquafeed ingredient (Makkar *et al.* 2015). Besides differences among species, seaweeds chemical content varies with season, geographical location and environmental factors (Balboa *et al.* 2013). In addition, seaweeds produced using integrated multi-trophic aquaculture systems (IMTA) are considered richer than seaweeds harvested in the wild (Pereira *et al.* 2012; Marinho *et al.* 2013). Several studies report dietary seaweed supplementation in aquafeeds as species and dose dependent (Araújo *et al.* 2015; Pereira *et al.* 2012). Rainbow trout (*Oncorhynchus mykiss*) juveniles fed diets containing *Gracilaria* sp. at 5 % supplementation level exhibited higher growth performance and immunity enhancements than the control and a 10 % supplemented diet (Araújo *et al.* 2015). In contrast, the dietary incorporation of *Porphyra* sp. showed no differences in trout growth at 5 and 10 % supplementation levels, however caused a decrease in final weight in groups fed a 15 % supplementation level (Soler-Vila *et al.* 2009). In seabream an inverse linear relationship between growth and dietary *Gracilaria* sp. supplementation was found when testing 5, 15 and 25 % supplementation levels. Conversely, growth performance of this same species revealed no differences when testing *Ulva* sp. supplementation at levels ranging from 4 to 25 % (Vizcaíno *et al.* 2015; Emre *et al.* 2013). The beneficial effect of dietary seaweed supplementation has also been analysed in seabass, testing *Gracilaria* sp., *Ulva* sp. and *Fucus* sp. at 2.5 and 7.5 % supplementation levels, resulting in similar growth performance for all

dietary treatments when compared with the control diet (Peixoto *et al.* 2016a). In another study with seabass, the dietary incorporation of *Ulva* sp. and *Pterocladia* sp. at 5 % level improved growth performance and stress responsiveness against air exposure respectively. Seabass fed seaweed supplemented diets showed higher survival rates and recovered their normal posture faster (Wassef *et al.* 2013). In a recent study by Shapawi and Zamry (2016), *Kappaphycus* sp., *Eucheuma* sp. and *Sargassum* sp. were used as dietary ingredients, at 5% incorporation level, using Asian seabass (*Lates calcarifer*) as model. Fish growth performance and feed conversion rations were not affected by any of the seaweed meals. However, *Sargassum* sp. caused higher feed intake and ash content when compared to the other diets (Shapawi and Zamry 2016). These studies indicate that seaweed supplementation provides a tool to wield fish growth performance and enhance aquaculture production.

Primarily, the current study aimed to investigate the effects of *Gracilaria* sp. and *Alaria* sp. supplementation at 5 % level on meagre growth performance and aerobic scope. Specifically, we hypothesized that dietary seaweed supplementation modulates this species metabolic rates, shifting energy allocation towards growth. Secondly we aimed to test if meagre immune and antioxidant responses are modulated by seaweeds supplementation and are associated with higher protection against infection. Therefore, a bacterial infection was performed, using *Phdp* as a biotic stressor, to assess the effects of seaweed supplementation in meagre immune status, antioxidant capacities and plasma bioindicators.

Material and Methods

The current study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and in agreement with the guidelines on the protection of animals used for scientific purposes according to European Union directives (2010/63/UE).

Seaweeds and experimental diets

The seaweeds used in this work, from the phyla Phaeophyta (*Alaria* sp.) and Rhodophyta (*Gracilaria* sp.), were produced at ALGAPlus, Lda. (Ílhavo, Portugal) and at Daithi O'Murchu Marine Research Station (DOMMRS, Cork, Ireland) facilities. *Gracilaria* sp. was produced by ALGAPlus in a land based IMTA system, similar to Abreu *et al.* (2011). *Alaria* sp. was produced by DOMMRS in an IMTA system consisting of long lines installed in the vicinity of salmon production cages. The seaweeds were dried and added as separate supplements to the experimental diets at concentrations of 5 % adjusted for dry matter content (DM). Seaweed mineral and proximal compositions are presented in Table 1.

Table 1 Proximate and mineral composition of the used seaweed meals. N = 3 per seaweed.

	Limit Detection (LD)	*Gracilaria* sp.		*Alaria* sp.	
Mineral composition. (mg. kg^{-1} dry tissue)		*Mean*	*SD*	*Mean*	*SD*
Lead (Pb)	0.004	1.1	0.1	< LD	
Mercury (Hg)	0.005	< LD		< LD	
Cadmium (Cd)	0.031	0.2	0.0	1.1	0.2
Tin (Sn)	0.005	0.4	0.0	1.1	0.2
Arsenic (As)	0.074	< LD		< LD	
Iron (Fe)	1.021	511.0	31.0	74	17
Zinc (Zn)	1.908	43.0	1.0	34	6
Copper (Cu)	0.206	1.5	0.1	< LD	
Selenium (Se)	0.832	6.5	0.3	3.1	1.1
Mineral composition (%)					
Potassium (k)	0.034	13.1	0.5	6.5	0.4
Sodium (Na)	0.025	11.7	1.6	7.0	0.3
Magnesium (Mg)	0.001	0.3	0.0	1.4	0.0
Calcium (Ca)	0.002	0.2	0.0	0.6	0.0
Phosphorus (P)	0.006	0.3	0.1	0.5	0.0
Proximate composition (%DM)					
Dry matter		93.4	0.0	88.0	0.1
Crude protein		25.9	0.2	14.3	0.3
Ash		34.3	0.2	31.0	0.3
Crude fat		1.1	0.7	1.3	0.1
Gross energy (kJ g^{-1} DM)		12.8	0.2	12.2	0.1

Each experimental diet was tested in triplicate and formulated to meet meagre nutritional requirements described by Chatzifotis *et al.* (2011a). The experiment comprised three isoenergetic (22 kJ g^{-1} DM), isoproteic (44 %DM) and isolipidic (16 %DM) diets, covering a control diet (CTR) without seaweed supplementation, a *Gracilaria* sp. supplemented diet (GRAC) and an *Alaria* sp. supplemented diet (ALAR). All the ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 2.0 mm pellet size). Lastly, diets were dried at 45 ºC for 12 h and stored at 4 ºC until used. Diets were analyzed in triplicates for dry matter (105ºC for 24 h), protein (Kjeldahl; N x 6.25) after acid digestion, fat content as described by Folch *et al.* (1957) and gross energy content by adiabatic bomb calorimeter (IKA-Werke C5000). Ash content was determined by combustion in muffle furnace (550 °C for 12 h). Detailed diets formulation and proximate composition are presented in Table 2.

Table 2 Feed formulation and proximate composition of the experimental diets.

	Dietary Treatments		
	Control	*Gracilaria* sp.	*Alaria* sp.
Feed ingredients (%DM)			
Na_2CO_3	0.25	0.25	0.25
Diamol	2.75	2.75	2.75
Wheat	27.20	27.20	27.20
Wheat gluten	13.00	13.00	13.00
Fish meal (RE > 680)	13.00	13.00	13.00
Fish oil	14.00	14.00	14.00
Soya protein concentrate	13.00	13.00	13.00
Pea protein concentrate	13.00	13.00	13.00
Lysine HCL	0.30	0.30	0.30
DL-methionine	0.50	0.50	0.50
Monocalcium phosphate	1.50	1.50	1.50
CaCO3	0.50	0.50	0.50
Yttrium oxide	0.01	0.01	0.01
Premix	1.00	1.00	1.00
Gracilaria sp.		5.0	
Alaria sp.			5.0
Proximate composition (%DM)			
Dry matter	93.03	92.34	92.43
Ash	11.21	12.25	15.01
Crude protein	45.02	43.28	42.91
Crude fat	15.34	15.95	15.73
Gross energy (kJ g^{-1} DM)	22.04	22.58	21.79

Fish and rearing conditions

The growth trial was conducted at the Aquatic Engineering laboratory of ICBAS (Porto, Portugal). Meagre juveniles were obtained from a commercial fish farm (MARESA, Spain). Fish were acclimated for two weeks prior to experimentation. Afterwards, 180 meagre (39.70 ± 0.33 g) were randomly distributed in 9 rectangular fiberglass tanks (80 L volume). Each experimental diet was tested in triplicate tanks, with 20 fish per tank. The tanks were connected to a closed recirculation seawater system ensuring similar quality parameters for all replicates. The water flow rate was adjusted to 90 L h^{-1}, while temperature was maintained at 21.5 ± 1.0 ºC and salinity at 29.0 ± 1.0 ‰. Oxygen levels, total ammonium, nitrite, nitrate and pH levels were monitored twice weekly and kept in optimal values for marine species (Tucker Jr 1998) during the entire experiment. Fish were maintained on a 12 hour photoperiod and manually fed until apparent satiety, 2 times a day (at 10:00 h and 15:00 h) for a total of 69 days.

After feeding on the experimental diets for 69 days, 10 fish per treatment were selected for measurements of metabolic rates (respirometry; see below). Body mass and length were

measured to calculate growth parameters. A subset of fish was transported to MARE (Peniche, Portugal), where a sub-lethal infection trial was performed.

Growth performance calculations

Growth performance was analysed based on weight gain, daily growth index (DGI), feed conversion ratio (FCR) and protein efficiency ratio (PER).
Daily growth index (DGI) was calculated as = 100 × [(FBW)$^{1/3}$ – (IBW) $^{1/3}$] × trial duration in days (TD), where FBW and IBW are the final and initial average body weights (g); Feed conversion ratio (FCR) was calculated as = FI (g) / weight gain (g), whereas FI is feed intake on dry matter basis; protein efficiency ratio (PER) was calculated as = wet weight gain (g) / crude protein intake (g). Data were corrected for the slight variation in crude protein between diets adjusting for the dry matter content of each diet. The voluntary feed intake (VFI) (% body weight day^{-1}) was calculated as 100 × [FI (g) / ABW (g) / TD (days)], where ABW is (IBW + FBW) / 2, FI is feed intake and TD is trial duration in days.

Respirometry

Metabolic rates were measured by intermittent flow respirometry using established protocols (Genz *et al.* 2013; Peixoto *et al.* 2015; Rosewarne *et al.* 2016). Static respirometers (each 0.99 L) were submerged in aerated water (≥ 95 % air saturation) and employed to measure rates of oxygen consumption (MO_2; mg O_2 kg^{-1} h^{-1}) in individual fish. A temperature controlling instrument (TMP-REG; Loligo Systems; Tjele, Denmark) was used to maintain water temperature at 21.5 ± 0.1 °C. Oxygen partial pressure (kPa) inside the respirometers was measured using fiber optic sensor technology (PreSens, Regensburg, Germany). The software AutoResp (Loligo Systems Aps, Tjele, Denmark) collected the data and calculated MO_2 from measurements of oxygen content inside the respirometers. Values of MO_2 for individual fish were adjusted to a common body mass of 82.1 g (study average) using a mass exponent of 0.8.
After the growth trial, 10 meagre from each experimental diet treatment were selected randomly and fasted for 24 h to ensure a post-absorptive state. A standard chasing protocol (3 min) was then used to measure maximum metabolic rate (MMR). Following exhaustion, and a 1 min air exposure to further elevate oxygen requirements, fish were transferred to the respirometers where MO_2 recordings started immediately. MMR was the highest of three consecutive measures of MO_2. Next, fish were left undisturbed in the respirometers for 24 h and MO_2 values were logged continuously. Standard metabolic rate (SMR) was estimated as the average of the 10 % lowest MO_2 values (Peixoto *et al.* 2015; Baktoft *et al.* 2016). Aerobic

metabolic scope (AMS) was calculated as the difference between MMR and SMR (in mg O_2 kg^{-1} h^{-1}). Routine metabolic rate (RMR) was estimated as the average MO_2 during the last 4 h of respirometer confinement (i.e. between 09:00 and 13:00 h the day after fish were transferred to the respirometers).

Infection trial

Acclimatization period

Before the pre-test infection trial, meagre were acclimated for 15 days, in rectangular fiberglass 500L tanks, connected to a closed recirculating seawater system. Salinity (35.0 ± 1.0 ‰), temperature (21 ± 1 °C) and photoperiod (12 hour light: 12 hours dark) were kept constant. Oxygen, total ammonium and nitrite levels were kept at optimal values recommended for the species (Tucker Jr 1998).

Preparation of pathogen suspension

The *Photobacterium damselae* subsp*. piscicida* (*Phdp*) strain AQP17.1 was used in the present study. *Phdp* were cultured at 22 °C in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Scharlau) supplemented with NaCl to a final concentration of 1.5 ($g.ml^{-1}$) (TSA-1.5 and TSB-1.5, respectively). To prepare several inocula for infection, bacteria were cultured for 48 h at 22 °C on TSA-1.5 and then inoculated into TSB-1.5 and cultured overnight at the same temperature, with continuous shaking (100 rpm; Stuart). Exponentially growing bacteria were collected by centrifugation at 3500g for 30 min and resuspended in Hanks buffer saline solution (HBSS- Gibco, Sigma). Plating serial dilutions of the suspensions onto TSA-1.5 plates and counting the number of colony forming units (CFU) following incubation at 22 °C for 48 h confirmed bacteria concentration of the inocula. Bacterial turbidity, expressed as absorbance (A), was measured by a spectrophotometer at 600 nm, assuming 1 x 10^8 CFU ml^{-1} = 1A. Once the bacterial concentration was adjusted to 1.0 x 10^8 CFU ml^{-1}, 10-fold serial dilutions were performed down to 1.0 x 10^4 CFU ml^{-1}.

Infection pre-test trial

To estimate the 50 % lethal dose (LD_{50}) of *Phdp*, seventy two fish (28.3 ± 10.9g) were anesthetized by immersion in 0.5ml L^{-1} 2-phenoxietanol (Sigma-Aldrich), before injection with *Phdp* or HBSS. Fish were randomly distributed in four groups of independent closed seawater systems (1 control group and 3 test concentrations: 1.0 x 10^4, 1.0 x 10^5 and 1.0 x 10^6 CFU ml^{-1}). Each group had triplicated aquaria containing 6 meagre. The fish were injected

intraperitoneally (i.p.) with 100µl volumes of *Phdp* bacterial suspensions. Control triplicated aquaria were subjected to i.p. injection with the same volume of HBSS. The survival rate was recorded daily for ten days post-injection. The median lethal dose (LD_{50}) was calculated using the method described by Reed and Müench (1938). Samples were taken aseptically from the kidney of the infected fish and inoculated in TSA-1.5 plates, followed by incubation at 22˚C for 48 hours. The derived LD_{50} value (CTR fish; Table 2) was used in the infection trial described below and covering all diets (CTR, GRAC and ALAR).

Infection trial

Ten fish from the growth trial (body weight: 78.69 ± 24.04 g) from each dietary treatment (i.e. CTR, GRAC and ALAR) were randomly distributed in two independent closed seawater systems (one infected and one non-infected system). Fish were anesthetized by immersion in 0.5 ml L^{-1} 2-phenoxyethanol (Sigma-Aldrich) before injection. The infected fish were injected intraperitoneally (i.p.) with 100µl of *Phdp* bacterial suspension at a concentration of 2.29 x 10^5 CFU ml^{-1}. The non-infected fish were subjected to i.p. injection with the same volume of sterile HBSS, without the *Phdp*. Blood and livers samples were collected at the end of the infection trial from ten fish from each treatment for haematocrit analysis, plasma biochemical and oxidative stress analyses.

Haematocrit

Blood samples (approximately 1 ml) were collected by caudal puncture from six fish per treatment. Prior to blood collection, syringes and tubes were sandblasted with Ethylenediamine tetraacetic acid (EDTA, 0.8M). Haematocrit measurements were performed using 45 µl of blood following previous studies (Soares *et al.* 2012).

Plasma parameters

Plasma samples were aliquoted and stored at -80 ºC until analysed. Plasma analyses were performed using comercial kits adapting the original protocol to microplate assays. Each sample was tested in duplicated and all protocols were performed using one blanc and one calibator in order to standardize results.

Lactate measurements were performed using D-/L-Lactic acid, UV method Kit (NZYTech, Genes and Enzymes) reducing the original protocol volumes 10 times. All procediment followed the original protocol and the absorbance was read at 340 nm after 30 min of reaction. Cholesterol levels in the plasma were quantified using Cholesterol-LQ Kit

(SPINREACT,S.A./S.A.U.) and performed by addition of 1 ml of reagent to 10 µl of plasma sample. After 10 min of dark incubation at 22˚C, 300 µl of solution were transferred to a 96-well plate for further absorbance reading at 505 nm. Glucose concentration was determined using Glucose-RTU kit (SPINREACT,S.A./S.A.U.) adding 250 µl of reagent to 10 µl of plasma sample. The mix was incubated for 20 min at 22˚C and the absorbance read at 505 nm. Triglycerydes measurement was performed using Triglycerides–LQ kit (SPINREACT,S.A./S.A.U.) by addition of 1 ml of reagent to 10 µl of plasma sample. After 10 min of dark incubation at 22˚C, 300 µl of mix were transferred to a plate and the absorbance was read at 505 nm.

Oxidative stress

To analyse the hepatic biomarkers of oxidative stress, livers were homogeneized using phosphate buffer (0.1 M pH 7.4) in a proportion of 1:5 in relation to liver weight. The protein content was determined according to previous studies (Bradford 1976) and used to standardize antioxidant enzymes activities. Lipid peroxidation (LPO) was determined by quantifying the presence of thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.* 1979). Cholinesterase (ChE) activity was evaluated according to Ellman *et al.* (1961), using acetylcholine as substrate. Catalase (CAT) activity was studied based on Clairborne (1985), with hydrogen peroxyde (30 %) as substrate. Glutathione peroxidase (GPx) and Glutathione reductase (GR) were evaluated based on NADPH oxidation at 340 nm (Cribb *et al.* 1989; Mohandas *et al.* 1984). Total (TG) and oxidized (GSSG) glutathiones were evaluated at 412 nm by the formation of 5-thio-2-nitrobenzoic acid, as detailed in Baker *et al.* (1990) and reduced glutathione (GSH) was estimated by the difference between TG and GSSG. Glutathione s-transferase (GST) was determined by absorbance at 340 nm, using 1-chloro-2,4-dinitrobenzene as substrate as Habig *et al.* (1974).

Immune status

Meagre innate immune parameters were accessed by plasma lysozyme and peroxidase activity levels, as well as by alternative complement pathway response. Lysozyme was measured by turbidimetric assay based on *Micrococus lysodeikicus* lysis and using hen egg white lysozyme (Sigma-Aldrich) as standard (Valero *et al.* 2014). Peroxidase activity levels were determined by 3,3', 5,5' – tetramethylbenzidine hydrochloride (Sigma-Aldrich) reduction (Quade and Roth 1997). Hemolytic activity of the alternative complement system was assayed as described by Sunyer and Tort (1995) via rabbit red blood cells agglutination.

Statistical analyses

Prior to analysis, data were scrutinized for ANOVA assumptions including normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test). The growth and respirometry data were analysed applying one-way-ANOVA tests using the dietary treatments as independent variable. Oxidative stress and plasma data were analyzed by two-way-ANOVA using dietary treatments and infection as factors. When the ANOVA test showed significant differences, Tukey's test was applied to compare individual means. Significant differences were considered when $P < 0.05$. When ANOVA assumptions were not satisfied, data were subjected to non-parametric Kruskal-Wallis tests and further analyzed using Spearman's correlation test to identify interactions between factors (McDonald 2014). Specifically, when Spearman's correlation test revealed a correlation coefficient of 1.0, the significant interaction suggested that both dietary treatment and infection had a significant effect. The statistical analyses were carried out using SigmaPlot 11.0 (Systat Software Inc, USA).

Results

Growth performance

In all dietary treatments, meagre exhibited approximately a 2 fold increase in body weight after being fed the experimental diets for 69 days (1.79 - 1.82). Meagre fed the *Alaria* sp. supplemented diet exhibited the higher DGI and VFI values, 1.09 and 1.14 % day^{-1}, respectively, although no significant differences were found in comparison with the remaining groups ($P > 0.05$) (Table 3). As a consequence, no significant differences were evidenced between dietary treatments concerning FCR and PER (Table 3).

Table 3 Growth performance parameters in meagre fed the experimental diets for 69 days. Results are presented as mean ± SD. N = 20 fish per dietary treatment. No growth parameters differed significantly ($P > 0.05$) between dietary treatments.

	Dietary Treatments		
Growth	Control	*Gracilaria* sp.	*Alaria* sp.
Weight gain (g)	31.39 ± 1.77	31.68 ± 2.23	32.43 ± 3.98
Final body weight (g)	70.79 ± 2.23	71.96 ± 2.90	71.84 ± 4.92
Daily growth index (DGI)	1.06 ± 0.06	1.06 ± 0.07	1.09 ± 0.14
Feed conversion ratio (FCR)	1.28 ± 0.12	1.47 ± 0.19	1.33 ± 0.14
Protein efficiency ratio (PER)	1.75 ± 0.16	1.53 ± 0.19	1.68 ± 0.16
Voluntary feed intake (VFI; % day^{-1})	1.05 ± 0.07	1.11 ± 0.03	1.14 ± 0.02

Respirometry

Maximum metabolic rate (MMR) was significantly higher in meagre fed the ALAR diet, when compared to the control ($P = 0.023$) (Fig. 1). MMR related to the GRAC diet was elevated compared to the CTR diet (Fig. 1) however the difference was not statistically significant ($P > 0.05$).

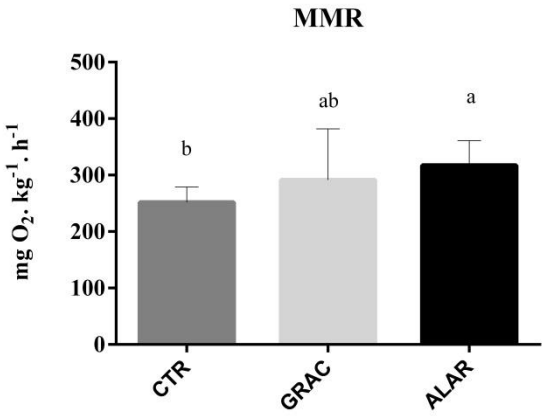

Fig. 1 Maximum metabolic rate (MMR; mg O_2 kg^{-1} h^{-1}) in meagre fed the experimental diets (Table 2) for 69 days. Different letters indicate statistically significant differences ($P < 0.05$). Values are presented as mean ± SD. N = 10 for each dietary treatment.

As showed in Table 4, meagre fed GRAC and ALAR diets revealed no statistically significant findings SMR and AMS appeared elevated among the fish that received the GRAC and ALAR diets. In contrast RMR seemed lower among the fish that received the ALAR diet compared to the GRAC and CTR diets.

Table 4 Standard metabolic rate (SMR), aerobic metabolic scope (AMS) and routine metabolic rate (RMR) in juvenile meagre fed the three experimental diets (Table 2) for 69 days. N = 10 per dietary treatment. Values presented as mean ± SD (mg O_2 kg^{-1} h^{-1}). No statistically significant differences were observed among the dietary treatments. Measures of maximum metabolic rate (MMR) are presented in Fig.1.

	Dietary Treatments		
Metabolic rates	Control	*Gracilaria* sp.	*Alaria* sp.
Standard metabolic rate (SMR)	99.08 ± 30.68	121.08 ± 45.35	117.79 ± 20.47
Aerobic metabolic scope (AMS)	155.65 ± 35.41	170.45 ± 68.31	199.30 ± 38.77
Routine metabolic rate (RMR)	157.46 ± 78.75	166.27 ± 86.90	136.37 ± 24.83

Post-Infection results

Haematocrit

Results for haematocrit levels after infection with *Phdp* are presented in Table 5. The results showed no significant effects ($P > 0.05$) of the dietary supplementations.

Table 5 Haematocrit levels in post-infection meagre. No statistical differences between dietary treatments were observed (P > 0.05). Values are presented as mean ± SD. N = 10 per dietary treatment.

	Dietary Treatments		
Blood	Control	*Gracilaria* sp.	*Alaria* sp.
Haematocrit (%)	23.29 ± 0.87	20.74 ± 0.87	22.50 ± 0.94

On the other hand, pathogen infection significantly affected the haematocrit level despite the dietary treatment. Haematocrit decreased significantly in fish infected with *Phdp* when compared with non-infected (non-infected) group (P = 0.001) (Fig. 2).

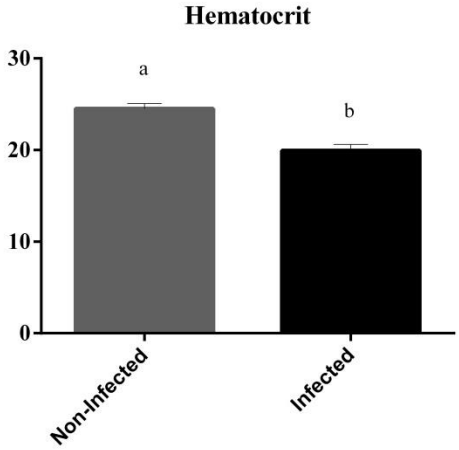

Fig. 2 Haematocrit levels of juvenile meagre subjected to a sub-lethal infection with *Phdp*. Letters indicate statistical differences between infected and non-infected groups (P < 0.001) Values are presented as mean ± SD. N = 10 per dietary treatment.

Two-ANOVA analysis showed no interaction between infection and diet on haematocrit levels (P = 0.428). Means ± S.D. of haematocrit levels in fish subjected to infection and the corresponding non-infected treatment for each diet are shown in Table 6.

Table 6 Haematocrit levels in juvenile meagre. Values presented in percentage as mean ± SD. N = 10 per dietary treatment. No significant interactions between diet and infection were observed (P > 0.05).

		Dietary Treatments		
Blood		Control	*Gracilaria* sp.	*Alaria* sp.
Haematocrit (%)	Non-infected	24.90 ± 2.81	23.20 ± 2.97	25.40 ± 3.24
	Infected	21.80 ± 4.71	18.40 ± 2.76	19.60 ± 2.80

Plasma biochemical parameters

Post infection levels of plasma cholesterol (Fig. 3a), glucose (Fig. 3b) and triglycerides (Fig. 3d) did not vary among the dietary treatments ($P > 0.05$). The findings indicate that these plasma bioindicators are unaffected by seaweed supplementation, even after long term feeding. Plasma lactate levels (Fig. 3c) increased significantly, however, in fish fed ALAR supplemented diet when compared to control diet ($P = 0.004$), suggesting that seaweed supplementation may alter the dynamics of anaerobic pathways.

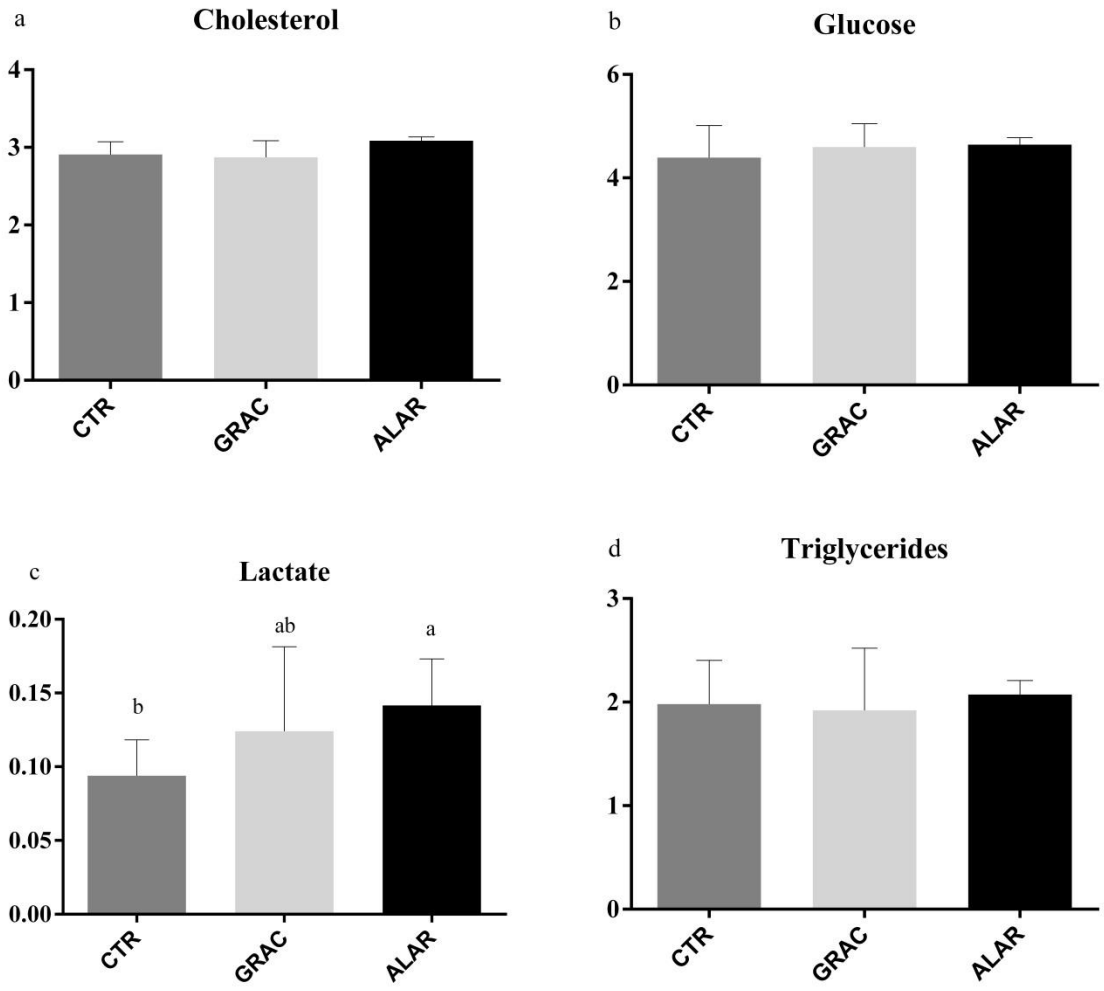

Fig. 3 Plasma parameters, expressed in $nmol.L^{-1}$, in meagre fed the experimental diets and subjected to a sub-lethal infection with *Phdp*. Different letters indicate significant differences at $P < 0.05$. Values are presented as mean ± SD. N = 6 per dietary treatment.

When analyzing the plasma parameters considering only the effect of infection (Table 7), no significant differences were found between groups ($P > 0.05$).

Table 7 Plasma parameters in juvenile meagre, expressed in nmol.L^{-1}. No significant differences (P < 0.05) between non-infected and infected groups were found. Values presented as mean ± SD. N = 6 per dietary treatment.

	Cholesterol	Glucose	Lactate	Triglycerides
Non-Infected	2.93 ± 0.20	4.56 ± 0.52	0.11 ± 0.02	2.16 ± 0.38
Infected	2.92 ± 0.19	4.50 ± 0.43	0.13 ± 0.06	1.69 ± 0.47

Results obtained when analyzing the correlation between diet and infection, are provided in Table 8. No significant differences were found in the analysis (P > 0.05).

Table 8 Plasma parameters in juvenile meagre, expressed in nmol.L^{-1}. Data revealed no statistical differences for the interaction between diet and infection (P > 0.05). Values are presented as mean ± SD. N = 6 per dietary treatment.

		Dietary Treatments		
Hepatic Biomarkers		Control	*Gracilaria* sp.	*Alaria* sp.
Cholesterol	Non-infected	2.92 ± 0.26	2.92 ± 0.25	3.06 ± 0.00
	Infected	2.92 ± 0.22	2.81 ± 0.11	3.10 ± 0.06
Glucose	Non-infected	4.80 ± 0.73	4.57 ± 0.73	4.68 ± 0.16
	Infected	4.40 ± 0.51	4.72 ± 0.23	4.53 ± 0.05
Lactate	Non-infected	0.10 ± 0.03	0.11 ± 0.02	0.10 ± 0.01
	Infected	0.09 ± 0.02	0.10 ± 0.07	0.17 ± 0.01
Triglycerides	Non-infected	2.05 ± 0.03	2.25 ± 0.45	2.09 ± 0.19
	Infected	2.05 ± 0.61	1.50 ± 0.39	2.28 ± 0.35

Oxidative stress

Lipid peroxidation (LPO), revealed highly significant differences between dietary treatments when fish were fed diets containing seaweeds and infected with *Phdp* (Fig.4). Meagre fed the CTR diet showed much higher LPO levels than fish fed GRAC and ALAR diets (P < 0.001). The lowest LPO level was detected when meagre were fed the GRAC diet. These findings indicate that seaweed supplementation may mitigate hepatic lipid peroxidation, particularly with GRAC supplementation.

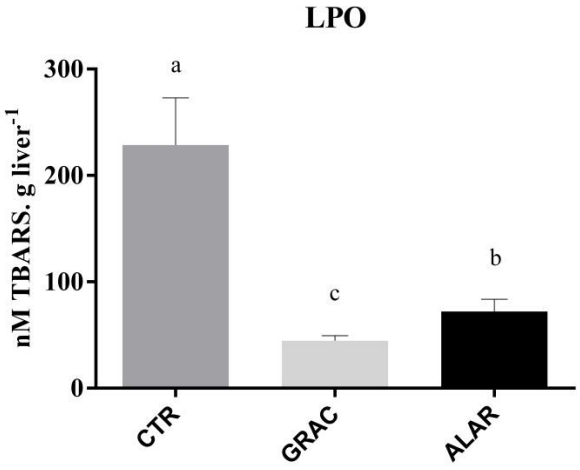

Fig. 4 Lipid peroxidation (LPO) measured in liver of juvenile meagre fed different diets (Table 2) and infected with *Phdp*. Different letters indicate significant (P < 0.05) differences between the dietary treatments. Values are presented as mean ± SD. N = 6 liver per dietary treatment.

Analysis of liver antioxidant enzymes (Table 9) revealed no significant differences between dietary treatments (P > 0.05) when meagre were infected with Phdp. Seaweed supplementation indicated no effect on the meagre antioxidant system.

Table 9 Levels of antioxidant enzymes activities measured in liver of juvenile meagre after infection with *Phdp*. No statistical differences (P < 0.05) between dietary treatments after the sub-lethal infection were detected. Values are presented as mean ± SD. N = 6 per dietary treatment. Units are nmol min^{-1} mg^{-1} protein, unless stated otherwise.

	Dietary Treatments		
Hepatic Biomarkers	Control	*Gracilaria* sp.	*Alaria* sp.
Acetylcholinesterase (AChE)	0.48 ± 0.18	0.50 ± 0.20	0.45 ± 0.21
Catalase (CAT)*	25.93 ± 8.72	24.56 ± 11.17	23.65 ± 7.94
Glutathione peroxidase (GPx)	2.27 ± 0.89	2.01 ± 0.55	1.77 ± 0.46
Glutathione Reductase (GR)	1.34 ± 0.55	1.74 ± 0.88	1.43 ± 0.69
Reduced Glutathione (GSH)	0.10 ± 0.04	0.11 ± 0.03	0.10 ± 0.02
Oxidized Glutathione (GSSG)	0.18 ± 0.06	0.16 ± 0.05	0.16 ± 0.08
Glutathione s-transferase (GST)	158.55 ± 52.05	124.82 ± 45.28	128.34 ± 46.50
Total Glutathione (TG)	0.28 ± 0.05	0.27 ± 0.08	0.26 ± 0.09

*The unit of CAT is µmol min^{-1} mg^{-1} protein.

In terms of the effects of the sub-lethal bacterial infection with *Phdp*, the results for antioxidant enzymes activities revealed several significant findings (Fig. 5). A higher value was observed in non-infected fish for the enzymes CAT (P = 0.016), GPx (P = 0.009), GR (P = 0.003), GSSG (P < 0.0001) and TG (P < 0.0001) (Figs. 5b, c, d and f). These enzyme activities

decreased approximately 1.5 fold when meagre were subject to infection. In contrast, infection had no significant effect on AChE, GSH and GST activities (Fig. 5a, e, g) ($P > 0.05$). These results indicate a notorious influence of infection over the antioxidant status.

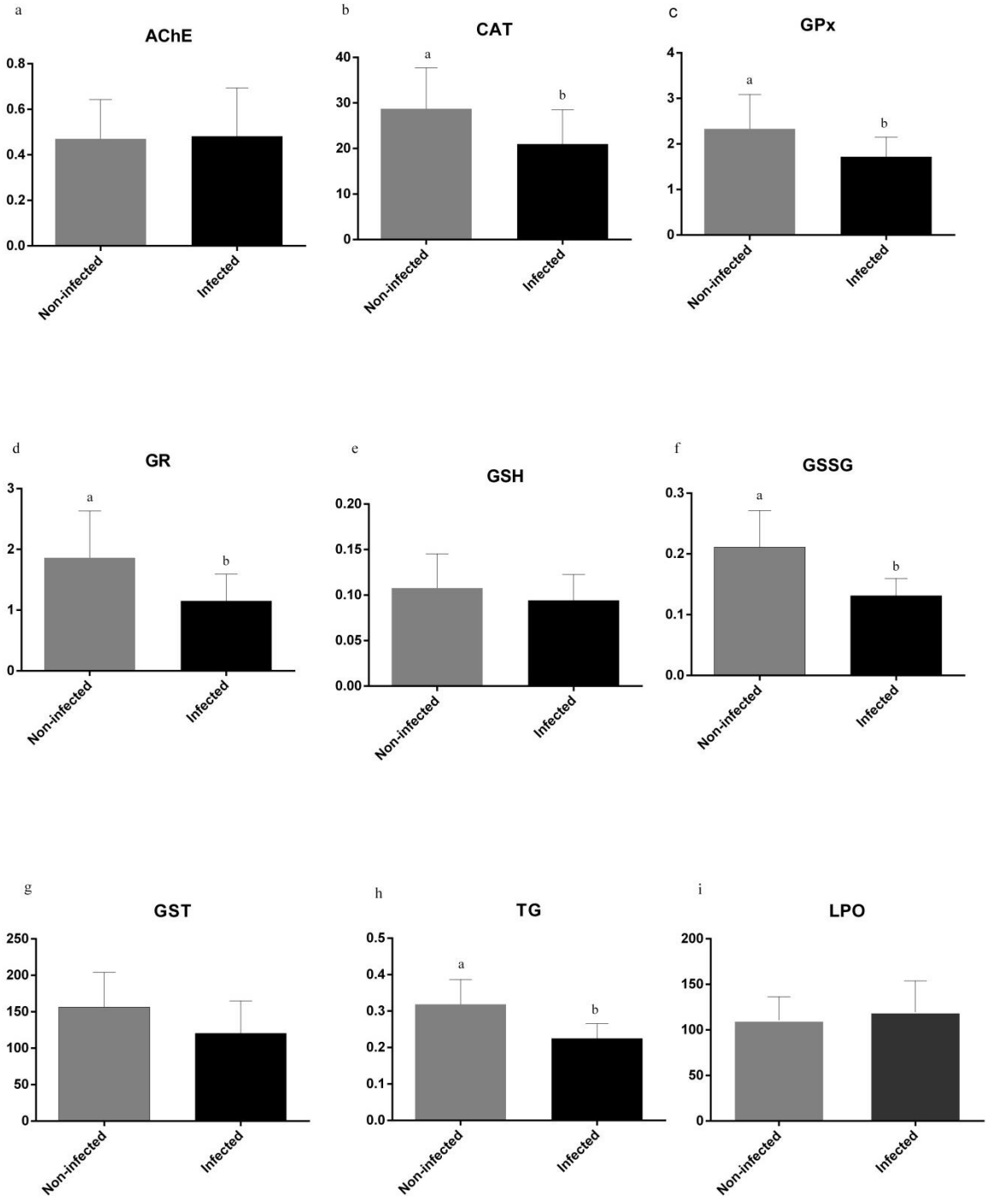

Fig. 5 Hepatic levels of acetylcholinesterase (**a**), catalase (**b**), glutathione peroxidase (**c**), glutathione reductase (**d**), reduced glutathione (**e**), oxidized glutathione (**f**), Glutathione s-transferase (**g**), total glutathione (**h**), lipid peroxidation (**i**) of juvenile meagre subjected to a sub-lethal infection with *Phdp*, regardless of the dietary treatment. Letters indicate statistical ($P < 0.05$) differences between infected and non-infected groups. Values are presented as mean ± SD. N = 6 per dietary treatment. Units are nmol min^{-1} mg^{-1} protein except for catalase which is µM min^{-1} mg^{-1} protein and lipid peroxidation which is nmol TBARS. g $liver^{-1}$.

Analyzing the interaction between diets and infection for antioxidant enzymes activities (Table 10), significant differences were found for GPx and GSH within the CTR diet. GPx level of activity decreased in infected fish when fed CTR diet (P = 0.014) compared to the other diets. GSH activity was also affected by the interaction between factors, as its activity increased in infected fish fed with CTR diet (P = 0.009). These findings indicated that fish fed the CTR diet were more susceptible to infection than the fish fed seaweed supplemented diets. In contrast, AChE, CAT, GR, GSSG and TG activities, and LPO levels, were not significantly affected by the interaction between diets and infection (P > 0.05) (Table 10).

Table 10 Levels of antioxidant enzymes activities measured in liver of juvenile meagre after infection with *Phdp*. Letters indicate statistical (P < 0.05) differences for the interaction between diet and infection. Values presented as mean ± SD. N = 6 per dietary treatment. Units are nmol min^{-1} mg^{-1} protein except for catalase which is µM min^{-1} mg^{-1} protein and lipid peroxidation, which is nmol TBARS . g $liver^{-1}$.

		Dietary Treatments		
Hepatic Biomarkers		Control	*Gracilaria* sp.	*Alaria* sp.
Acetylcholinesterase (AChE)	Non-infected	0.37 ± 0.11	0.54 ± 0.05	0.52 ± 0.24
	Infected	0.59 ± 0.17	0.47 ± 0.27	0.39 ± 0.17
Catalase (CAT)	Non-infected	32.47 ± 6.37	30.25 ± 13.34	23.77 ± 6.21
	Infected	19.39 ± 4.88	20.00 ± 7.59	23.54 ± 10.17
Glutathione peroxidase (GPx)	Non-infected	3.02 ± 0.53[a]	2.04 ± 0.58	1.88 ± 0.63
	Infected	1.52 ± 0.33[b]	1.98 ± 0.59	1.65 ± 0.23
Glutathione Reductase (GR)	Non-infected	1.62 ± 0.68	2.44 ± 0.70	1.63 ± 0.79
	Infected	1.05 ± 0.15	1.18 ± 0.55	1.22 ± 0.59
Reduced Glutathione (GSH)	Non-infected	0.08 ± 0.04[b]	0.14 ± 0.02	0.11 ± 0.03
	Infected	0.11 ± 0.04[a]	0.08 ± 0.02	0.09 ± 0.01
Oxidized Glutathione (GSSG)	Non-infected	0.22 ± 0.05	0.20 ± 0.03	0.20 ± 0.09
	Infected	0.14 ± 0.02	0.13 ± 0.04	0.12 ± 0.02
Glutathione s-transferase (GST)	Non-infected	154.33 ± 73.47	151.98 ± 45.42	161.86 ± 19.27
	Infected	162.77 ± 25.56	103.10 ± 35.03	94.83 ± 41.06
Total Glutathione (TG)	Non-infected	0.30 ± 0.06	0.34 ± 0.03	0.32 ± 0.10
	Infected	0.25 ± 0.03	0.22 ± 0.05	0.21 ± 0.03
Lipid peroxidation (LPO)	Non-infected	147.12 ± 27.04	43.22 ± 4.45	74.51 ± 49.35
	Infected	146.25 ± 76.66	34.23 ± 2.75	69.83 ± 21.38

Discussion

This study is the first study to evaluate the application of dietary seaweed supplementation in meagre. Our findings suggest that seaweed supplementation may be used to modulate fish performances to match aquaculture rearing conditions. Improved fish performance is associated with elevated survival during rearing and reduced stress related to fish holding conditions, ultimately providing a better commercial product. Growth parameters after 69 days of feeding (Table 3) showed a similar weight increase for all dietary treatments, suggesting that seaweeds can be included in meagre diets without compromising growth rates. Additionally, the 2 fold increase in body mass reported here is in accordance with the results provided by Emre *et al.* (2015) when feeding meagre a 46 % crude protein diet, for 69 days at 17.40 ± 0.07 °C. The results reported in our work for DGI, FCR and PER are in agreement with previous data reported for meagre juveniles when fed diets with equivalent basal composition (43-47 % crude protein, 14-18 % crude lipids) (Chatzifotis *et al.* 2010; Emre *et al.* 2015). Similar values have also been reported for other carnivorous fish species such as seabass (Peixoto *et al.* 2016a), seabream (Peres *et al.* 2011) and Senegalese sole (*Solea senegalensis*) (Conde-Sieira *et al.* 2016), fed to satiety under cultivation conditions. Furthermore, VFI in supplemented diets was similar to control, suggesting that seaweed inclusion is palatable and readily accepted.

Fish growth rates involve significant energy costs, constraining fish energy budgets, which results in dynamic energetic trade-offs between life history related traits such as self-maintenance, immunity, sexual maturity and locomotion (Arendt 1997; Arnott *et al.* 2006). With these purposes, oxygen consumption rates of meagre fed diets supplemented with seaweeds were used as proxy for metabolic rates (i.e. ATP turn-over rate; (Salin *et al.* 2015). Although studies have reported that fish diet may influence metabolic performance (Vagner *et al.* 2014), our results suggest that seaweed supplementation has little or no effects on SMR, RMR and AMS, indicating that these metabolic traits may be persistent over time, even if diet is changing. Our study is the first to measure metabolic rates in meagre; however, it is important to highlight that our metabolic rate data are within expected values considering what is described in the literature for other fish species, including European seabass (Jourdan-Pineau *et al.* 2010; Peixoto *et al.* 2015) tested at similar temperatures. Factors causing variation in MMR are uncertain and remain a subject of recent interest (Van Leeuwen *et al.* 2011; Killen *et al.* 2014; Hedrick *et al.* 2015; Killen *et al.* 2016; Metcalfe *et al.* 2016). The results observed when measuring MMR (Fig. 1) indicate an influence of dietary seaweed supplementation, since both supplemented diets showed higher values than control, although only the *Alaria* sp. supplemented diet showed significant differences. The increase in MMR suggests higher capacity for fish to deal with stressful situations (Norin and Clark 2016), indicating that meagre

fed supplemented diets exhibit improved capacity to adapt to aquaculture conditions. The physiological basis of the relationship between diet and MMR remains uncertain and deserves further research. Additionally, oxygen consumption rates should be measured after a pathogen infection to analyze any possible modulation on the energetic scope caused by a biotic stressor.

In order to ascertain possible immune and anti-oxidant benefits for meagre by dietary seaweed supplementation, and to better understand this species when dealing with biotic stressors in aquaculture, an infection trial was performed using *Photobacterium damselae* subsp*. piscicida* (*Phdp*). The most common innate immune parameters were tested including lyzozyme, peroxidase and alternative complement pathway (ACH50). Interestingly, the levels detected for these parameters, in undiluted plasma, were low and highly variable. A similar result was obtained by Soares *et al.* (2012) when analyzing lyzozyme in meagre infected by *Vibrio* sp.. These authors suggested that meagre may activate diverse defence mechanisms against different stresses. Haematocrit level is another parameter analysed in infected fish since alterations in haematocrit levels often represent a response from fish to pathogens and has been pointed as an indicator of stage of disease development (Olsen *et al.* 1992). In the present work, when analyzing haematocrit levels in relation to diets (Table 5), no differences were found for meagre fed diets supplemented with seaweeds. Also, when testing for an interaction between diet and infection (Table 6) no differences were found in haematocrit two weeks post-infection. According to Frisch and Anderson (2000), haematocrit values respond to stimulli for a short period of time (30 to 60 minutes) and return to normal levels afterwards, even if the stimulli remains present. This hypothesis indicates that differences in haematocrit levels associated with dietary seaweed supplementation may not be persistent after long-term feeding. The haematocrit analyzed, and considering only infection revealed lower levels in infected animals (Fig. 2), consistent with the results found in Rainbow trout after a viral infection (Rehulka 2003) and Caspian salmon (*Salmo trutta caspius*) after a fungal infection (Jamalzadeh *et al.* 2009). Another study using Atlantic salmon (*Salmo salar*) injected with an organ homogenate from individuals of the same species suffering from infectious salmon anaemia, revealed low haematocrit levels in infected fish associated with elevated lactate levels (Olsen *et al.* 1992). In the present study, lactate levels showed no direct relation to haematocrit levels, and no differences were detected when analyzing data regarding infection (Table 7) or the interaction between diets and infection (Table 8). However, dietary seaweed supplementation affected lactate levels, with meagre fed *Alaria* sp. diet presenting significantly higher lactate than the control and *Gracilaria* sp. diets (Fig. 3). It is well known that lactate levels in fish are described to increase as a result of muscle anaerobic metabolism, for example after exercise (Olsen *et al.* 1992; Kieffer 2000). Interestingly, both lactate level and MMR were elevated in fish fed *Alaria* sp. diet, indicating that the two variables are related. The higher

MMR values detected in meagre fed *Alaria* sp. diet may, therefore, be associated with higher physical activity and higher recruitment of white muscle fibres, which induces glycogen breakdown and possibly explains the higher lactate levels detected in meagre fed seaweed supplemented diets. Other plasma metabolites such as cholesterol, triglycerides and glucose are known to respond to dietary modulation involving lipid and starch contents (Du *et al.* 2005; Hamre *et al.* 2004). In our study, the basal formulation of the three tested diets was the same, which may explain the absence of statistical differences between dietary treatments (Figs. 3 a, b and d) as well as the absence of interaction between diet and infection (Table 8). Several studies with fish fed diets containing high plant derived protein sources revealed low choleterol and lipid derivatives levels (Laporte and Trushenski 2012; Maita *et al.* 1998), which have been associated with higher susceptibility to disease (Maita *et al.* 1998; Deng *et al.* 2013). On the contrary, the substitution of fish meal for soybean meal in sunshine bass (*Morone chrysops x M. saxatilis*) exposed to an abiotic stress, revealed elevated glucose levels associated with diet, stress and the interaction between both factors, 45 minutes after exposure (Laporte and Trushenski 2012). The results reported in the present work for glucose may therefore be explained by the sampling time, since meagre blood samples were collected 2 weeks after the infection. A recent study with seabream showed decreased plasma lipid content and glucose levels when fish were fed diets supplemented with *Ulva* sp. and *Gracilaria* sp. at 25 % (Vizcaíno *et al.* 2015). On the contrary, Nile tilapia (*Oreochromis niloticus*) fed diets supplemented with spirulina (*Arthrospira fusiformis*) revealed increased levels in these parameters, as well as increased growth (Belal *et al.* 2012). In our work, depite the absence of statistical differences, the observed values for cholesterol, triglycerides and glucose are in accordance with the previously reported values for meagre fed diets with similar protein and lipid contents (Chatzifotis *et al.* 2010).

The effects of bacterial infection on the health status of cultured fish can be investigated using various biomarkers of oxidative stress such as lipid peroxidation (LPO), which is considered a biomarker not only for oxidative stress, but also as an indicator of stress in general (Marcogliese *et al.* 2005). In the present research, LPO levels decreased in meagre fed diets supplemented with seaweeds (Fig. 4), revealing a positive effect on cell walls lipids integrity. Moreover, meagre fed with *Gracilaria* sp. showed the lowest LPO levels, highlighting the pronounced antioxidant properties associated with red seaweeds. These results are in accordance with the MMR measurements (Fig. 1), since dietary seaweed supplementation allowed for higher energy allocation in biologically challenging conditions, without compromising cell walls integrity. In contrast, no other oxidative stress biomarker showed differences between dietary treatments (Table 9). This absence of differences may be associated with no critical changes in their catabolism, as indicated by the absence of differences between meagre basal metabolism (SMR, Table 4) for all diets. On the other hand,

when considering the effects of the infection (Fig. 5), differences in antioxidant enzymatic activities were detected when comparing infected with non-infected meagre, suggesting declined enzymatic activities in infected groups. An earlier study with seabream showed that *Phdp* infection led to the accumulation of granulocytes, monocytes and eosinophilic granular cells in the peritoneal fluid, as well as vasodilatation and edema on the muscle adjacent to the site of the injection (Noya *et al.* 1995). In addition, the authors reported that macrophages exhibited phagocytized bacteria, often intact and even dividing. Skarmeta *et al.* (1995) analyzed the macrophage found in rainbow trout, seabass and seabream infected with *Phdp*, and revealed that these had significant oxygen reactive species (ROS) production released in the respiratory burst. Kurhalyuk and Tkachenko (2011) found similar macrophage response in brown trout (*Salmo trutta*) infected with ulcerative dermal necrosis, followed by a decrease in the main anti-oxidant enzymes (SOD, CAT, GPx and GR). The authors suggested that ROS damage on structural proteins may relate to anti-oxidant activity decrease. In fact, proteins modified by oxidation were greatly increased in the liver. In the present work fish infected with *Phdp* had significant lower antioxidant enzymes activities when compared to non-infected groups (CAT, GPx and GR). Considering that *Phdp* affects the fish liver, causing tissue enlargement and color changes (Stephens *et al.* 2006; Rivas *et al.* 2013), it is possible to infer that ROS production against *Phdp*, may be the reason for anti-oxidant enzymes decrease. Altogether, these authors' findings explain the efficiency of the infection by *Phdp* and offer an hypothesis for the enzymes activity levels detected in our work. Despite a clear effect of the bacterial infection, no interactions were found between dietary seaweed supplementation and infection.

Conclusions

The results analyzed in this work have implications to the aquaculture sector since dietary seaweed supplementation may be used to improve meagre energetic metabolism, antioxidant capacities and overall health status. Dietary seaweed supplementation with 5 % of *Gracilaria* sp. or *Alaria* sp. in practical diets for meagre have no impact on growth performance after 69 days despite triggering higher maximum metabolic rates. The lack of response to the most common protocols for immune indicators leaves a gap in understanding how seaweeds may help meagre cope with both biotic and abiotic stressors. The infection trial with *Phdp* showed to affect antioxidant enzymes activities despite little influence from diet. In summary, our results indicate that seaweed could be used as supplement in aquafeeds as a tool to modulate specific traits in order to enhance meagre health status.

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CHAPTER V

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Effects of dietary supplementation of *Gracilaria* sp. extracts on fillet quality, oxidative stress, and immune responses in European seabass (*Dicentrarchus labrax*)

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CHAPTER V

EFFECTS OF DIETARY SUPPLEMENTATION OF *GRACILARIA* SP. EXTRACTS ON FILLET QUALITY, OXIDATIVE STRESS AND IMMUNE RESPONSES IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*).

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Abstract

The current study evaluated the effects of two different fractions derived from the methanolic extraction of the red seaweed *Gracilaria* sp. supplemented in European seabass (*Dicentrarchus labrax*) diets. With that purpose, three experimental diets were prepared: a basal diet (control), a control supplemented with the methanolic extract (GE, 0.5% diet) and a control supplemented with the insoluble residue of the GE extraction (GR, 4.5%). Seabass with an average initial weight of 16.5±0.6 g were fed the experimental diets for 42 days and the following parameters were evaluated: growth indicators, digestive enzymes activities, immune and oxidative stress responses, fillet pH and color (L*, a* and b* values) and skin color. The dietary supplementation of GE or GR had no effect on growth performance, digestive enzymes activities, fillet pH and color. Skin color was significantly lighter (L*) in fish fed GE (83.9±1.9) and GR (84.3±2.3) diets when compared with the control group (81.9±3.8). The dietary treatments did not affect the oxidative stress biomarkers. Alternative complement pathway (ACH50) was significantly higher in fish fed GE diet (168.2±13.4 EU.mL^{-1}) than in the control diet (113.1±31.4 EU.mL^{-1}). No dietary effect was observed on peroxidase and lysozyme activities. The current study indicates that dietary supplementation of *Gracilaria* sp. methanolic extracts may have little influence on the innate immune system and skin color in seabass.

Keywords: *Dicentrarchus labrax*; *Gracilaria* sp. extract; fillet quality; dietary seaweed supplementation; fish welfare.

Introduction

Seaweeds are an abundant source of bioactive compounds, such as polysaccharides and pigments, which are extremely valuable for the food and pharmaceutical industries (Cardoso *et al.* 2014). The potential applicability of the resulting seaweed by-products generated by these industries has evoked considerable interest. Previous studies using fish species relevant for aquaculture have shown that seaweeds could be a valuable source of nutrients for aquafeeds (Ramalho Ribeiro *et al.* 2015; Soler-Vila *et al.* 2009; Valente *et al.* 2006). In fact, dietary seaweed supplementation has been associated with improvements in fish growth performance (El-Tawil 2010), immune response (Raghunathan *et al.* 2014), and antioxidant capacity (Peixoto *et al.* 2016), together with changes in flesh color (Soler-Vila *et al.* 2009). Conversely, Déléris *et al.* (2016) reported that the presence of anti-nutritional factors in seaweeds causes digestive enzymes inhibition *in vitro*. This suggests possible negative effects on the fish gastrointestinal tract, which can consequently reduce growth performances. Dietary inclusion of 9-13.5% of crude *Gracilaria arcuata*, in African catfish *Clarias gariepinus* resulted in decreased growth (Al-Asgah *et al.* 2016). In European seabass, the dietary inclusion of 10% of *Gracilaria bursa-pastoris* or 5% of *Gracilaria cornea* had a negative impact on growth performance (Valente *et al.* 2006). On the contrary, the dietary addition of 2.5-7.5% of *Gracilaria* sp. had no negative effect on growth performance and digestive capacity of seabass (Peixoto *et al.* 2016). Considering such, and in accordance with Evans and Critchley (2014), it is advised to limit seaweed supplementation to a maximum of 10% level in fish diets. However, the previous studies on how the dietary inclusion of seaweeds affects fish performance are inconclusive and seem dependent on seaweeds processing, species and dietary level of inclusion.

Gracilaria sp., a red seaweed (Rhodophyta), is an important source of polysaccharides mainly applied in the phycocolloids (agar) industry. This seaweed is also rich in polyphenols and sulphated polysaccharides, both compounds observed to reduce the attack of free radicals in tissues, minimizing oxidative stress (Fidelis *et al.* 2014). Moreover, Araújo *et al.* (2015) suggested that *Gracilaria* sp. supplementation in diets could help to prevent the oxidation of packaged fish, even after freezing, increasing its shelf-life. Rhodophyta species are characterized by their photosynthetic pigments, such as chlorophyll a, carotenoids (β-carotene, lutein and zeaxanthins), and phycobilins (R-phycocyanin and R-phycoerythrin) (Cardoso *et al.* 2014). These natural pigments may enhance the potential of seaweed inclusion in fish feeds, replacing or reducing the use of artificial colorants currently applied in the industry (Nickell and Bromage 1998). Additionally, *Gracilaria* sp. have high content of mineral and bioactive compounds, essential components in aquafeeds. Furthermore, red seaweeds contain lectins, alkaloids, glycosides, terpenoids, flavonoids, saponins, tannins and phytic acid

(Sangeetha *et al.* 2014). These compounds however need further consideration as, at high dietary levels, may act as anti-nutritional factors interfering with bioavailability and/or digestibility of nutrients (Al-Asgah *et al.* 2016).

To summarize, due to the natural bioactive compounds present in seaweeds, they should be considered as valuable dietary supplements, which may help to decrease the susceptibility of fish to stressors. Fish vulnerability to biotic and abiotic stressors is a well-documented restrictive factor in intensive aquaculture production, including the European seabass. This carnivorous species has a great relevance for the Mediterranean aquaculture sector, being the second most cultivated species in Portugal.

To our knowledge, the current study is the first attempt to evaluate the use of dietary supplementation of *Gracilaria* sp. extracts in fish fillet quality and digestive capacity. To better access the impact of dietary *Gracilaria* sp. supplementation on seabass welfare, were analyzed selected biomarkers from the innate immune and oxidative stress systems.

Materials and Methods

All procedures were conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE. The experiment took place at the aquaculture facilities of the Institute for Marine Resources and Ecosystem Studies (IMARES; now Wageningen Marine Research – WMR, Yerseke, The Netherlands) complied with the current laws of the Netherlands and were approved by the animal experimental committee (DEC no. 2014085).

Preparation of Gracilaria sp. methanolic fractions

The seaweeds used in this work were produced and supplied by ALGAPlus (Ílhavo, Portugal). The mineral composition of *Gracilaria* sp is depicted in Table 1. Seaweed was harvested, washed, dried and grinded and the resulting powdered material (about 3 Kg) was percolated with methanol, at room temperature, until exhaustion. The methanolic solution was evaporated, under reduced pressure, to give a crude methanol extract (382 g).

Table 1 Mineral composition of the experimental seaweed. Values presented as Mean±Standard Deviation (SD), for n=3.

Sample (dry tissue)	**Detection Limit (DL)**	**GE**	**GR**
Pb (mg/kg)	0.0045	0.43 ± 0.02	0.85 ± 0.02
Hg (ug/kg)	71.3	< DL	< DL
Cd (ug/kg)	4.97	6.1 ± 1.2	23.5 ± 1.3
Sn (mg/kg)	0.040	0.26 ± 0.01	0.20 ± 0.03
As (ug/kg)	74.2	< DL	< DL
Fe (mg/kg)	0.206	1978 ± 233	2310 ± 160
Zn (mg/kg)	0.043	9 ± 1	11 ± 2
Cu (mg/kg)	0.040	3.23 ± 0.26	1.06 ± 0.12
Se (mg/kg)	0.832	3.4 ± 0.8	2.9 ± 0.6
K (%)	0.057	0.84 ± 0.10	0.10 ± 0.02
Na (%)	0.032	1.08 ± 0.11	0.44 ± 0.05
Mg (%)	0.002	0.18 ± 0.01	0.23 ± 0.01
Ca (%)	0.007	0.13 ± 0.02	0.08 ± 0.01
P (%)	0.004	0.82 ± 0.18	0.88 ± 0.03

Part of the crude methanol extract (88 g) was applied over a Silica Gel column (0.2-0.5 mm, 376 g) and eluted with mixtures of hexane, EtOAc and Me_2CO, wherein 250 mL fractions (frs) were collected as follows: frs 1-91 (hexane-EtOAc, 12:1), frs 92-126 (hexane-EtOAc, 10:1), frs 127-161 (hexane-EtOAc, 7:1), frs 162-221 (EtOAc), frs 222-234 (EtOAc-Me_2CO, 1:1), frs 235-239 (Me_2CO). Frs 31-41 were combined (590 mg) and crystallized in methanol to give 516.0

mg of cholesterol (soluble extract - GE). Frs 61-141 were combined (590 mg) and applied on a Silica gel column (0.2-0.5 mm, 13 g) and eluted with mixtures of petroleum ether, $CHCl_3$ and Me_2CO, 100 mL fractions were collected as follows: frs 1-8 (petroleum ether $CHCl_3$, 7:3), 9-34 (petroleum ether -$CHCl_3$, 1:1), 35-48 (petroleum ether $CHCl_3$, 3:7), 49-90 (petroleum ether - $CHCl_3$, 1:9). Frs 20-25 were combined (42.5 mg) and crystallized in MeOH to give 10.0 mg of palmitic acid (insoluble residue - GR). Overall, fractionation of the crude methanolic extract of *Gracilaria* sp. by column chromatography of silica gel with mixtures of hexane, ethyl acetate and acetone, followed by crystallization, led to the isolation of cholesterol and palmitic acid as major compounds. Their structures were determined by analysis of their ^{1}H and ^{13}C NMR data as well as High Resolution Mass Spectrometry. Phenolic compounds were detected by characteristic UV absorption and a positive $FeCl_3$ test.

Experimental diets

Three diets were manufactured by SPAROS Lda. (Olhão, Portugal) and supplied to seabass for 42 days. The three experimental diets were isoproteic (50% crude protein), isolipidic (20% crude lipid) and isoenergetic (23 $Kj.g^{-1}$ gross energy). The diets were formulated as follows: without seaweed supplementation (control diet), with 0.5% *Gracilaria* sp. extract (GE diet) or with 4.5% *Gracilaria* sp. residue (GR diet) (Table 2). The percentage of supplementation of the extract and residue were based on previous study carried out from our research group (Peixoto *et al.* 2016), indicating that the inclusion of 5% of raw *Gracilaria* meal was adequate for seabass feed. In the current study, *Gracilaria* meal had an 10% yield of extract material (E) and 90% of residue (R), thus the experimental diets were prepared with 0.5% extract or 4.5% residue to simulate the proportions in the raw seaweed meal. Powder ingredients (Table 2) were grounded to below 100 micra in a micro-pulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain) and the mixture was humidified with 25% water.

Table 2 Ingredients used in the experimental diets formulation (%). CTRL: diet without seaweed; GE: CTRL diet supplemented with 0.5% *Gracilaria* sp. extract; GR: CTRL diet supplemented with 4.5% *Gracilaria* sp. residue.

Dietary treatments			
Ingredients	**CTRL**	**GE**	**GR**
Fishmeal Standard	10.0	10.0	9.6
Fishmeal SOLOR	20.0	19.9	19.1
Soy protein concentrate (Soycomil)	11.8	11.7	11.3
Wheat gluten	4.0	4.0	3.8
Corn gluten	8.0	8.0	7.6
Soybean meal 48	12.0	11.9	11.5
Rapeseed meal	5.0	5.0	4.8
Wheat meal	9.2	9.2	8.8
Peas gelatinized (Aquatex 8071)	3.2	3.2	3.1
Fish oil - COPPENS	6.5	6.5	6.2
Soybean oil	4.0	4.0	3.8
Rapeseed oil	4.0	4.0	3.8
Vit & Min Premix PV01	1.0	1.0	1.0
Binder (Kieselghur)	0.5	0.5	0.5
Monocalcium Phosphate	0.5	0.5	0.5
L-Lysine	0.2	0.2	0.2
DL-Methionine	0.1	0.1	0.1
Gracilaria extract		0.5	
Gracilaria residue			4.5

Seaweed extracts were dissolved in absolute ethanol and mixed in the oil fraction prior to the incorporation in the feed mash. Diets were extruded to a pellet size of 2.0 mm by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 3 hours at 45°C. The chemical composition of the diets (Table 3) was analyzed according to Horwitz *et al.* (2006). Briefly, the humidity percentage was calculated after lyophilization. The crude protein (N × 6.25) was determined using a Leco N analyzer (Model FP-528, Leco Corporation, St. Joseph,USA). Crude lipid content was determined by extraction using petroleum ether at 40–60◦C (SoxtecTM2055 Fat Extraction Sys-tem, Foss, Hilleroed, Denmark). The ash content was analyzed by combustion, 6 h at 500◦C, in a muffle furnace (NabertherмL9/11/B170, Bremen, Germany). Finally, the gross energy was quantified in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Staufen,Germany).

Table 3 Chemical composition of the experimental diets (%, unless otherwise stated).

Chemical composition **(DM%)**	**CTRL**	**GE**	**GR**
Dry matter	93.1	93.5	94.5
Crude protein	50.1	50.2	49.1
Crude lipid	20.1	20.2	18.9
Ash	8.9	8.8	9.1
Gross Energy (kJ g^{-1})	22.7	23.0	22.6

Fish and rearing conditions

Juvenile seabass (average weight of 4 g) were housed for 12 weeks in a holding tank, connected to a flow through system of filtered seawater. Seabass were automatically fed *ad libitum* throughout the day (09:30 – 16.30), with a commercial diet (MAR-Perla MP-T, Trouw France), abundantly dispersed across a feeding belt. Two weeks prior to the feeding trial, seabass (average weight 16.5±0.6 g) were randomly distributed into the experimental tanks (120 fish per tank, 360 fish per treatment). The experimental setup (Fig. 1) consisted of 3 recirculation water systems (RAS) with 3 tanks each, ensuring that each RAS had a tank of each of the experimental diets. The tanks had 900 L capacity and the water flow rate per tank was set at 1 m^3 h^{-1}.

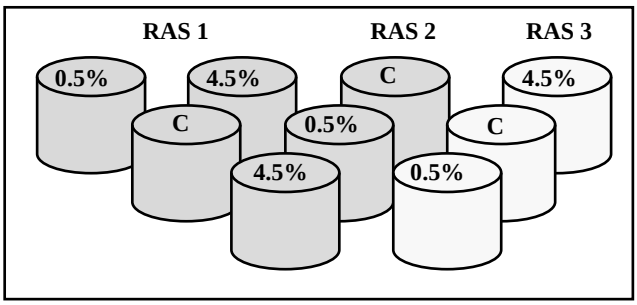

Fig. 1 Experimental set up showing the recirculating water systems (RAS) organization and experimental diets distribution.

The feeding schedule was daily adjusted for expected growth and fish mortality and the amount of diet set at 2% (DM) of average body weight. Several times per day, the tanks were inspected for left-over feed. The observed mortality during the experiment was 3.89% for control, 1.94% for GE and 3.61% for GR.

The physical-chemical parameters of the rearing water, such as O_2 level (85±10 % saturation), pH (7.9±0.6), temperature (20.4±0.6 ºC) and salinity (30 ppm) were monitored and adjusted during the entire trial. Total ammonium and nitrates maintained at residual levels. The photoperiod was set for 12 h light:12 h dark.

At the end of the feeding trial, 22 fish per treatment were anaesthetized with clove oil (0.4 mL L^{-1}), weighted and measured for the calculation of the growth performance parameters. Blood

samples were withdrawn from the caudal vein and then centrifuged (5 min, 10.000 rpm at 4 ºC), and plasma stored at -80 ºC, until immune parameters analyses. Additionally, liver and intestine were weighed and stored at -80 ºC, pending oxidative stress and digestive enzymes analyses. Fillet samples from ten fish per treatment were sampled and stored at 4 ºC, for color and pH measurements.

Growth Performance

Weight gain (WG) was calculated as $W_F - W_I$, where W_I is the initial body weight at the start of the feeding trial and W_F is the final body weight. Daily growth index (DGI, % body weight day^{-1}) was calculated as: $100 \times [(FBW)^{1/3} - (IBW)^{1/3}] \times$ days, where IBW and FBW are the initial and the final average body weights (g) respectively. Voluntary feed intake (VFI, % body weight/day) was calculated as: VFI = 100 × [feed intake (g) / ABW (g) / trial duration (days)], where ABW (average body weight) is (IBW + FBW) / 2. Feed conversion ratio (FCR) was calculated as: feed intake (FI) (g) / weight gain (g). The hepatosomatic index (HSI) was calculated as 100 × [liver weight (g)/fish weight (g)].

Digestive enzymes activities

Digestive enzymes activities were measured with whole intestine, homogenized in 50 mM Tris-HCl and 200 mM sodium chloride buffer (pH 8.0). The activity of α-amylase followed the increase in maltose procedure, by the hydrolysis of α-D (1,4) glycosidic bond in polysaccharides, and stained with 3.5-dinitrosalicylic acid (DNS). Lipase activity assay was performed using ρ-nitrophenyl as substrate. Proteolytic enzymes, trypsin and chymotrypsin assays used L-BAPNA (N-benzoyl-L-arginine-p-nitroanilide) and SAAPFpNA (succinyl-Ala-Ala-Ala-Pro-Phe-p-nitroanilide) respectively. Formation of nitroaniline was then accounted as proxy for activity measures. Specific enzyme activities were calculated with total protein content, which was analyzed according to the folin phenol method in the same intestine homogenate.

Fillet Quality

Fillet quality analysis was performed based on pH and flesh color measurements, for a period of seven consecutive days. Fillets were cut from the right lateral side and stored in plastic bags in an ice box which was placed in a climate controlled room set at 4 ºC. Fillet color and pH were measured once per day for seven days. The pH was assessed at the mid-axial with a calibrated spear electrode PH62 (WTW, Weilheim, Germany). The color at the anterior dorsal

section was determined on the visceral part of the fillets at the mid-axial area. Color measurements at the posterior region were determined near the tail peduncle (Fig. 2a). All color measurements were performed using a chroma meter (measurement area: ⌀ 8 mm; 0° viewing angle; Konica Minolta type: CR-400/410) and the L*, a* and b* values recorded after flashing. Measurements were executed according to the CIE 1976 L*a*b* color scheme. Measures of L*, a*, and b* values reflect the human eye response to light and color perception (Fig. 2b). L* value represents light/luminosity ranging from 100 (white) to 0 (black). a* represents the red/green length, with positive values for red and negative ones for green. b* represents the yellow/blue length, with positive values for yellow and negative ones for blue. Additionally, at day seven, skin color measurements were performed based on the same methodology as fillet color.

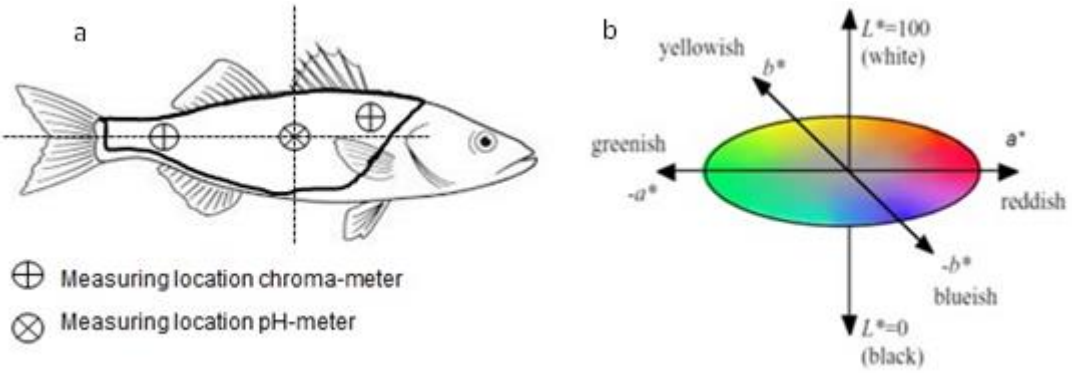

Fig. 2 Measurement locations of colour and pH on seabass fillets.

Oxidative stress biomarkers

Oxidative stress biomarkers were determined in liver after the homogenization in phosphate buffer (0.1 M pH 7.4). The protein content of each sample was determined by Bredford method and used to standardize enzymes activities. Lipid peroxidation (LPO) was determined measuring the by-products formed by malondialdehyde and thiobarbituric acid. The resulting substances, thiobarbituric acid reactive substances (TBARS), form a pink chromophore measured spectrophotometrically at 535 nm. Catalase (CAT) activity was analyzed at 240nm. Glutathione peroxidase (Gpx) and glutathione reductase (GR) were evaluated, at 340 nm, based on NADPH oxidation. Total glutathione (TG) was evaluated by the formation of 5-thio-2-nitrobenzoic acid, at 412 nm .

Immune-related parameters

Immune status was assessed through the determination of key innate immune parameters, namely lysozyme, peroxidase and the alternative complement pathway levels. Lysozyme activity was evaluated as the amount of enzyme required to catalyze a bacterial lysis that produces a decrease of 0.001 units min^{-1} in absorbance. Hen egg white lysozyme (Sigma, Germany) was used as standard and *Micrococcus lysodeikticus* (0.5 mg mL^{-1}; 0.05 M sodium phosphate buffer; pH 6.2) as bacterial suspension. Peroxidase activity (U mL^{-1} plasma) was determined as the reduction of 3,3, 5,5—tetramethylbenzidine hydrochloride (Sigma, Portugal),considering that a change in one unit of absorbance corresponds to one enzymatic unit. The alternative complement pathway (ACH50) was analyzed using a concentration of 2.8 × 10^8 cells mL^{-1} rabbit red blood cells (RaRBC). The reciprocal of the plasma dilution giving 50% hemolysis of RaRBC equals one ACH50 unit.

Statistical analysis

All data was scrutinized for normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test). The analysis of variance was performed applying one-way-ANOVA test using the dietary treatments as dependent variable. Significant differences were considered when $P<0.05$. The statistic software package used was SigmaPlot 11.0 (Systat Software Inc, USA). As for fillet characteristics, Mann-Whitney U tests were used for the statistical analysis of the L*, a* and b* colour measurements and ANOVA for differences in pH.

Results

Growth performance data

Juvenile seabass grew up to 2.5 fold from initial weight in 42 days of feeding trial. Weight gain (WG: 22.2-24.7 g), daily growth index (DGI: 2.0-2.1% BW day^{-1}), voluntary feed intake (VFI: 1.9-2.0% BW day^{-1}) and feed conversion ratio (FCR: 0.8-0.9), showed no differences between groups, regardless of the dietary treatment (Table 3).

Table 3 Growth performance of seabass fed the experimental diets for 42 days.

	Dietary treatments			***p* value**
	Ctrl	**GE**	**GR**	
Initial Body W (g)	16.97 ± 0.69	16.36 ± 0.41	16.27 ± 0.27	0.37
Final Body W (g)	41.09 ± 0.65	38.60 ± 1.99	40.98 ± 1.49	0.24
WG (g)	24.12 ± 0.17	22.24 ± 1.58	24.71 ± 1.42	0.19
DGI	2.10 ± 0.04	2.00 ± 0.09	2.17 ± 0.09	0.17
VFI (% body weight day^{-1})	2.00 ± 0.05	2.00 ± 0.06	1.89 ± 0.04	0.07
FCR	0.87 ± 0.04	0.90 ± 0.05	0.79 ± 0.03	0.10
HSI (%)	1.67 ± 0.16	1.84 ± 0.09	1.55 ± 0.15	0.10

Values presented as Mean±Standard Deviation (SD). Absence of letters indicates absence of significant differences between treatments (P<0.05) for n=3, p values are given.

WG – Weight Gain;

DGI – Daily Growth Index = 100 x ((Final body weight)1/3 - (Initial body weight)1/3) / days

VFI – Voluntary Feed Intake = 100 × [FI (g) / ABW (g) / TD (days)], where ABW is (IBW + FBW) / 2

FCR – Feed Efficiency Ratio = dry feed intake / weight gain

HSI – Hepatosomatic Index = 100 × [liver weight (g)/fish weight (g)]

Digestive enzyme activities in intestine

Seabass showed no significant differences in their ability to digest dietary carbohydrates since amylase activity did not vary between the dietary treatments (Table 4). In addition, lipase, trypsin and chymotrypsin activities (Table 4) did not differ between the dietary treatments (P>0.05).

Table 4 Digestive enzyme activities in intestines of seabass fed the experimental diets for 42 days.

	Dietary treatments			***p* value**
	CTRL	**GE**	**GR**	
Amylase (U. mg protein^{-1})	278.44 ± 34.34	299.97 ± 71.74	309.35 ± 42.74	0.49
Lipase (mU. mg protein^{-1})	90.60 ± 27.84	108.88 ± 26.80	99.79 ± 22.14	0.38
Trypsin (U. mg protein^{-1})	288.77 ± 158.80	190.60 ± 86.23	75.83 ± 180.50	0.14
Chymotrypsin (U. mg protein^{-1})	232.16 ± 77.01	199.59 ± 63.01	210.23 ± 67.16	0.64

Values presented as Mean±Standard Deviation (SD). Absence of letters indicates non-significant differences between treatments (P<0.05) for n=9 fish per treatment.

Fillet quality

No significant differences were observed for fillet pH between the dietary groups (Table 5) during the measurement period. In addition, no dietary effect was observed for the anterior and posterior measurements of L* between dietary treatments. Fish fed GE diet showed significant differences in b* value (P=0.028) for the posterior measurement at day 2. Also, at day 5, the a* value (P=0.026) for the anterior measurement showed differences between GE and CTRL fed fish.

Fish fed GR diet showed a significant difference from CTRL fed fish in the posterior measurement of the a* value (P=0.018) on day 3 (Table 5).

Skin color measurements on day 7 revealed significantly higher a* (P=0.012) and L* (P=0.020) values in fish fed GE diet when compared with fish fed the CTRL diet. Fish fed GR diet had a significant higher a* (P=0.035) and showed a tendency of higher L* (P=0.077) when compared to fish fed CTRL diet.

Table 5 Fillet pH and colour, and skin colour of seabass fed the experimental diets for 42 days.

Diet	Parameter		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
CTRL	pH		6.31±0.20	6.28±0.10	6.32±0.08	6.29±0.06	6.33±0.08	6.32±0.07	6.34±0.07
	Anterior	*L**	71.9±0.9	73.0±0.8	72.8±0.8	73.4±0.8	72.7±0.9	71.9±0.8	72.5±0.8
		*a**	11.2±0.3	11.1±0.2	11.2±0.2	10.9±0.2	10.8±0.2*	10.9±0.2	11.4±0.2
		*b**	-6.9±0.5	-7.2 ±0.5	-7.1±0.5	-7.0±0.8	-6.8±0.6	-6.8±0.6	-6.5±0.6
	Posterior	*L**	73.7±0.9	74.9±1.0	75.6±1.0	76.7±1.2	75.8±1.0	74.7±0.7	75.8±1.1
		*a**	12.1±0.6	12.3±0.5	12.5±0.5+	12.3±0.5	12.1±0.6	12.1±0.4	12.8±0.5
		*b**	-5.8±0.9	-6.2±0.6*	-5.9±0.6	-5.6±0.8	-5.7±0.8	-5.9±0.6	-5.2±0.7
	Skin	*L**	-	-	-	-	-	-	81.9±3.8*
		*a**	-	-	-	-	-	-	10.9±0.3*+
		*b**	-	-	-	-	-	-	-4.8±0.9
GE	pH		6.32±0.14	6.30±0.12	6.36±0.10	6.30±0.10	6.36±0.09	6.36±0.09	6.39±0.09
	Anterior	*L**	71.9±1.0	72.8±0.9	72.8±0.9	73.5±1.0	72.6±1.0	71.8±0.9	72.4±0.9
		*a**	11.2±0.3	11.1±0.3	11.3±0.2	11.0±0.3	10.9±0.2*	11.0±0.2	11.0±2.0
		*b**	-6.9±0.5	-7.1±0.6	-7.0±0.6	-6.8±0.7	-6.7±1.0	-6.7±0.7	-6.5±0.7
	Posterior	*L**	74.2±1.1	75.0±1.0	75.3±1.2	76.6±0.8	75.6±1.0	74.7±1.2	75.8±1.0
		*a**	12.2±1.1	12.4±0.4	12.3±0.5	12.1±0.4	12.1±0.5	12.0±0.5	12.7±0.4
		*b**	-5.6±0.9	-5.8±0.6*	-5.9±0.7	-5.6±0.6	-5.7±0.7	-5.8±1.0	-4.9±1.1
	Skin	*L**	-	-	-	-	-	-	83.9±1.9*
		*a**	-	-	-	-	-	-	11.1±0.3*
		*b**	-	-	-	-	-	-	-4.8±1.3
GR	pH		6.30±0.17	6.29±0.09	6.36±0.19	6.26±0.07	6.33±0.08	6.32±0.07	6.34±0.08
	Anterior	*L**	72.0±0.9	72.9±0.7	72.8±0.8	73.5±0.8	72.7±0.8	71.9±0.8	72.6±0.9
		*a**	11.1±0.2	11.1±0.3	11.2±0.2	10.9±0.3	10.8±0.3	10.9±0.2	11.3±0.2
		*b**	-6.9±0.5	-7.2±0.5	-7.1±0.5	-6.9±0.5	-6.8±0.6	-6.8±0.6	-6.6±0.6
	Posterior	*L**	74.2±0.5	75.3±1.0	75.4±0.4	76.7±0.8	75.9±1.0	74.8±0.8	75.6±1.0
		*a**	11.8±0.5	12.3±0.5	12.2±0.4+	12.2±0.4	12.0±0.4	12.0±0.8	12.6±0.4
		*b**	-5.7±0.8	-5.9±0.7	-5.6±2.4	-5.7±0.6	-5.7±0.7	-5.9±0.4	-5.3±0.6
	Skin	*L**	-	-	-	-	-	-	84.3±2.3
		*a**	-	-	-	-	-	-	11.1±0.3+
		*b**	-	-	-	-	-	-	-4.4±1.0

Color was quantified as L* (light/luminosity), a* (red/green) and b* (yellow/blue) values at an anterior and posterior location of the fillet and skin. Fillet pH and color were determined over 7 days, while skin color was only determined on day 7. n=30, Mean±Standard Deviation (SD). Significant differences (P<0.05) between GE and CTRL are signaled with *. Significant differences (P<0.05) between GR and CTRL are signaled with +.

Oxidative stress biomarkers in liver

The results to assess the hepatic antioxidant status are presented in Table 6. Lipid peroxidation levels (LPO), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and total glutathione (TG) did not vary between the dietary treatments (P<0.05).

Table 6 Enzymatic and non-enzymatic parameters of hepatic antioxidant responses in seabass fed the experimental diets for 42 days.

	Dietary treatments			***p* value**
	CTRL	**GE**	**GR**	
LPO	4.20 ± 1.06	5.52 ± 3.27	5.55 ± 2.50	0.41
CAT	155.51± 16.79	158.88 ± 23.60	146.72 ± 17.67	0.45
GPx	1.07 ± 0.50	2.79 ± 2.18	2.17 ± 1.69	0.21
GR	2.89 ± 1.45	2.79 ± 1.16	3.34 ± 0.57	0.62
TG	0.27 ± 0.10	0.19 ± 0.04	0.37 ± 0.18	0.62

Values presented as Mean±Standard Deviation (SD). Absence of letters indicates absence of significant differences between treatments (P<0.05) for n=9 fish per treatment.

LPO – Lipid Peroxidation (nmol TBARS. g tissue-1)

CAT – Catalase (µmol, min-1.mg protein-1)

GPx – Glutathione Peroxidase (nmol. min-1. mg protein-1)

GR – Glutathione Reductase (nmol. min-1. mg protein-1)

TG – Total Glutathione (nmol. min-1. mg protein-1)

Immune-related parameters in plasma

Plasma immune parameters are depicted in Fig. 3. Seabass fed GE diet showed higher alternative complement activity (ACH50) when compared to the CTRL diet (P=0.042). Plasma peroxidase and lysozyme did not vary among the dietary treatments.

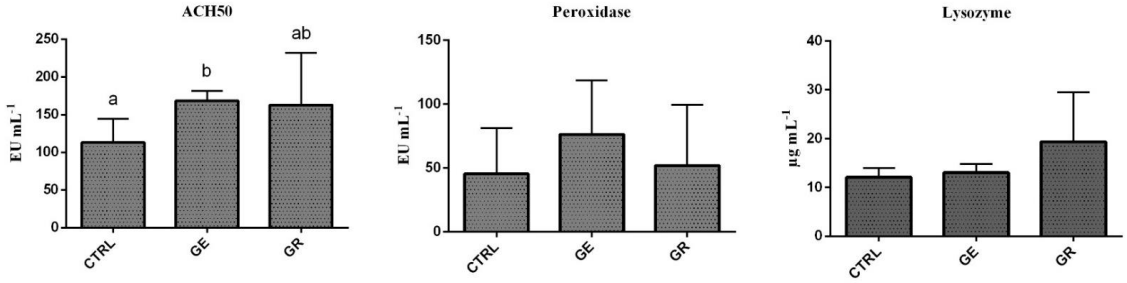

Fig. 3 Alternative complement pathway (ACH50), peroxidase and lysozyme activities analysed in plasma from seabass fed the experimental diets for 42 days. Presence of letters indicates significant differences between treatments (P<0.05) for n=9 fish per treatment.

Discussion

This research aimed to evaluated seabass performance, welfare and fillet quality when fed diets supplemented with *Gracilaria* sp. methanolic fractions. The experiment was designed to test the use of *Gracilaria* sp. extracts, with different biochemical properties, as dietary additive (functional feed) for a period of 42 days. The results demonstrated that *Gracilaria* sp. extract supplementation does not alter seabass growth performance, digestive enzyme activities, fillet pH, or oxidative stress biomarkers. However, significant differences were detected for fillet color and innate immune parameters, namely, the alternative complement pathway. These results suggest that seaweed extracts may simultaneously act as a nutrient supply, namely pigments, and facilitate the incorporation of valuable marine bioactives in fish and human nutrition. Accordingly, previous studies demonstrated that dietary seaweed supplementation might influence seabass market value and modulate fish immune response (Valente *et al.* 2015).

Earlier studies with seabass demonstrated that the dietary inclusion of crude *Gracilaria* sp. as a protein replacement (Valente *et al.* 2006) and as dietary supplementation (Peixoto *et al.* 2016) do not alter fish growth performance. In agreement with those findings, in the present study the methanolic *Gracilaria* sp. extract and residue were used as dietary supplements without causing growth impairment. By fixing the feeding level, a similar macronutrient availability was achieved for all dietary treatments. In addition, since feeding fish *ad libitum* could lead to biased conclusions, a fixed rate prevented differences associated with feed intake from being expressed. In this study, fish from all dietary treatments presented a two-fold increase in weight, which establishes the acceptability and suitability of the diets. Additionally, DGI, VFI and FCR were also similar between dietary treatments. It should be noted that the growth data presented in this work are in agreement with previous results reported for seabass reared at 20 ºC and fed diets containing 50% protein (Peres and Oliva-Teles 1999).

Fish metabolic capacity and health condition may be appraised by the hepatosomatic index (HSI) (Huggett 1992). An abnormal HSI value could be associated with toxicity, due to pollutants exposure (Focardi *et al.* 2005), or xenobiotics contained in feed ingredients. Xenobiotics are capable of modifying liver weight by altering enzymatic activities and lipid deposition, which disrupts several endocrine pathways (Kime 2012). Nonetheless, HSI values determined in this work revealed no differences between control and *Gracilaria* sp. supplemented diets, displaying similar values as previous studies in seabass (Wassef *et al.* 2016). These results suggest that the compounds present in the methanolic fractions do not hinder seabass hepatic functions, at least when included in diets up to 5% supplementation level.

The activity level of digestive enzymes is associated with an organism potential to digest a meal (German *et al.* 2004) which can be altered in the presence of anti-nutritional factors (ANFs) (Oliveira *et al.* 2009), or morphological alterations (Araújo *et al.* 2015). Seaweeds have been previously scrutanized for ANFs since a high-level supplementation (above 10%) has been associated with digestive constraints (Valente *et al.* 2006). In our trial, however, enzymatic activities for carbohydrates digestion (α-amylase), lipids (lipase) and proteins (trypsin and chymotrypsin) showed no significant differences between the experimental diets (Table 6). The authors suggest that such similarity in digestive enzymes activity levels was related with low levels of ANFs present in *Gracilaria* sp. methanolic extracts. Moreover, the absence of differences in digestive enzymes activity between supplemented diets and CTRL indicates that the extraction process had no interference on seabass gut morphology in 42 days of feeding. The long-term application of extract-supplemented diets however requires deeper investigation.

The pH and color of fish fillets provides a measure of flesh freshness, ultimately affecting the consumer perception of quality (Roth *et al.* 2009). Fillet color is often a subjective indicator used by consumers to evaluate fish quality (Vanhonacker *et al.* 2013). In the present work, there were no relevant differences in fillet pH and color between diets supplemented with different *Gracilaria* sp. extracts. pH values were stable throughout the measurement period of seven days, which is consistent with the reported results for seabass fillets stored at 4 ºC (Bojanić *et al.* 2009). Similarly, the inclusion of the red seaweed *Palmaria palmata* in Atlantic salmon (*Salmo salar*) diets revealed no pH differences measured in fillets after 15 days of storage (Moroney *et al.* 2015). Only few significant differences were found in flesh color, but none of them were consistent over days or visible for the human eye. Overall, our data suggests that seaweed supplementation has no relevant effect over fillet muscle quality. However, seven days after filleting, there was a difference in skin color. Fish from the GE and GR groups had lighter skin color than fish from the CTRL group suggesting that *Gracilaria* sp. supplementation affects skin pigmentation in both fractions (GE and GR) after a 7-day 4ºC storage period. These differences of 2.0 and 2.4 are visible for the human eye and may therefore have possible effects on consumer preferences. Valente el at. (2015) reported lighter fillets when rainbow trout were fed diets containing 5% *Gracilaria* sp. supplementation and an overall preference from consumers.

Oxidative stress is a chain reaction process characterized by repetitive formation of oxygen reactive species (ROS) (Halliwell 2006) linked to changes in biochemical parameters and antioxidants activities. ROS accumulation can lead to the oxidation of the fatty acids that compose cell membranes, forming lipid peroxides, a process known as lipid peroxidation (LPO) (Repetto *et al.* 2012). In the present work, no influence of dietary *Gracilaria* sp. supplementation was found in the lipid peroxidation of seabass liver when fed the different

experimental diets. This result is contrary to what was expected since polyphenolic compounds derived from the methanol extraction of red and brown seaweeds have been described to decrease lipids peroxidation (Wang *et al.* 2009). Dietary seaweed supplementation has also been evaluated as a vector for molecules with antioxidant properties, such as vitamins or halogenated minerals (Moroney *et al.* 2015; Valente *et al.* 2015). The present work evaluated the activity levels of the hepatic antioxidant enzymes CAT, GPx, GR and TG and found no differences between dietary treatments. However, solvent fractions obtained from the methanolic extraction of different red seaweeds, including *Gracilaria edulis*, were characterized has having antioxidant properties (Ganesan *et al.* 2008). Nonetheless, Ganesan *et al.* (2008) also reported that antioxidant activities of methanol extracts from different seaweeds display dose dependency, increasing at higher extract concentrations. The low concentration and/or availability of the compounds with antioxidant properties may be the reason for the lack of differences in the present work. The authors suggest that the potential protective role of antioxidant compounds present in *Gracilaria* sp. extracts should be ascertain in future studies by investigating their effect *in vitro* and *a posteriori* in seabass at different concentrations.

Immunostimulants supplementation to aquafeeds has been reported as an effective method to boost fish defenses against stressors (Ringø *et al.* 2012). According to Ramalho Ribeiro *et al.* (2015), seaweeds should be applied in aquaculture as part of a dietary bio-fortification strategy. Deoxyprostaglandins, ceramides and oxygenated fatty acids present in seaweeds are described as having anti-inflammatory, anti-bacterial and immunostimulant properties. These compounds have been shown to be concentrated in methanolic fractions by extraction processes (Dang *et al.* 2008). A previous study from our research group have shown that dietary supplementation of raw Gracilaria meal altered fish immune status (Peixoto *et al.* 2016). However, in the present study, lysozyme and peroxidase levels were not altered by dietary supplementation of the methanolic fractions of Gracilaria extraction. These results may be related with the potential alteration of bioactives compounds contained in crude Gracilaria sp. during the extraction process. Both, bacterial and seaweed cell walls are composed of polysaccharides that may stimulate immune defense responses (Lee *et al.* 2013; Vera *et al.* 2011), and it has been shown that these compounds can trigger an immune response, involving phagocytosis by neutrophils and macrophages (Uribe *et al.* 2011). These cells possesses peroxidase activity that halogenate bacterial walls for their subsequent hydrolization by lyzozymes (Uribe *et al.* 2011). It is plausible to infer that the process of Gracilaria sp. extraction may have caused polysaccharides cleavage, allowing supplementation to seabass diets without triggering an immune response. However, higher complement activity (ACH50) was measured in the seabass group fed *Gracilaria* sp. extract (GE diet) compared to the control (CTRL diet). In fish fed the residue fraction (GR diet), the ACH50 activity was not significantly different from the values detected in fish fed the GE diet

or CTRL. In preceding works (Peixoto *et al.* 2015) the authors found similar levels of ACH50 in control diets, however, in diets containing 7.5% *Gracilaria* the ACH50 level was lower. That result was associated with detrimental effects caused by the level of supplementation and associated with an inhibition of the protein cascade that composes de alternative pathway of the complement system. Whether *Gracilaria* sp. extracts act as immunostimulant should be further analyzed exposing fish to immune challenges.

Overall, the presented results indicate that *Gracilaria* sp. methanolic fractions can be included in seabass diets without affecting growth performance or digestive enzyme capacities. The fillets pH and color from fish fed with *Gracilaria* sp. extract or residue were not different from the diet without supplementation over seven days. Skin color of these groups after seven days was slightly lighter which may influence market value. Concerning the implications of dietary seaweed extract supplementation in seabass welfare, oxidative stress and immune-related biomarkers analyzed suggest that seaweed supplemented diets have no negative effects. Future studies should investigate a possible beneficial role of bioactive compounds with potential antioxidant and immune-stimulant properties in fish under challenging conditions.

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CHAPTER VI

OXIDATIVE STRESS AND IMMUNE RESPONSES GENES HAVE SIGNIFICANT ALTERED EXPRESSION IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) FED *GRACILARIA* SP. SUPPLEMENTED DIET.

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PAPER IN EDITION

Protective effects of seaweed supplemented diet on antioxidant and immune system in European seabass (Dicentrarchus labrax) subjected to bacterial infection.

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Dietary seaweed (*Gracilaria* sp.) supplementation modulated European seabass (*Dicentrarchus labrax*) antioxidant capacity, immunity and resistance against *Photobacterium damselae* subsp. p*iscicida* (Phdp) infection.

Here we reported that seaweed supplementation decreased mortality rates in infected fish. Moreover, *Gracilaria* sp. decreased peroxidase activity and increased lysozyme activity in both infected and non-infected fish. Conversely, lipid peroxidation decreased in fish fed seaweed supplemented diet, evidencing cell protection against free radicals. Hepatic catalase activity and glutathione transferase increased with seaweed supplementation, with no clear effect of infection. Dietary seaweed supplementation had a stronger effect on gene expression levels than infection. Seaweed caused a down regulation of hepatic heat shock proteins (*grp-78*, *grp-170*, *grp-94*, *grp-75*), *prdx1*, *sirt5*, *gr* and *sirt1* genes. Bacterial infection caused a down-regulation of *prdx4* and *mn-sod* genes. Diet and infection interaction down-regulated hepatic *cs*, *prdx5* and *gpx4* transcription levels. Considering immunity, seaweed supplementation caused an up-regulation of *il34*, *ccr9*, *cd33* and a down-regulation of *mif*, *il1b*, *defb*, *a2m*, *myd88* genes. Additionally, infection resulted in a clear up-regulation of IgMs and a down regulation of leap2.

Overall, *Gracilaria* sp. supplementation evidenced a determinant role of this seaweed in the up-regulation of immune and antioxidant related pathways.

Introduction

The strong intensification of aquaculture practices involves rearing animals at high densities in enclosed spaces. These conditions may result in poor water quality, a suitable environment for bacterial proliferation [1,2]. The suppression of fish immunity and the disruption of their antioxidant systems increases the susceptibility to infectious agents [3] and potentiate pathologies during infection. This vulnerability to pathogens, often leads to disease outbreaks that culminate in massive death rates with subsequent economic losses for the producers [2].

Fish photobacteriosis, inflicted by the Gram-negative halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (Phdp), may induce acute septicaemia in young fish or in older fish may form granulomatous lesions which result in massive mortalities [4]. Nowadays, to supress these infections, in detriment of treatment, preventive techniques are being applied in aquaculture to [5]. One of the most promising methods for the control of cultured fish diseases, at a cost effective and promising eco-friendly alternative to chemotherapy and vaccines, is that of strengthening fish defence mechanisms through the prophylactic administration of immunostimulants [6]. Since nutrition can modulate life-traits, the dietary supplementation, with immunostimulant and antioxidant nutraceuticals, is a promising tool for boosting fish resistance to disease. Such approach appears to be a good alternative to other methodologies currently employed, since natural components (e.g. polysaccharides) are less likely to interfere with fish homeostasis or disrupt the environment [5,7]. Thus, seaweeds, containing bioactive molecules with immunostimulant and antioxidant properties, are in the spotlight to improve robustness of farmed fish. Moreover, the nutritional necessities associated with fish immunocompetence are diverse and linked with growth requirements [8]. Considering this, seaweeds may be useful to modulate fish defences without compromising growth.

Several seaweed species have been found to produce or contain secondary metabolites with antiviral, antibacterial, anti-inflammatory and antioxidant properties [9]. Therefore, small amounts of seaweeds in aquafeeds may prove to be of considerable benefit in increasing disease resistance. Seaweed polysaccharides which are known for stimulating non-specific host immunity have also been shown to inhibit bacterial activity as well as act as probiotic, thus improving gut health and potentiating fish digestive capacities [10]. Specifically, ***β***-glucans derived from various seaweed species appear to stimulate the immune system through the rapid release of reactive oxygen species (ROS) and signalling proteins in fish. On the other hand, seaweeds phenolic compounds may wield their scavenging effect reducing ROS formation in fish tissues. Similarly, red

seaweeds are very rich in arachidonic acid that is metabolized into eicosanoids (prostaglandins, thromboxanes and leukotrienes), which act as inflammation mediators [11]. These chemotactic lipids control key immune responses such as chemotaxis, phagocytosis and antigen presentation [12].

European seabass (*Dicentrarchus labrax*) is a species of established importance in the European aquaculture sector. Several studies have investigated how dietary changes modulate seabass immune system, including partial replacement of fish oil and fish meal [13-15]. However, long-term feeding with plant derived proteins and oils upheld controversial due to the existence of anti-nutritional factors [16]. High soybean meal diets, for example, have been associated with the activation of T cell mediated processes such as the up-regulation of interleukins 18 and 22 as well as the transcription of genetic markers of inflammation, namely Tumor Necrosis Factor (TNF-α) and Factor Nuclear Kapa (NF-kB) [17].

Despite unveiling a negative influence of the inclusion of plant proteins in fish health, these studies have ascertained that stress responsiveness and susceptibility could be nutritionally regulated [18]. Nevertheless, little is known about the mechanisms by which functional foods affect fish metabolism and immune system, both locally at infection sites and systemically. In this context, it is of great interest for the aquaculture sector to understand how ingredients derived from marine sources, such as seaweeds, may be applied in aquafeeds to improve fish immunity.

The objective of this study was to evaluate the effect of dietary supplementation with 5% *Gracilaria* sp. aqueous extract in seabass when challenged with *Photobacterium damselae* subsp. *piscicida*. Specifically, we aimed to determine how *Gracilaria* sp. supplementation alters seabass survival rates, growth parameters, plasma bioindicators, immune and antioxidant parameters, as well as immune and antioxidant gene transcription levels in liver, spleen and head kidney in response to infection.

Discussion

The present work aimed to evaluate the contribution of *Gracilaria* sp. dietary aqueous extract supplementation in seabass immune and antioxidant capacities when challenged with a bacterial infection.

Dietary seaweed supplementation, in particular *Gracilaria* sp. was previously tested in seabass. Valente, et al. [19] described the application of two different *Gracilaria* species (*G. bursa-pastoris* and *G. cornea*) very interesting ingredients for seabass juveniles. However, the authors emphasise the decrease in growth performance and digestibility in individuals fed the diet supplemented with *G. cornea* at 10% level, recommending that its supplementation should not exceed the 5% level. The results obtained in the present research revealed no significant differences in growth parameters between dietary groups, meaning that the application of the aqueous extract of *Gracilaria* sp., at 5% level of supplementation, is possible without affecting *D. labrax* performance. Thus, we emphasize the use of dietary seaweed supplementation as immune enhancer in detriment of its use as protein source.

In the last years, multiple researches have provided insights about the improvements on the immune system achieved through seaweeds application. Castro, et al. [20] demonstrated an increase in the respiratory burst of turbot (*Scophthalmus maximus*) phagocytes when using water-soluble extracts from eight seaweed species *Ulva* sp, *Enteromorpha* sp., *Codium* sp., *Fucus* sp., *Pelvetia* sp., *Dictyota* sp., *Chondrus* sp. and *Porphyra* sp.. In shrimp (*Fenneropenaeus chinensis*), the application of *Sargassum* sp. polysaccharide extracts as dietary supplement resulted in enhanced immune activity and improved resistance to vibriosis [21]. Our results highlight *Gracilaria* sp. supplementation as an effective participant delaying *P. damselae* sp. infection. In fact, mortalities in fish infected with Phdp fed the CTRL diet occurred earlier than in GRA groups. Moreover, at day 10 post-infection, the mortality in GRA groups is significantly lower than CTRL. These results are in accordance with Van Doan, et al. [22] when feeding basa fish (*Pangasius bocourti*) with diets supplemented with agar. Similarly, lower mortality was observed after a challenge with *Streptococcus iniae* in olive flounder (*Paralichthys olivaceus*) fed diets supplemented with *Hizikia fusiformis* [23].

The results regarding plasma biochemistry, namely glucose and triglycerides levels revealed no dietary influence in seabass challenged with Phdp. However, when analysing dietary influence within placebo groups, TGA levels were lower in GRA group. These results suggest that dietary *Gracilaria* sp. supplementation alters seabass physiological condition, either by regulating or promoting fatty acid metabolism. Lower cholesterol and triglyceride levels were also observed in Japanese flounder (*Paralichthys*

olivaceus) fed diets supplemented with *Eucheuma denticulatum* [24]. Moreover, the antihyperlipidemic effects of seaweed dietary supplementation have also been previously demonstrated in rats [25] and chickens [26] by causing a reduction in cholesterol and triglycerides levels.

Plasma bioindicators of innate immunity analysed in this study, lysozyme and peroxidase activities, were included to evaluate if dietary seaweed supplementation might confer fish some resistance to infection. Phagocytes immediately respond to bacterial infections by releasing the peroxidases stored in their cytoplasmic granules [27]. This causes an initial increase in plasma peroxidase activity, which decreases over time after the initial exposure to the pathogen [28]. Our results indicate that plasma peroxidase levels decrease in both infected and placebo groups fed GRA diet. Therefore, we hypothesize that dietary *Gracilaria* sp. may be eliciting an immediate response by these peroxidase enzymes which was no longer detectable 10 days post infection. Leukocytes also respond to bacterial infection by realising lysozyme, an enzyme that possesses lytic activity against pathogens [27]. For example, plasma lysozyme activity increases in the presence of microbial agents and/or after immunization procedures [29]. Increased lysozyme activity has also been described in response to polysaccharides from seaweed cell walls [30]. Results in our study have shown that lysozyme activity in seabass fed GRA diet was higher than fish fed CTRL diet, suggesting a potential effect of polysaccharides present in the seaweed-supplemented diet. Taken together, our results indicate that *Gracilaria* sp. may alter seabass immune system, eliciting a primary response, which may be useful when fish become in contact with pathogens.

Upon infection, phagocytes activation is followed by an increase in oxygen consumption, which results in higher ROS production. These ROS have potent microbicidal properties that will aid fighting infection, in a process designated respiratory burst [20]. However, excessive ROS concentration leads to oxidative stress in cells, phenomena previously described in infection scenarios [31]. Seaweed application as supplement in fish diets has been associated with antioxidant activities as well as with protection against oxidative stress [32]. In our results, lipid peroxidation in infected fish fed GRA diet was lower than in fish fed CTRL diet. Accordingly, a decrease in lipid peroxidation products was obtained in rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with *Gracilaria pygmaea* [33]. Glutathione s-transferase (GST) is an enzyme complex that acts by conjugation with other molecules in order to increase their solubility hence helping detoxification processes [34]. In that process, these enzymes also bind with peroxidised lipids catalysing their breakdown. In our results, an increase GST activity was observed in fish fed GRA diet, in both infection and placebo, when compared to CTRL. The increase in GST may be associated with upregulated detoxifying activity in

seabass fed supplemented diets. On the other hand, catalase (CAT) acts defensively against free radicals, breaking down hydrogen peroxide [35]. Our results depict an increase in CAT in seabass fed GRA diet within placebo groups. This suggests that the metabolic cleanse in seabass fed diets supplemented with *Gracilaria* sp. is higher in common everyday conditions but not when fighting infection.

Plasma bioindicators, protein levels and enzymes activities are useful biomarkers of the effect of dietary seaweed supplementation on fish immunity and antioxidant systems. However, a more profound evaluation was necessary to understand the modulation of *Gracilaria* sp. supplementation in seabass immune and antioxidant responses. Therefore, we analysed gene expression levels in relevant tissues (HK and spleen for immunity and liver for antioxidant processes), factoring *Gracilaria* sp. supplementation and Phdp infection.

In our results, the transcription levels analysed in liver of seabass fed with *Gracilaria* sp. supplemented diets and infected with Phdp revealed a significant difference from fish fed CTRL diet. The heat shock proteins and molecular chaperones *grp94, grp170, grp78, grp75* and *mthsp10*, which are established markers of fish response to a stressor [36], evidenced a clear influence of diet in response to infection since in seabass fed the supplemented diet *gprs* and *mithsp10* chaperons expression was decreased when compared to CTRL fish. Besides their involvement in stress responses, heat shock proteins have also been linked with fish immunity [37,38]. These molecules have a major role in the immune system, mediating the development of inflammation and the specific and non-specific immune responses to bacterial and viral infections, through apoptosis and the inflammatory process [39]. In a smaller degree, also the antioxidant enzymes expression such as peroxiredoxins (*prdx*), glutathione peroxidases (*gpx*), glutathione reductase (*gr*) and catalase (*cat*) revealed decreased expression in fish fed *Gracilaria* sp. when compared to CTRL. Considering their function in cell defence mechanisms as modulators of inflammation and cells protection [40,41] the down-regulated expressions observed in our results suggests a protective role of *Gracilaria* sp. in seabass response to pathogens. Moreover, fish sirtuins (*sirt*) were already demonstrated to respond to dietary changes [42] and xenobiotics [43]. In mammals *sirt1* is a nuclear protein involved in cell survival, metabolism regulation, inflammation and oxidative stress response, whilst *sirt5* is mitochondrial, participating in its regulation, including ATP production, metabolism, apoptosis, and intracellular signalling [44]. In our results, *sirt1* and 5 expression levels showed a reversed pattern between dietary groups infected with Phdp. These results seem to follow the same mechanistic regulation as in mammals, where *sirt1* inhibition accompanies *sirt5* overexpression and is associated with the resolution of inflammation [44]. Globally, genes encoding for antioxidant enzymes and redox

homeostasis were down-regulated in liver of seabass fed GRA diet, suggesting the contribute of *Gracilaria* sp. to the antioxidant processes.

Head-kidney (HK) is the major endocrine and haematopoietic-lymphoid organ in teleost fish, being the equivalent to bone marrow for vertebrates [45]. Our results demonstrated that dietary supplementation with *Gracilaria* sp. significantly altered the expression levels of cytokines in HK, particularly when fish were infected with Phdp. Cytokines are key participants of the immune system acting as anti, pro or pre inflammatory, regulating the immune response in order to resolve infection [46]. The interleukin IL-1β is a powerful pro-inflammatory cytokine that increases when macrophages recognize molecular motifs present in pathogens (PAMPs) [47]. IL-1β has been reported to enhance macrophage functions including the production of superoxide anion in seabass infected with *Vibrium anguillarum* immediately after infection [48]. This cytokine acts as a chemoattractant for leucocytes, which also involves the recruitment of other interleukins [49]. In our results, *il-1β* transcription levels in seabass fed GRA diet decreased at 10 dpi. A decrease in *il-1β* levels in rainbow trout (*Oncorhynchus mykiss*) head kidney was also detected at days 0, 2, 4, 6 and 8 post-exposure to *Aeromonas salmonicida*, despite increased in the proximal intestine [50]. However, in our work, other interleukins expression levels, such as anti-inflammatory *il-10* and *il-20* increased in seabass fed GRA in response to infection. IL-20 in fish has been identified as belonging to the IL-10 family, and both these cytokines have been observed to increase in fish macrophages after infection with *Yersinia ruckeri* [51]. Moreover, the transcription levels of *il-34* together with *csf1r*, and *cd33* increased in infected seabass fed GRA diet suggesting higher involvement of macrophages in Phdp infection resolution. The influence of *Gracilaria* sp. dietary supplementation in seabass is also observable by the increase in the expression levels of the lymphokine *mif*, the chemokine receptor *ackr4* and the antimicrobial peptide *defb*, all related with the involvement of chemokines mechanisms of immunosuppression [49]. Immunoglobulins (Ig) which are involved in both innate and adaptive immunity showed increased expression in infected fish fed GRA diet. IgM has been described to activate complement and lysozyme for the lysis and opsonization of pathogens [45,52]. It also mediates agglutination, phagocytosis and pathogen removal [53]. Therefore, the increase observed in the expression levels of *igm* together with the increased lysozyme activity; further substantiate the hypothesis that dietary *Gracilaria* sp. supplementation positively regulates seabass immune response against pathogens. Overall, the up-regulated expression in immune related genes observed in seabass fed GRA diet when compared to CTRL suggests heightened immunity against infection with Phdp.

The contribution of *Gracilaria* sp. supplementation in seabass diets was determinant for the resolution of the infection with *P. damselae* by shifting blood biochemistry, enzymatic

activities and transcriptional levels of genes related to both immune and antioxidant processes.

Overall, the results presented in this work constitute an important step in comprehending how dietary seaweed supplementation affects seabass in infection scenarios. The dietary supplementation of 5% aqueous extract of *Gracilaria* sp. is possible for this carnivore fish species without compromising growth or affecting feed consumption rates. Moreover, when infected with *P. damselae*, seabass fed GRA diet demonstrated lower susceptibility to the pathogen, which resulted in higher survival rates, point of major importance for the aquaculture industry. Triglycerides levels hint on a more resourceful energetic metabolism by fish fed *Gracilaria* sp. supplemented diet. Additionally, innate immune parameters, lysozyme and peroxidase, in seabass fed GRA diet revealed increased resistance to pathogen proliferation. Regarding peroxidation processes in liver, the protective role of *Gracilaria* sp. supplementation reflects lower LPO levels and increased GST activity, which suggest increased hepatic capacity to respond to higher ROS levels produced during inflammation. Altered expression levels of key genes involved in the immune and antioxidant systems were observed in seabass fed GRA diet when compared to the CTRL. In particular, a shift in the contribution of determinant genes for the inflammatory process was observed in GRA groups evidencing a determinant role of *Gracilaria* sp. supplementation in the up-regulation of immune and antioxidant related pathways.

Methods

Experimental diets

Two isoproteic (50% DM) and isolipidic (19%) diets were tested in quadruplicates: a control diet (CTRL) and a supplemented diet with 5% *Gracilaria* sp. aqueous extract (GRA). *Gracilaria* sp. was produced by ALGAPlus in a land based IMTA system [54]. The seaweed was dried and thermally processed, using hot water at 83°C for 160 min. After filtration, the resulting agar was recovered through a freeze-thawing process. The final solid product was washed, dehydrated with ethanol and dried at 60°C, overnight, under vacuum. The extract was then added as separate supplement to the experimental diet (GRA) at 5 % w/w base, adjusted for dry matter content (DM). All ingredients were finely grounded (hammer mill, 0.8 mm sieve), mixed and then extruded (twin screw extruder, 2.0 mm pellet size, SPAROS, Portugal). Diets were finally dried at 45ºC for 12 h and stored at 4ºC until used. The detailed mineral and chemical compositions of the diets are presented in Table S1 of supplementary materials.

Fish and rearing conditions

Fingerlings were purchased from MARESA (Spain) and transported to the Aquatic Engineering laboratory of ICBAS (Porto, Portugal). Seabass were then acclimated to the experimental conditions for two-weeks while fed the CTRL diet. Thereafter, fish were individually weighed (initial body weight: 11.95 ± 0.34 g) and distributed in eight circular tanks of 80 L capacity with 30 fish per tank. For the feeding trial, tanks were connected to a closed recirculation seawater system ensuring similar quality parameters for all replicates. After infection tanks were individualized to prevent cross contamination. Water conditions were optimized and monitored for 30 ‰ salinity and 22 ± 0.5 ºC temperature.

Bacterial suspension and dose validation

Photobacterium damselae subsp. p*iscicida* (Phdp), strain SK-223/04, was purchased from CECT (Valencia, Spain). The strain was activated in tryptic soy broth (TSB; Biokar Diagnostics, France) and marine agar (Conda S.A., Spain). The bacteria were grown in TSB for 48 h at 22 ºC until reaching the exponential phase. The inocula were then centrifuged at 3500 g, for 30 min at 22 ºC. The resulting pellet was resuspended in NaCl

0.9% (Sigma-Aldrich). Serial dilutions were performed, incubated for 48 h at 22 ºC, and the number of colony forming units (CFU) was counted. To perform a correspondence of CFU counts with bacterial turbidity, the serial dilutions were spectrophotometrically read at 600 nm.

A dose validation trial was performed to establish an appropriate concentration for the challenge. Seabass (10 fish per tank) were randomly distributed in tanks, anesthetized by immersion in 0.5 ml. l^{-1} of 2-phenoxietanol (Sigma-Aldrich) and intraperitoneally (i.p.) injected with 100 µl of saline solution (negative control) or Phdp suspension (3 test concentrations: 1.0 x 10^4, 1.0 x 10^5 and 1.0 x 10^6 CFU ml^{-1}). The survival rate was monitored for 7 days post-injection, period that corresponds to the second consecutive day without mortalities. The method described by Reed and Muench [55] was used to calculate endpoints. Samples were taken aseptically from the kidney of the infected fish and inoculated in marine agar plates, followed by incubation at 22˚C for 48 hours.

For ethical reasons concerning animal welfare guidelines, the concentration of the inocula for the bacterial infection was calculated to achieve a lethal dose (LD) of 30 to 40% efficiency.

Bacterial infection

After 80 days, 30 fish per tank were anesthetized and intraperitoneally injected with either saline solution or Phdp suspension, similarly to the dose validation protocol described above. The Phdp inocula concentration was determined by absorbance (OD) as 5.93 x 10^6 CFU.ml^{-1}.

In the following 10 days, tanks were monitored to account for mortality and feed intake.

After 10 days of infection trial, eight fish per tank were anesthetized, blood was collected from the caudal vein and plasma obtained by centrifugation (5 min, 10000 g), aliquoted and stored at -80 ºC.

Liver, head kidney and spleen were collected, immediately frozen in liquid nitrogen and stored at -80 ºC.

Fish Growth Performance

Growth performance parameters were calculated using the following formulas:

Daily growth index (DGI) = 100 × [(FBW)1/3− (IBW)1/3] × TD, whereas FBW and IBW are the final and initial average body weights (g) and TD the trial duration (90 days).

Feed conversion ratio (FCR) = FI (g)/weight gain (g), whereas FI is feed intake on dry matter basis over 90 days.
Voluntary feed intake (VFI) (% body weight/day) = 100 × [FI (g) / ABW (g) / TD (days)], where ABW is ((IBW + FBW) / 2).

Plasma metabolites analysis

Plasma glucose (Glucose-RTU kit, Spinreact) and triglycerides (Triglycerides–LQ kit, Spinreact) concentrations were measured from 10 µL of plasma using commercial kits adapted to microplate format, according to the recommendations of the manufacturer. Each plasma sample was tested in duplicate and blanks were performed for standardization.

Immune plasma parameters

Plasma lysozyme activity was evaluated by turbidimetric assay, according to Ellis [56], based on the addition of the samples to a standard bacterial suspension of *Micrococcus lysodeikicus*. The absorbance decrease caused by bacterial lysis was measured by readings at 0.5 min and 4.5 min after addition. Values were standardized using hen egg white lysozyme (Sigma, Portugal).
Plasma peroxidase levels were determined by the chemical reduction of 3,3_, 5,5_— tetramethyl benzidine hydrochloride (Sigma, Portugal), according to Quade and Roth [57].

Liver enzymatic assays

Livers were homogenized in phosphate buffer, 0.1 M pH 7.4. Part of the homogenates were used for analysis of thiobarbituric acid reactive substances (TBARS). The remaining portion was centrifuged at 10000 g for 20 min and supernatants used for analysis of protein content, catalase (CAT) and glutathione transferase (GST). Lipid peroxidation was measured by TBARS using methods described by [58]. Results were reported as nmol. g^{-1}. The protein content of homogenates was measured using methods described in Bradford [59]. CAT activity was determined according to methods described by Clairborne [60] with hydrogen peroxide (30%) as substrate. GST activity was determined spectrophotometrically at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate, according to the method described by Habig, et al. [61].

Gene expression analyses

Total RNA from target tissues (liver, head kidney and spleen), was extracted with a MagMax-96 for microarrays total RNA isolation kit (Life Technologies, Carlsbad, USA) after tissue homogenization in TRI reagent. RNA quantity and purity were determined by Nanodrop (Thermo Scientific) with absorbance ratios at 260 nm/280 nm above 1.9. Reverse transcription (RT) of 500 ng of total RNA was performed with random decamers, using the High-Capacity cDNA Archive Kit (Applied Biosystems, USA). RT reactions were incubated for 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without reverse transcriptase. Real-time quantitative PCR (qPCR) was performed using an Eppendorf Mastercycler Ep Realplex Real-Time PCR Detection System (Eppendorf, Germany), using 96-well PCR array layouts designed for the simultaneous profiling of 19 genes in liver (Table S2) and 29 genes in head kidney and spleen (Table S3).

Genes selected for analysis in liver were focused on oxidation-reduction processes, cell redox homeostasis, response to oxidative stress and cellular respiration. Genes selected for immune response evaluation in head kidney and spleen were involved in response to bacterium, cytokine-cytokine receptor interaction, cytokine signalling, response to cytokines and cell proliferation. Primers were designed to obtain amplicons of 50-150 bp in length. The arrays included 23 new sequences for seabass, already represented in the IATS-nutrigroup seabass transcriptomic database (www.nutrigroup-iats.org/seabassdb; [62]) and uploaded to GenBank with the accession numbers MG596338-MG596342, MG596345, MH138003-MH138019 (Table S4). Among them, 16 are full codifying sequences. Diluted RT reactions were used in combination with SYBR Green Master Mix (Bio-Rad) and specific primers (final concentration of 0.9 µM), in a final volume of 25 µl. The PCR program used included an initial denaturation step (95 °C for 3 min), followed by 40 cycles of denaturation (15 s at 95 °C) and annealing/extension for 60 s at 60 °C. All pipetting operations were performed by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Germany) to improve data reproducibility. PCR efficiency (>90 %) and reactions specificity were verified by melting curve analysis (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C) and linearity of serial dilutions of RT reactions. Each reaction was performed in triplicate and the fluorescence data acquired during the extension phase was normalized by the delta–delta Ct method [63] using β-actin as the housekeeping gene.

Statistical analysis

Data was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test). The analysis of variance was performed applying two-way ANOVA test, with Diet (CTRL and GRA) and Infection (*Phdp*) as independent factors. Significant differences were considered for $p<0.05$. The statistic software package used was SigmaPlot 12 (Systat Software Inc., U.S.A.). Multivariate analysis (Partial Least Squares-Discriminant Analysis; PLS-DA) was also performed to find discriminative features among groups by means of the EZ-Info software (Umetrics, Sweden). The contribution of genes in the PCR-arrays to the PLS-DA models was assessed by means of variable importance in projection (VIP) measurements. A VIP score > 1 was considered an adequate threshold to determine discriminant variables in the PLS-DA model [64,65].

Results

Mortality

After infection seabass mortalities were recorded daily (Table 1). Placebo fish presented no mortalities, i.e. 100% survival rate, despite the dietary treatment. Infected fish fed the CTRL diet registered the first mortalities, namely at day one and three post-infection. At day four, mortalities were observed in both dietary groups infected with Phdp. Overall, deaths occurred within the predicted time-line for bacterial infections, between days three and seven post inoculation.

Table 1 - Mortality and survival percentage (b) recorded for 10 days post-infection in seabass fed the experimental diets (CTRL or GRA) and subjected to *Phdp* infection. N = 30 for each group. Superscript letters indicate significant differences between diets in infected groups p<0.05.

	DAYS POST-INFECTION											
DIET_INFECTION	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total Dead**	**% survival**
CTRL_PLACEBO	0	0	0	0	0	0	0	0	0	0	0	100.00
CTRL_PHDP	0	0	2	0	1	2	1	0	0	0	6	80.00[a]
CTRL_PHDP	1	0	1	2	1	0	2	0	0	0	7	76.67[a]
CTRL_PLACEBO	0	0	0	0	0	0	0	0	0	0	0	100.00
GRA_PLACEBO	0	0	0	0	0	0	0	0	0	0	0	100.00
GRA_PHDP	0	0	2	0	0	0	0	0	0	0	2	93.10[b]
GRA_PHDP	0	0	0	2	0	0	4	0	0	0	6	80.00[b]
GRA_PLACEBO	0	0	0	0	0	0	0	0	0	0	0	100.00

Growth parameters

Table 2 portrays the biometric data analysis for seabass which revealed no significant differences between dietary groups. Daily growth index revealed no statistical differences ($p>0.05$) between groups for diet, infection or their interaction. Feed conversion ratio and voluntary feed intake showed no differences between dietary groups or infection ($p>0.05$).

Table 2: Growth performance of seabass fed the experimental diets (CTRL or GRA) subjected infection with *Phdp*. Values presented as mean ± standard deviation. N=30 for each group. No significant differences were found for diet, infection or their interaction in two-way ANOVA for p<0.05.

	CTRL				*GRA*				*p < 0.05*		
	Placebo		*Phdp*		*Placebo*		*Phdp*				
	AVG	*STD*	*AVG*	*STD*	*AVG*	*STD*	*AVG*	*STD*	*Diet*	*Infection*	*D x I*
IBW	11.92	0.63	12.20	1.16	11.64	0.26	12.05	0.43	0.44	0.22	0.83
FBW	28.00	1.57	26.83	4.55	28.58	0.19	28.43	2.49	0.23	0.35	0.44
DGI	0.84	0.02	0.77	0.10	0.88	0.03	0.84	0.07	0.10	0.18	0.54
FCR	1.98	0.07	2.24	0.18	1.93	0.03	2.01	0.02	0.25	0.43	0.14
VFI	1.77	0.07	1.87	0.06	1.80	0.08	1.81	0.08	0.92	0.52	0.38

IBW, Initial body weight (g); FBW, Final body weight (g); DGI, Daily growth index; FCR, Feed conversion ratio; VFI, Voluntary feed intake ($g^{-1}.kg^{-1}.$ $days^{-1}$)

Plasma bioindicators

The evaluation of glucose levels (Fig. 1a) revealed no differences between diets, infection or the interaction between both factors (p>0.05).

On the contrary, triglycerides levels (Fig. 1b) showed differences between diets within placebo groups (p=0.018), with GRA diet presenting lower TGA levels than CTRL. However, no differences were detected between diets within Phdp infected fish (p>0.05). In addition, highly significant differences were detected when comparing placebo and Phdp infected seabass in each diet (p<0.001), with lower levels detected in infected fish. No statistical significance was found for the interaction between both factors in the TAG levels (p>0.05).

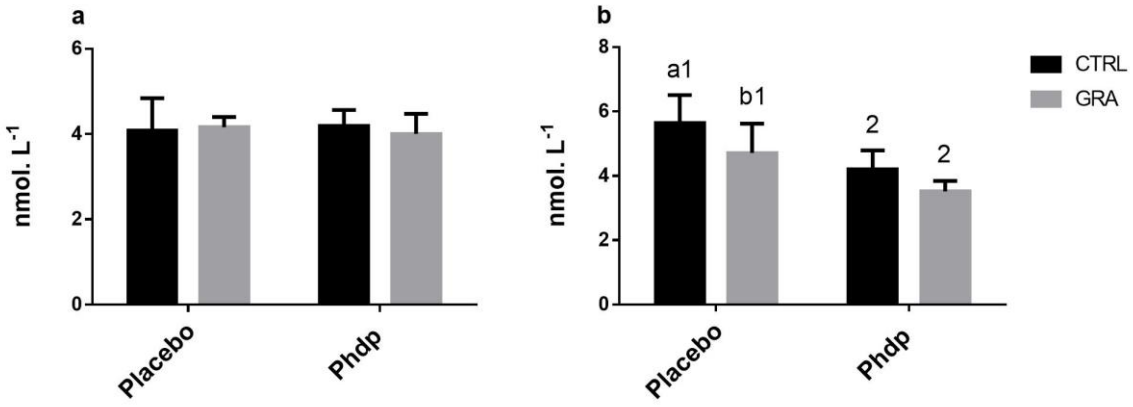

Figure 1 – Glucose (1a) and Triglycerides (1b) levels determined in plasma of seabass fed the experimental diets (CTRL or GRA) and subjected to *Phdp* infection. Results presented as mean ± standard deviation. N = 8 fish per group. Different letters indicate significant differences between diets and different numbers indicate differences between infection and placebo ($p<0.05$).

Immune parameters in plasma

The measurement of the levels of circulating peroxidase activity (Fig. 2a) revealed differences between the dietary treatments ($p<0.05$) and an interaction between diet and infection ($p<0.05$). The GRA groups presented lower peroxidase levels in both placebo and Phdp groups.

Lysozyme activity (Fig. 2b) showed statistical differences between dietary groups with higher levels observed for GRA diet ($p<0.001$). Considering infection, lower levels of lysozyme were detected in Phdp groups ($p=0.004$). Additionally, the interaction of both factors (diet and infection) revealed significant differences, with seabass fed the CTRL diet demonstrated lower lysozyme activity ($p=0.005$) than GRA.

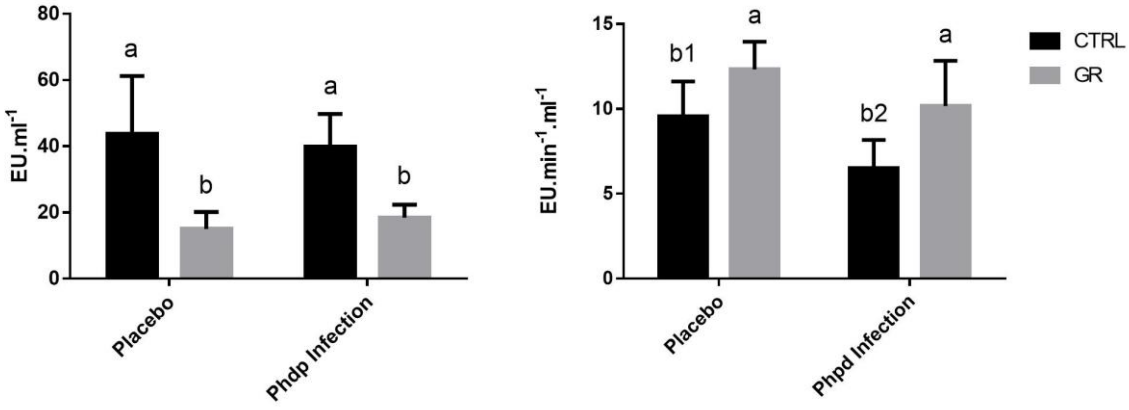

Figure 2 - Peroxidase (2a) and lysozyme (2b) activities determined in plasma of seabass fed the experimental diets (CTRL or GRA) and subjected to *Phdp* infection. Results presented as mean ± standard deviation. N = 8 fish per group. Superscript letters indicate significant differences between diets and different numbers indicate differences between infection for $p<0.05$.

Antioxidant parameters

Lipid peroxidation (Fig. 3a) displayed significant differences between diets within the Phdp infected groups, with lower peroxidation in GRA diet ($p=0.018$). Considering the CTRL diet alone, seabass infected with Phdp revealed higher lipid peroxidation for $p=0.002$.

Fish fed GRA diet demonstrated higher Catalase activity (Fig. 3b) within the placebo groups ($p=0.002$). No statistical differences were detected between diets in Phdp infected groups or the interaction of both diet and infection.

Glutathione s-transferase activity (Fig. 3c) showed highly significant statistical differences ($p<0.001$) between dietary treatments within placebo and Phdp groups, with GRA presenting higher GST activity levels than CTRL diet.

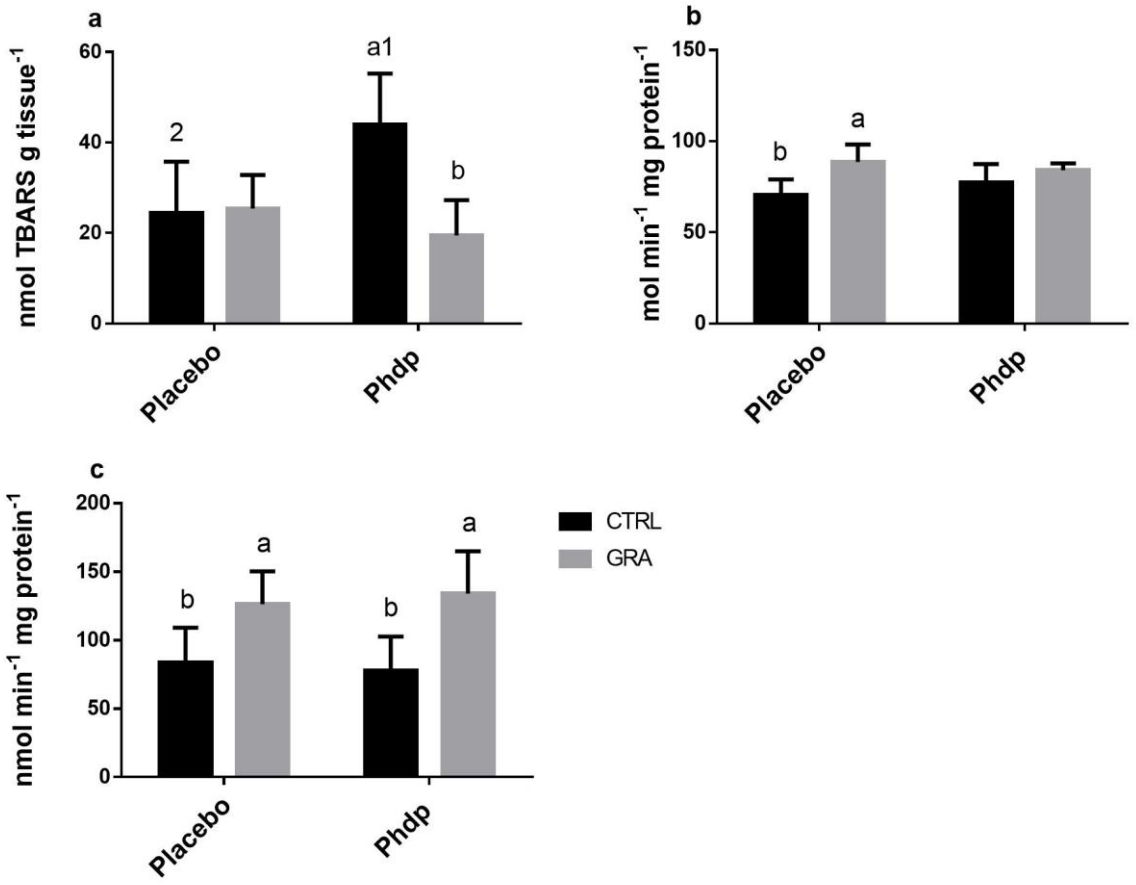

Figure 3 - Lipid peroxidation (3a), Catalase (3b) and Glutathione s-transferase (3c) activities determined in liver of seabass fed the experimental diets (CTRL or GRA) and subjected to *Phdp* infection. Results presented as mean ± standard deviation. N = 8 fish per group. Different letters indicate significant differences between diets and different numbers indicate differences between infection for $p<0.05$.

Gene Expression analysis

The effects of diet and infection were assessed for each tissue by means of specific PCR-arrays focused on genes related to cell redox homeostasis and response to oxidative stress in liver, or immune response and cell proliferation in head kidney and spleen.

Expression results for each experimental group and tissue, with the corresponding two-way ANOVA analysis can be found in Supplemental Tables S2-S4. Interestingly, in two

of the three tissues (liver and head kidney), the effect of diet was more evident than that of the Phdp infection challenge. In liver, 13 out of 19 genes were found to be differentially expressed by diet, whereas only 6 were differentially expressed with infection, and 7 by the combined of diet x infection effect. For head kidney, diet differences drove a differential expression for nine out of 29 genes, four genes were affected by the infection challenge, and the combination of diet and infection contributed to the differential expression of 7 genes. The relative effect of diet was less evident in the spleen, as the same number of genes, 7, was differentially expressed by diet or infection, whereas their combination affected 3 genes.

Multivariate analysis approach of tissue raw data contributed to a clearer visualization of the complex interplay between diet and infection. For instance, in liver, 77% of total variance was explained by four components, with the three main components explaining more than 67% of variance (Figure 4A). Seabass fed CTRL and GRA diets were clearly separated within the first component (26.10% of total variance), whereas separation along component 2 (18.87% of total variance, Figure 4B) and component 3 (22.23% of total variance, Figure 4C) contributed to the differentiation of infected and non-infected individuals within each dietary group. This approach also served to highlight and classify genes with the higher contribution to variation (VIP≥1, Figure 4D). According to this, the marked hepatic down-regulation, in seabass fed GRA diet, of heat shock proteins (*grp-78*, *grp-170*, *grp-94*, *grp-75*), *prdx1*, *sirt5*, *gr* and *sirt1* were the main factors contributing to component 1, that reflected diet separation. VIP contribution after 2 components highlighted that the differential response to Phdp infection was mainly due to the down-regulation of *prdx4* and *mn-sod*. Separation along component 3 revealed the contribution of *cs*, *prdx5* and *gpx4*. For the two later genes, no significant effect of diet or infection was found, but a significant diet x infection response was evidenced.

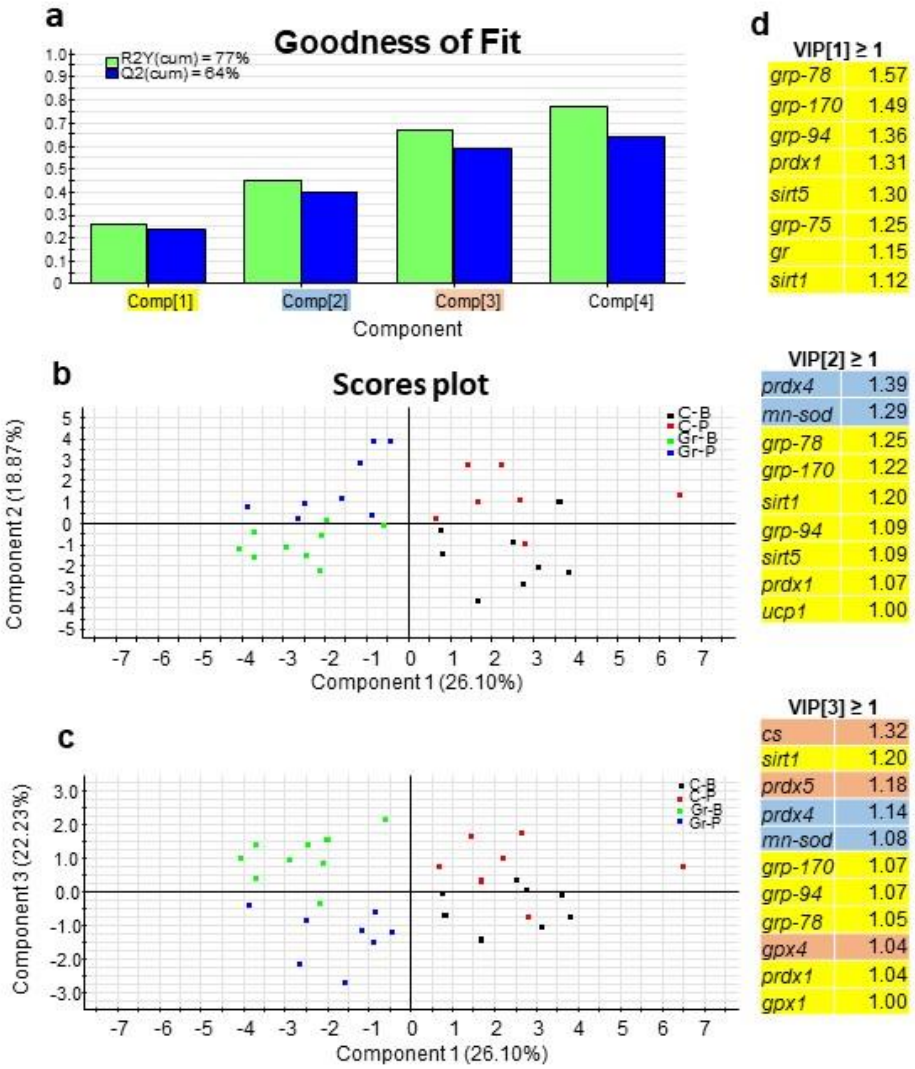

Figure 4 - Discriminant analysis (PLS-DA) of liver molecular signatures of seabass altered by dietary Gracilaria sp. supplementation and/or Phdp infection. (a) Cumulative coefficients of goodness of fit (R2, white bars) and prediction (Q2, grey bars) by each component; 77% of total variance is explained by four components. (b) PLS-DA score plot of acquired data from challenged individuals along component 1 and 2. (c) PLS-DA score plot of acquired data from challenged individuals along component 1 and 3. (d) Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation. Markers with VIP values > 1 after the first, second and third components are highlighted in yellow, blue and orange, respectively.

PLS-DA analysis of transcriptional response in head kidney also highlighted a separation of animals fed the CTRL or GRA diet along component 1 (40.82% of total variance) (Figure 5B). Scores plot of component 1 and component 2 allowed the discrimination among infected and non-infected individuals fed the CTRL diet (Figure 5C), although this separation was better accomplished along component 3, explaining 23.05% of total variance (Figure 5D). Interestingly, for animals fed the GRA diet, infected and non-infected individuals overlapped in all scores plots combinations, and for this reason these two groups were grouped together for analysis. With this approach, the three components explained 78% of total variance (Figure 7A). The most relevant VIP in component 1 revealed the contribution of several genes in group separation via up-regulation (*il34*, *ccr9*, *cd33*) or down-regulation (*mif*, *il1b*, *defb*, *a2m*, *myd88*) when fed GRA diet. After two components, VIP analysis highlighted the role of *g8x1* and *mmd*, which were down-regulated with infection in individuals fed CTRL diet. This separation was more evident with component 3, in which a clear up-regulation of *IgMs* in infected fish was evident in both dietary groups. Down-regulation of *leap2* with infection was also revealed as a relevant contribution for VIP after 3 components, regardless of the low expression level of this gene in head kidney.

Regarding spleen, PLS-DA analysis only found two components, with a very low explained (30%) or predicted (11%) variance, with no clear separation among experimental groups in the scores plot. Therefore, these results are presented in (Supplemental Figure S1).

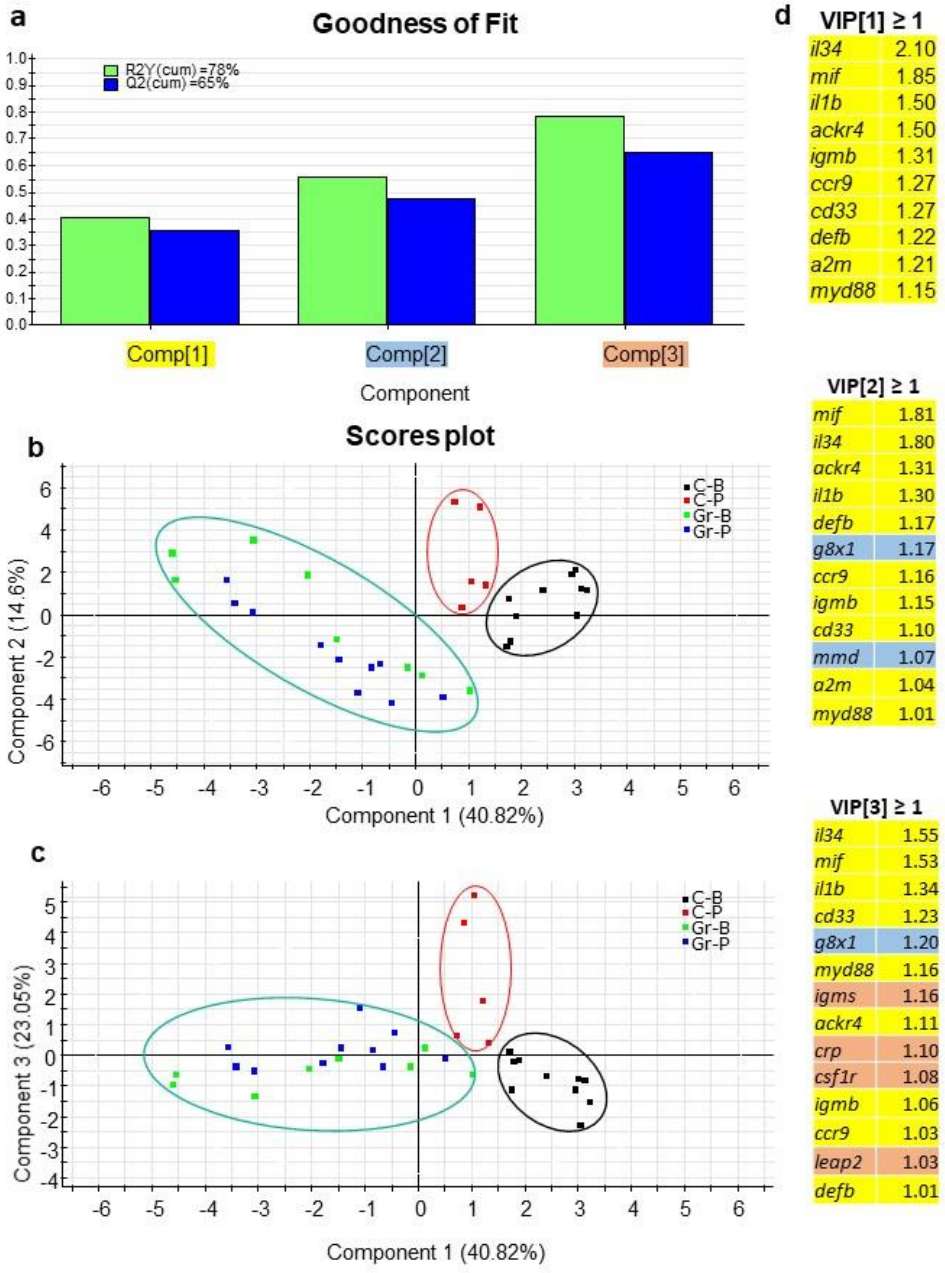

Figure 5 - Discriminant analysis (PLS-DA) of head kidney molecular signatures of seabass altered by dietary Gracilaria sp. supplementation and/or Phdp infection. (a) Cumulative coefficients of goodness of fit (R2, white bars) and prediction (Q2, grey bars) by each component; 78% of total variance is explained by four components. (b) PLS-DA score plot of acquired data from challenged individuals along component 1 and 2. (c) PLS-DA score plot of acquired data from challenged individuals along component 1 and 3. (d) Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation. Markers with VIP values > 1 after the first, second and third components are highlighted in yellow, blue and orange, respectively.

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Ethical statement

All procedures were conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE. The experiment took place at the Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto (Portugal). This study was approved by the ORBEA (Organismo Responsável pelo Bem-Estar dos Animais), the Institutional Animal Care and Use Committee (IACUC) of the ICBAS.

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Authors contributions

M.J. Peixoto wrote the main manuscript text. M.J. Peixoto and R.O.A. Ozório carried out the biochemical analyses and were responsible for figures 1, 2 and 3 and the tables 1, 2, S1 and S5. R.O.A. Ozório, L.J. Magnoni and M.J. Peixoto were responsible for the experimental design. R. Ferraz, J. Calduch-Ginerd and J. Pérez-Sánchez carried out the gene expression analyses and prepared figures 4, 5 and S1 and tables S2, S3, S4, S6, S7 and S8. J.F. Gonçalves were responsible for the coordination and preparation of the in vivo fish trial. R. Pereira were responsible for the seaweed production and processing. All authors reviewed the manuscript.

Data Availability Statement

The datasets generated during and/or analysed during the current study are available in the IATS-nutrigroup seabass transcriptomic database repository, www.nutrigroup-iats.org/seabassdb.

Competing Interests Statement

The authors declare no competing interests.

Supplementary Materials

Table S1 Proximate and chemical composition of the experimental diets.

Ingredients (%)	**CTRL**	**GRA**
Fishmeal 60	22.00	22.00
CPSP 90	3.00	3.00
Feather meal hydrolysate	5.00	5.00
Haemoglobin powder	5.00	5.00
Poultry meal 65	20.00	20.00
Pea protein concentrate	3.50	3.50
Soybean meal 48	9.00	9.00
Rapeseed meal	3.50	3.50
Wheat meal	14.80	14.80
Fish oil – SAVINOR	12.00	12.00
Vit & Min Premix PV01	1.00	1.00
Lutavit E50	0.03	0.03
Choline chloride	0.07	0.07
Betaine HCl	0.10	0.10
Binder (Kieselghur)	0.30	0.30
Antioxidant	0.20	0.20
Sodium propionate	0.10	0.10
L-Lysine	0.20	0.20
DL-Methionine	0.10	0.10
L-Taurine	0.10	0.10
Gracilaria sp.	0.00	0.05
Chemical composition	**CTRL**	**GRA**
Dry matter (%)	93.9 ± 0.0	93.5 ± 0.2
Crude protein (%DM)	51.4 ± 0.0	50.3 ± 0.1
Crude fat (%DM)	19.1 ± 0.3	18.2 ± 0.2
Gross Energy (kJ/g DM)	23.6 ± 0.1	23.4 ± 0.1
Ash (%DM)	10.2 ± 0.2	10.1 ± 0.1

* *Gracilaria* sp. extract was added as separate supplement to the basal diet at 5 % w/w base, adjusted for dry matter content (DM).

Table S2. Forward and reverse primers for the oxidative stress pathway focused PCR-array.

Gene name	Symbol		Primer sequence (5'-3')
β-actin	*actb*	F	TCC TGC GGA ATC CAC GAG A
		R	AAC GTC GCA CTT CAT GAT GCT
Citrate synthase	*cs*	F	GTG TAT GAG ACC TCC GTG TTG G
		R	AGC AAC TTC TGA CAC TCT GGA ATG
Cytochrome c oxidase subunit I	*coxi*	F	ATA CTT CAC ATC CGC AAC CAT AA
		R	AAG CCT CCG ACT GTA AAT AAG AAA
Mitochondrial respiratory uncoupling protein 1	*ucp1*	F	CGA TTC CAA GCC CAG ACG AAC CT
		R	TGC CAG TGT AGC GAC GAG CC
Sirtuin 1	*sirt1*	F	GGT GGA CCT CTT GAT TGT CAT TGG CTC TTC
		R	GGG ATG AGG GCA ACT GGT CGG ACT TTA
Sirtuin 5	*sirt5*	F	AGA CAC AGA TGA CGC AGA GAT
		R	TCA GGA GAC CGT GAC AGC
Catalase	*cat*	F	GCA TCA GGT GTC TTT CTT GTT CAG
		R	GGA GCC GTA GCC GTT CAT
Superoxide dismutase Mn	*mn-sod*	F	AGG CTA TCT GGA ATG TCA TCA ACT GGG AGA
		R	GCA GTC TGG AGA CGC TCG GTC AC
Glutathione peroxidase 1	*gpx1*	F	TGC CCA CCC TTT GTT TGT CTA TCT
		R	CCA TCA GGA CTG ACC AGG AAC T
Glutathione peroxidase 4	*gpx4*	F	GCA CGC CAA GTA CGC TGA GAG
		R	GCT CCT GGT TCC CAA ACT GGT TAG A
Glutathione reductase	*gr*	F	GGT GGA CTG TCT CCT GTG
		R	TCA TCT CGC CGA TGT TCA
Peroxiredoxin 1	*prdx1*	F	GTC TGG GTT CTA TGA AGA TTC CGC TGG TAT
		R	TTA GGA CGC CGT AAT CAG TGG AGA TG
Peroxiredoxin 4	*prdx4*	F	TGT TGA CTC CCA GTT CAC CCA CCT T
		R	CCA CCC TGC TTC CTC GGC GTA T
Peroxiredoxin 5	*prdx5*	F	TGT TGG TGG AAG ATG GAG TTG TGA AGA AGA
		R	CAG GTC AGC CCA GTG CCA TCA G
Peroxiredoxin 6	*prdx6*	F	CCA CCC AAG GGA CTT CAC
		R	CTG ATC CTA GCG GCA CAG
Glucose-regulated protein 94	*grp-94*	F	CGA TGG CAC AGT AGA AGA GGA CCT TGG TAA
		R	GCA CCG CCT CAT CAT CTG TTC TGG
Glucose-regulated protein 170	*grp-170*	F	CTA CGG CGA CCT CAG CTT CCT
		R	GTC AGG TTC AGA GAG CCA AAC ACA CT
Glucose-regulated protein 78	*grp-78*	F	GCC GAC GAC GAC GAT AAG AGG
		R	CCAG GTC GAT TCC AAC CAC AGT
Mitochondrial Hsp10	*mthsp10*	F	GCT TTA CTC CAG AGA CTG TGA CGA A
		R	CAG CAC CTT GCC TTG AGA CTT C
Mitochondrial Hsp70 mortalin	*grp-75*	F	GCT GCT GGT CGG AGG AAT GTC
		R	GTC CTG AAC CGT TTG CTG AAC CTT

Table S3. Forward and reverse primers for the immune pathway focused PCR-array.

Gene name	Symbol		Primer sequence (5'-3')
β-actin	*actb*	F	TCC TGC GGA ATC CAC GAG A
		R	AAC GTC GCA CTT CAT GAT GCT
Interleukin 1-beta	*il1b*	F	CAT GAG CGA GAT GTG GAG ATC CAA GAT
		R	CAT TGT CAG TGG GTG GTG GGT AAT C
Interleukin-8	*il8*	F	CAA TCA GCA GGG ACT ACA ACA CAC A
		R	CTG TCT GGA GGG ATG ATC CTT GAC T
Interleukin-10	*il10*	F	CAG TGC TGT CGT TTT GTG GAG GGT TTC
		R	TCT CTG TGA AGT CTG CTC TGA GTT GCC TTA
Interleukin-20	*il20*	F	GCT AGA AAT AAA GGA GGC GGC ACA GAA GG
		R	CAG TCC AGC ACA GTG TCC AGT TCT C
Interleukin-34	*il34*	F	AGA ACC CGA CAG AGT GCC AGA GT
		R	CAG GAG GGA TTT TGG GGA CGC ATA TC
Tumour necrosis factor α	*tnfα*	F	TCT ACA GCC AGG CGT CGT TCA G
		R	CCG CAC TTT CCT CTT CAC CAT CGT
C-C chemokine receptor type 3	*ccr3*	F	TGA CCT TCG ACC GAC ACC TA
		R	ACA ATA CAG GAG ACT ACC GCA TAG C
C-C chemokine receptor type 9	*ccr9*	F	CCT GTG TGT CTG GCT TGT TTC TAC TCT C
		R	TCG CTC TTC ACC TGG GCA AAG ATA AAC TC
Atypical chemokine receptor 4/C-C chemokine receptor type 11	*ackr4*	F	TAC TTC TCT TCA CCC TGC CTT TCT G
		R	GCT GCC GAA CCC AAC TTC CA
T-cell surface glycoprotein CD3 zeta chain	*cd247*	F	CTG ATG CGT CTG AAG AGA ATG GAG GC
		R	GTT CAA GCA CCT GGT AAG GAT CAG CAT C
T-cell surface glycoprotein CD8 beta	*cd8b*	F	AGT GAT CCC GCC AAC ATT ACC TCC TA
		R	TCT TCT TAG GGC AGC GAC AGA CT
Myeloid differentiation primary response protein MyD88	*myd88*	F	CCA ATT CAG GTT GAT GAG GTT GAC A
		R	TCC TCC AGG GTG ATA CCA ATC C
Myeloid cell surface antigen CD33	*cd33*	F	CTG TTC ATT CAC CCA TCC TAG AG
		R	GGT CGA ACG ATG CCA GAT T
Macrophage colony-stimulating factor 1 receptor	*csf1r*	F	CGG GCA GGA ACA GCT AAT CTA CCA
		R	ACT TGG GCT CAT CAC ACA CTT CAC
Macrophage migration inhibitory factor	*mif*	F	GCT CCC TCC ACA GTA TTG GCA AGA T
		R	TTG AGC AGT CCA CAC AGG AGT TTA GAG T
Monocyte to macrophage differentiation factor	*mmd*	F	GGT CAT CTA CTT CTT CAT CGC TGC CTC CTA
		R	CCA ACT CTC GCA GGT TCA ACC AAG GT
Interferon regulatory factor 8	*irf8*	F	TCT GAA GGC TGC CGA ATC TCC
		R	CTG TCT GAA CTG TAT AGG GCA CCA C
Nuclear factor NF-kappa-B p100 subunit	*nfkb2*	F	CTG GAG GAA ACT GGC GGA GAA GC
		R	CAG GTA CAG GTG AGT CAG CGT CAT C
Liver-expressed antimicrobial peptide 2	*leap2*	F	GGT TTG CTC CAA CGG ACC AA
		R	CAC AGG CTT CAT GCT GTT CCA
Lysozyme C	*lyz*	F	CGG AGC CAT CAA CCA CAA CAC TG
		R	GCC ATT ATT ACA CCA CCA GCG ACT GT
Beta-defensin	*defb*	F	GGG CTG AGC TTG GTT CTC CTT GT
		R	CCT CCC CAA CTG CGA GCA TCA
IgM membrane-bound	*igmb*	F	ACA GAG GAA GAT AAC ATG GCG GTG G
		R	TGG TTA CAA TGG TGA ACA GCA GAG TGAT

Table S4. Classification of new assembled sequences according to BLAST-X searches. [a] Gene identity determined through BLAST-X searches: *leap2*:liver-expressed antimicrobial peptide 2; *lyz*: lysozyme C; *defb*: beta-defensin; *igmb*: IgM membrane-bound; *a2m*: alpha-2 macroglobulin; *lgals8x1*: galectin-8-like isoform X1; *ucp1*: mitochondrial respiratory uncoupling protein 1; *sirt1*: sirtuin 1; *sirt5*: sirtuin 5; *cat*: catalase; *mn-sod*: superoxide dismutase Mn; *gpx1*: glutathione peroxidase 1; *gpx4*: glutathione peroxidase 4; *gr*: glutathione reductase; *prdx1*: peroxiredoxin 1; *prdx4*: peroxiredoxin 4; *prdx5*: peroxiredoxin 5; *prdx6*: peroxiredoxin 6; *grp-94*: glucose-regulated protein 94; *grp-170*: glucose-regulated protein 170; *grp-78*: glucose-regulated protein 78; *mthsp10*: mitochondrial Hsp10; *grp-75*: mitochondrial Hsp70 mortalin. [b] Best BLAST-X protein sequence match (lowest E value). [c] Expectation value. [d] Codifying sequence.

Contig	Size (nt)	Annotation[a]	Best match[b]	E[c]	CDS[d]	GenBank accession
L12_73806	1173	*leap2*	XP_023257275	1e-38	200-439	MG596338
L12_66506	950	*lyz*	KKF29953	7e-88	195-626	MG596339
L2_17762	328	*defb*	ADJ21805	7e-29	<1-226	MG596340
L12_87068	2152	*igmb*	ARC77253	0	139-1632	MG596341
L3_71324	569	*a2m*	XP_019123863	8e-90	<1->569	MG596342
L12_74857	1236	*lgals8x1*	ANN46245	0	43-999	MG596345
L12_80996	1495	*ucp1*	XP_023147774	0	162-1082	MH138003
L2_44162	707	*sirt1*	XP_018536491	1e-161	<1->681	MH138004
L12_83923	1727	*sirt5*	XP_008276398	0	65-982	MH138005
L12_87749	2310	*cat*	XP_022611780	0	<1-1047	MH138006
L3_68914	981	*mn-sod*	ANS56706	7e-164	51-728	MH138007
L12_69789	994	*gpx1*	XP_023265962	8e-124	227-655	MH138008
L3_67061	957	*gpx4*	XP_023118319	3e-122	54-629	MH138009
L12_86603	2069	*gr*	XP_018549649	0	83-1576	MH138010
L3_72270	1072	*prdx1*	XP_018532829	5e-136	61-657	MH138011
L12_66928	955	*prdx4*	XP_019941327	3e-172	92-883	MH138012
L12_67332	960	*prdx5*	XP_018547633	3e-129	131-703	MH138013
L3_49822	768	*prdx6*	XP_018525676	3e-155	79-744	MH138014
L12_89051	2765	*grp-94*	XP_023283565	0	104-2509	MH138015
L2_65026	450	*grp-170*	ADX97080	4e-77	<1->450	MH138016
L12_51413	785	*grp-78*	KKF16569	1e-150	165->785	MH138017
L3_44203	706	*mthsp10*	XP_011605755	8e-61	249-548	MH138018
L1_45931	726	*grp-75*	ABF70952	7e-145	<1->726	MH138019

Table S5. Growth parameters, plasma bioindicators, immune and antioxidant parameters in seabass fed the experimental diets for 90 days, following Phdp infection.

	CTRL				*GRA*				*p < 0.05*		
	Placebo		*Phdp*		*Placebo*		*Phdp*				
	AVG	*STD*	*AVG*	*STD*	*AVG*	*STD*	*AVG*	*STD*	*Diet*	*Infection*	*D x I*
IBW (g)	11.92	0.63	12.20	1.16	11.64	0.26	12.05	0.43	0.44	0.22	0.83
FBW (g)	28.00	1.57	26.83	4.55	28.58	0.19	28.43	2.49	0.23	0.35	0.44
WG (g)	16.09	0.94	14.63	3.39	16.94	0.45	16.39	2.06	0.12	0.18	0.47
DGI	0.84	0.02	0.77	0.10	0.88	0.03	0.84	0.07	0.10	0.18	0.54
Total Feed (g)	954.85	89.17	984.25	106.14	973.95	42.21	988.70	115.82	:::::::::::	:::::::::::	:::::::::::
ABW (g)	19.96	1.10	19.51	2.85	20.11	0.04	20.24	1.46	0.44	0.65	0.46
FCR	1.98	0.07	2.24	0.18	1.93	0.03	2.01	0.02	0.25	0.43	0.14
VFI (g/Kg/days)	1.77	0.07	1.87	0.06	1.80	0.08	1.81	0.08	0.92	0.52	0.38
Peroxidase (Eu.ml-1)	43.78	17.47	39.93	9.82	15.05	5.13	18.40	3.95	**<0,05**	0.48	**<0,05**
Lysozyme (Eu.min-1.ml-1)	9.56	2.06	6.51	1.66	12.34	1.62	10.17	2.66	**<0,001**	**0.00**	**0.01**
LPO (nmol TBARS g tissue−1)	24.38	11.38	44.05	11.20	25.40	7.43	19.50	7.76	**0.02**	0.15	**0.00**
CAT (mol min−1 mg protein−1)	70.63	8.43	77.42	9.98	88.58	9.59	84.08	3.72	**0.00**	0.74	0.21
GST (nmol min−1 mg protein−1)	83.51	25.82	77.87	24.96	126.45	23.98	134.11	30.98	**<0,001**	0.93	0.00
Glucose (nmol. L−1)	4.08	0.77	4.19	0.38	4.16	0.24	4.01	0.47	0.88	0.96	0.42
TGA (nmol. L−1)	5.64	0.87	4.20	0.60	4.71	0.91	3.51	0.33	**0.02**	**<0,001**	0.13

Significant differences (p<0.05) in bold.

Table S6 – Two-way ANOVA of gene expression levels in the head kidney of seabass fed the experimental diets for 90 days, following Phdp infection. Data are expressed as mean ± SD (N=10). All data are normalized to the expression level of *cd247* of fish fed control diet and non-infected, with an arbitrarily assigned value of 1. Significant differences (p<0.05, two-way ANOVA) are in bold.

	Dietary treatments								***p* value**		
	Control				***Gracilaria* sp.**						
	Placebo		**Infected**		**Placebo**		**Infected**				
Genes	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Diet**	**Infection**	**D x I**
a2m	0.76	0.31	1.59	0.64	0.14	0.05	0.11	0.03	**0.008**	0.940	0.938
ackr4	1.87	0.87	0.16	0.01	0.28	0.04	0.35	0.05	0.068	0.147	**0.018**
defb	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.00	**0.006**	0.275	0.285
ccr3	1.48	0.25	1.00	0.10	1.62	0.27	1.38	0.21	0.310	0.127	0.614
ccr9	1.39	0.29	0.84	0.07	2.01	0.44	2.48	0.59	**0.010**	0.910	0.223
c3	0.54	0.24	1.13	0.43	0.16	0.07	0.11	0.04	0.881	0.955	0.968
g8x1	4.22	0.60	2.84	0.18	2.74	0.31	3.10	0.42	0.134	0.211	**0.036**
igmb	25.59	8.79	10.68	1.24	32.83	6.57	29.83	8.70	0.066	0.205	0.397
igms	67.49	17.86	126.166	19.26	93.75	19.41	290.790	55.32	**0.010**	**< 0.001**	0.077
irf8	6.64	1.48	4.96	0.31	6.59	0.95	8.79	1.51	0.179	0.995	0.074
il1b	0.20	0.10	0.26	0.07	0.06	0.02	0.05	0.01	**0.002**	0.214	0.102
il10	0.17	0.03	0.17	0.02	0.15	0.02	0.22	0.03	0.690	0.163	0.192
il20	0.13	0.03	0.06	0.01	0.06	0.01	0.07	0.01	0.093	0.693	0.189
il34	0.48	0.09	0.22	0.02	0.73	0.08	0.58	0.07	**< 0.001**	**0.006**	0.400
il8	0.56	0.13	0.39	0.08	0.48	0.08	0.77	0.21	0.554	0.545	0.696
leap2	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.392	**0.010**	**< 0.001**
lyz	3.61	1.17	1.26	0.52	0.62	0.26	0.32	0.07	0.638	0.374	0.395
csf1r	3.59	0.78	3.42	0.35	3.40	0.63	6.06	1.24	0.144	0.137	0.093
mif	22.23	3.20	21.74	1.76	8.99	1.09	14.35	2.12	**< 0.001**	0.266	0.185
mmd	2.45	0.53	1.38	0.12	1.14	0.12	1.39	0.13	0.076	0.805	**0.007**
cd33	2.59	0.62	2.57	0.22	5.16	0.96	5.25	1.20	**0.038**	0.801	0.213
myd88	6.72	0.70	7.60	0.32	5.72	0.39	6.54	0.42	**0.036**	0.078	0.953
nfkb2	8.93	1.09	6.84	0.40	7.94	0.85	9.61	1.44	0.897	0.578	0.340
crp	41.80	8.13	51.20	4.32	37.11	4.41	41.81	3.77	0.191	0.190	0.659
sap	0.05	0.01	0.06	0.01	0.08	0.01	0.05	0.01	0.100	0.372	**0.022**
cd247	1.14	0.27	0.82	0.06	0.97	0.19	1.80	0.41	0.121	0.315	**0.030**
cd8b	0.79	0.17	0.33	0.05	0.57	0.07	1.20	0.33	0.875	**0.011**	0.737
trf	58.23	35.41	6.90	2.72	1.02	0.45	0.63	0.13	0.689	0.147	0.246
tnfα	0.34	0.06	0.20	0.01	0.22	0.03	0.31	0.05	0.953	0.930	**0.041**

Table S7– Two-way ANOVA of gene expression levels in the spleen of seabass fed the experimental diets for 90 days, following Phdp infection. Data are expressed as mean ± SD (N=10). All data are normalized to the expression level of *ackr4* of fish fed control diet and non-infected, with an arbitrarily assigned value of 1. Significant differences (p<0.05, two-way ANOVA) are in bold.

	Dietary treatments								***p* value**		
	Control				***Gracilaria* sp.**						
	Placebo		**Infected**		**Placebo**		**Infected**				
Genes	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Diet**	**Infection**	**D x I**
a2m	0.50	0.17	0.26	0.10	0.47	0.21	0.19	0.05	0.179	0.692	0.690
ackr4	1.06	0.11	0.77	0.07	1.21	0.09	1.07	0.10	**0.032**	**0.037**	0.996
defb	0.01	0.00	0.02	0.01	0.02	0.01	0.02	0.00	0.146	0.253	0.094
ccr3	0.49	0.08	0.59	0.09	1.04	0.14	1.04	0.17	**0.014**	0.172	0.684
ccr9	1.28	0.10	0.75	0.08	1.31	0.14	1.23	0.11	0.073	0.057	0.074
c3	0.44	0.14	0.27	0.12	0.60	0.21	0.17	0.06	0.189	0.449	0.528
g8x1	2.21	0.20	1.81	0.16	1.76	0.10	1.93	0.12	0.525	0.091	0.136
igmb	7.52	1.42	3.81	1.04	10.33	1.74	9.04	1.48	**0.026**	0.171	0.124
igms	29.65	2.70	68.88	20.17	58.48	11.75	75.037	11.40	0.078	0.076	0.234
irf8	8.36	0.63	6.99	0.65	7.53	0.51	7.58	0.32	0.396	0.376	0.368
il1b	0.06	0.02	0.12	0.03	0.07	0.04	0.02	0.01	**< 0.001**	0.708	0.059
il10	0.34	0.06	0.22	0.03	0.38	0.11	0.24	0.03	0.857	0.927	0.783
il20	0.15	0.02	0.10	0.02	0.17	0.03	0.14	0.02	0.262	0.188	0.241
il34	3.62	0.38	1.90	0.20	3.53	0.40	2.68	0.29	0.440	**0.001**	0.175
il8	0.41	0.04	0.33	0.06	0.27	0.04	0.39	0.07	0.295	0.248	0.305
leap2	0.02	0.01	0.01	0.00	0.04	0.01	0.02	0.00	**0.015**	**0.010**	**0.006**
lyz	1.20	0.43	0.29	0.18	2.08	0.86	0.46	0.15	0.624	**0.023**	0.559
csf1r	9.97	0.98	7.66	0.60	6.68	0.40	10.17	0.91	0.568	0.305	**0.038**
mif	4.81	0.41	4.58	0.42	4.66	0.37	4.81	0.91	0.416	0.189	0.885
mmd	0.56	0.04	0.63	0.06	0.50	0.04	0.54	0.03	0.261	0.786	0.706
cd33	17.78	1.40	16.63	1.12	16.11	1.21	13.90	1.35	0.185	0.082	0.299
myd88	3.52	0.36	3.72	0.23	3.21	0.25	3.02	0.18	0.178	0.729	0.606
nfkb2	5.80	0.35	4.41	0.38	6.01	0.62	5.12	0.29	0.292	**0.004**	0.111
crp	16.77	1.51	19.42	1.74	11.75	1.03	16.80	2.62	**0.005**	0.303	0.958
sap	0.01	0.00	0.01	0.00	0.02	0.01	0.02	0.01	0.816	0.590	0.514
cd247	2.09	0.24	1.22	0.14	3.11	0.79	2.01	0.18	0.908	0.795	**0.017**
cd8b	0.44	0.03	0.29	0.04	0.68	0.08	0.57	0.06	**< 0.001**	**0.009**	0.936
trf	3.81	1.57	1.90	0.72	2.85	0.93	2.00	0.57	0.768	0.862	0.542
tnfα	0.28	0.03	0.22	0.02	0.29	0.04	0.24	0.03	0.844	**0.012**	0.752

Table S8– Two-way ANOVA of gene expression levels on the liver of seabass fed the experimental diets for 90 days, following Phdp infection. Data are expressed as mean ± SD (N=10). All data are normalized to the expression level of *grp-78* of fish fed control diet and non-infected, with an arbitrarily assigned value of 1. Significant differences (p<0.05, two-way ANOVA) are in bold.

	Dietary treatments								***p* value**		
	Control				***Gracilaria* sp.**						
	Placebo		**Infected**		**Placebo**		**Infected**				
Genes	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Diet**	**Infection**	**D x I**
cs	0.70	0.06	0.67	0.05	0.57	0.08	0.58	0.03	**0.029**	0.810	0.531
coxi	94.74	15.01	72.11	9.33	65.72	12.33	45.88	6.57	**0.012**	0.103	0.940
ucp1	2.01	0.33	1.02	0.13	2.70	0.25	2.25	0.20	**< 0.001**	**0.011**	0.160
sirt1	0.04	0.00	0.04	0.00	0.02	0.00	0.03	0.00	**< 0.001**	0.053	0.355
sirt5	0.27	0.02	0.26	0.02	0.21	0.02	0.17	0.01	**< 0.001**	0.162	0.426
cat	8.21	0.39	6.95	0.92	9.05	0.88	6.18	0.31	0.951	**0.004**	0.244
mn-sod / sod2	0.69	0.08	0.40	0.05	0.89	0.12	0.535	0.05	**0.018**	**< 0.001**	0.858
gpx1	0.51	0.05	0.51	0.06	0.45	0.06	0.36	0.03	0.051	0.413	0.443
gpx4	26.36	2.56	22.17	2.15	18.88	1.19	23.23	1.67	0.111	0.969	**0.036**
gr	0.16	0.02	0.19	0.02	0.12	0.01	0.10	0.01	**0.001**	0.551	0.200
prdx1	1.90	0.62	1.55	0.22	0.87	0.09	0.52	0.08	**< 0.001**	**0.010**	**0.004**
prdx4	2.03	0.22	1.25	0.15	1.84	0.10	0.91	0.07	0.093	**< 0.001**	0.246
prdx5	0.14	0.01	0.20	0.03	0.22	0.02	0.12	0.01	0.671	0.281	**< 0.001**
prdx6	5.54	0.52	4.07	0.36	5.76	0.46	5.19	0.59	0.178	**0.043**	0.360
grp-94	2.37	0.39	3.44	0.52	1.36	0.19	0.59	0.10	**< 0.001**	0.705	**0.006**
grp-170	0.57	0.12	1.45	0.21	0.19	0.04	0.19	0.08	**< 0.001**	0.079	**0.009**
grp-78	1.29	0.26	3.22	0.60	0.33	0.07	0.23	0.04	**< 0.001**	0.092	**0.005**
mthsp10	0.47	0.06	0.65	0.11	0.50	0.08	0.30	0.04	**0.014**	0.529	**0.012**
grp-75	0.63	0.09	0.86	0.16	0.35	0.04	0.35	0.05	**< 0.001**	0.464	0.396

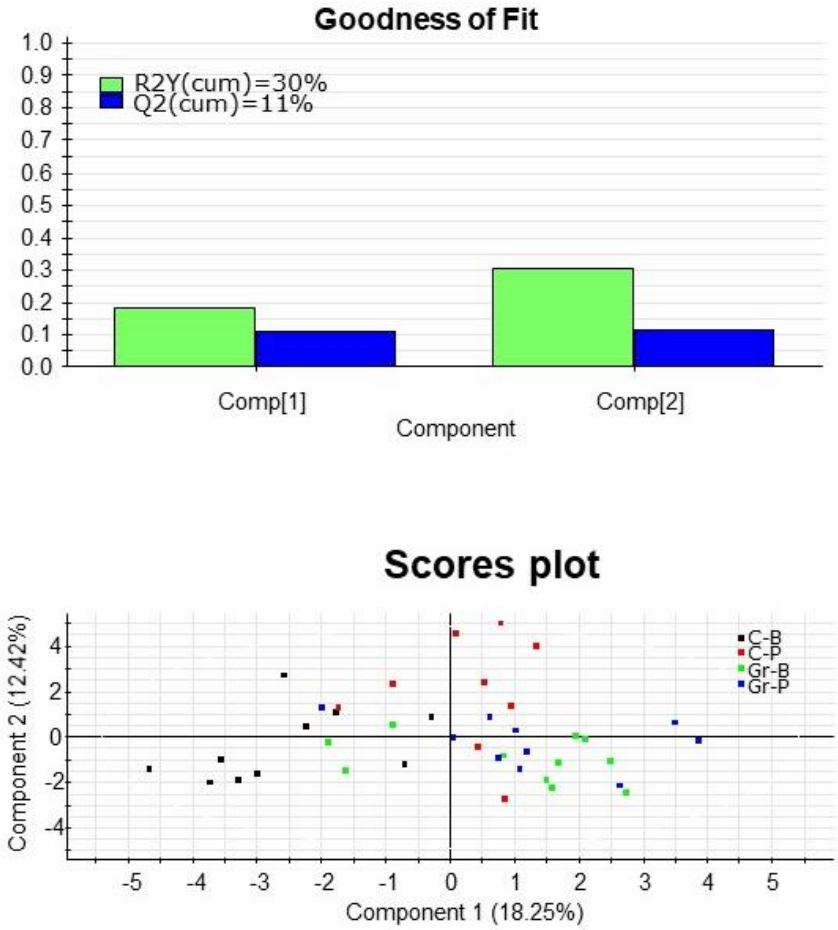

Figure S1 – Discriminant analysis (PLS-DA) of spleen molecular signatures of seabass altered by dietary *Gracilaria* sp. supplementation and/or Phdp infection.

CHAPTER VII

DISCUSSION

CHAPTER VII - DISCUSSION

GENERAL APPRECIATION

Aquaculture practices were mainly developed to ensure sustainability and worldwide availability of fish products (Subasinghe *et al.* 2009). However, the pressure over marine resources to "fuel" the sector has given a problematic connotation to this industry. The demand for fishmeal and oil reached a breaking point, which fisheries can no longer sustain. To address this issue, research in aquafeeds is mandatory. Consequently, several studies have addressed the issue of feedstuffs substitution by alternative ingredients, such as plant derived protein and oils (Naylor *et al.* 2009). Indications of improved immune and antioxidant bioindicators were also detected in these studies, increasing the interest in nutraceuticals or functional feeds, which could make way for the application of tailor-made diets for each aquaculture species or specific situation. In parallel, to reduce the need of fishmeal and oil, other marine sources began to be explored, specifically the inclusion of seaweeds in diets. However, the supplementation of seaweeds in aquafeeds needs to account for fish nutritional requirements, specifically in this case of seabass and meagre, fulfilling their protein and lipids necessities and at the same time improving their immune system.

The application of seaweeds revealed contradictory findings since their nutritional content (protein and lipid) was found to diverge with seasonality, temperature and salinity (Juneja *et al.* 2013; Marinho-Soriano *et al.* 2006). In addition, the level of the inclusion itself became a subject of interest since high levels of supplementation were associated with deleterious effects (Younis *et al.* 2018). The inclusion of *Gracilaria arcuata* at 20%, 30% and 40% in *Oreochromis niloticus* diets resulted in decreased final weight (FW), weight gain (WG), daily growth rate (DGR) and specific growth rate (SGR). In fact, the relation level of inclusion/growth parameters revealed to be inverse correlated (Younis *et al.* 2018). Moreover, seaweeds application hinted on immunomodulation with species-specific responses (Araújo *et al.* 2015; Soler-Vila *et al.* 2009b). In accordance with the seaweed nutritional and chemical profile their application elicited diverse responses associated with somatic growth, immunity modulation, antioxidant balance or gut flora (O'Sullivan *et al.* 2010). Additionally, distinct fish species metabolize seaweeds differently, leading to complex conclusions about seaweeds properties and possible uses in aquafeeds.

Thus, the general aim of this thesis was to evaluate the effects of seaweeds as feed supplement for seabass and meagre aquaculture (Table 4). This work was further supported through a close collaboration with innovative seaweed producers (ALGAPLUS, Portugal) that

provided extensive knowledge and seaweeds samples representative of the main phyla (Phaeophyta, Chlorophyta and Rhodophyta) which are known to have distinct composition (Rodrigues *et al.* 2015).

Table 4 – Summary of the seaweeds species and the parameters analyzed in seabass and meagre.

D. labrax	*Fucus* sp.	*Ulva* sp.	*Alaria* sp.	*Gracilaria* sp.		
				Crude	Methanolic extract	Aqueous extract
Growth	✔	✔	✗	✔	✔	✔
Digestive activity	✔	✔	✗	✔	✔	✗
Blood biochemistry	✗	✗	✗	✗	✗	✔
Immune responses	✔	✔	✗	✔	✔	✔
Oxidative responses	✔	✔	✗	✔	✔	✔
Oxygen consumption	✔	✔	✗	✔	✗	✗
Transcriptional levels	✗	✗	✗	✗	✗	✔
A. regius	*Fucus* sp.	*Ulva* sp.	*Alaria* sp.	*Gracilaria* sp.		
				Crude	Methanolic extract	Aqueous extract
Growth	✗	✗	✔	✔	✗	✗
Digestive activity	✗	✗	✔	✔	✗	✗
Blood biochemistry	✗	✗	✔	✔	✗	✗
Immune parameters	✗	✗	✔	✔	✗	✗
Oxidative responses	✗	✗	✔	✔	✗	✗
Oxygen consumption	✗	✗	✔	✔	✗	✗
Transcriptional levels	✗	✗	✗	✗	✗	✗

In view of previous publications that indicated the beneficial effects (dietary modulation, altered growth and immune capacities) of seaweeds supplementation in seabass (Kiron 2012), the first work in this thesis (**Chapter II)** aimed to investigate the role of seaweed dietary supplementation in growth performance, digestive capacity and immune and stress responsiveness of European seabass (*Dicentrarchus labrax*). Here the experimental procedure was designed to evaluate how fish responded to the diverse seaweed classes, and to assess if distinct levels of seaweed supplementation would lead to significantly different outcomes. Previously, seabass was found to present different nutrient utilization and digestibility when fed diets supplemented with green or red seaweeds, as well as at 5 or 10%

inclusion levels (Valente *et al.* 2006). In the present work (**Chapter II),** the dietary supplementation of *Fucus* sp., *Ulva* sp. and *Gracilaria* sp. using European seabass as target species revealed that seaweed supplementation in aquafeeds may be a valuable tool to increase the immunocompetency, with minimal modulation of digestive enzyme activities. Overall, the work presented in **Chapter II** revealed that *Fucus* sp., *Ulva* sp. and *Gracilaria* sp. could be supplemented to seabass diets without variations in growth parameters up to 7.5 % level. Our results are in agreement with the findings by Abdel-Warith *et al.* (2016) using *Ulva lactuca* dietary supplementation in African catfish, *Clarias gariepinus*. These authors found significant decreases in fish weight gain for 20 % and 30% supplementation, whilst 10 % supplementation level, despite decreasing growth, revealed no significant differences. *Ulva* sp. digestibility was shown to depend on fatty acid contents, which despite low in proportion, accounts for lower lipid digestion when high on carbon chains, and increased lipid digestion when rich in double bonds (Suryaningrum *et al.* 2017). Furthermore, 2.5 % seaweed supplementation with *Ulva* sp. showed a positive modulation in lysozyme activity, while *Gracilaria* sp. elicited an enhancement in seabass antioxidant capacities.

The results obtained in **Chapter II**, however raised the issue of whether the enhancements observed in immune and antioxidant parameters were substantiated by an underlying metabolic alteration or if the dietary supplementation did not influence fish energetic metabolism. For that, we selected a Mix diet (2.5 % of *Fucus* sp., 2.5 % of *Ulva* sp. and 2.5 % of *Gracilaria* sp.) plus a 7.5 % *Gracilaria* sp. supplemented diet and measured seabass oxygen consumption as proxy for metabolic parameters. Additionally, immune and antioxidant response parameters were measured attempting to understand their correlation with seabass metabolic rates. A similar approach was used by Zanuzzo *et al.* (2015) testing *Aloe vera* inclusion in *Oncorhynchus mykiss* diets, which resulted in altered metabolism but not in increased resistance against *Aeromonas salmonicida*. In this study (**Chapter III**), no differences were observed between dietary treatments for the lower (SMR-standard metabolic rate) or higher (MMR- maximum metabolic rate) metabolic boundaries. Therefore, the energetic budget (AMS- aerobic metabolic scope) of seabass was not altered by dietary seaweed supplementation. On the contrary, diets supplemented with 7.5 % *Gracilaria* sp. revealed higher routine metabolic rate (RMR) meaning that seabass routine activity level was modeled by *Gracilaria* sp. ingestion. This higher routine activity was not accompanied by alterations in growth, since no differences were found in growth performance between dietary treatments. The present results raised the intriguing possibility that *Gracilaria* sp. could be facilitating energy allocation for routine activities without altering seabass energetic balance. It is plausible to infer that the immune and antioxidant stasis of seabass may be affected by the extra energy allocated for routine metabolism since digestive enzymes activities remained

unaltered by dietary seaweed supplementation. The outcome of **Chapter III** suggested one of two possibilities. The augmented activity levels lead to an increase in the production of reactive oxygen species (ROS) in muscle cells, thus altering lipid peroxidation and antioxidants activity levels, similarly to the results presented by (Steinbacher and Eckl 2015). Alternatively, Torrecillas *et al.* (2015) showed that in seabass the dietary seaweed supplementation with concentrated mannan oligosaccharides inhibited lipid accumulation in tissues. Interestingly, the alternative complement pathway levels were lower in supplemented diets, fact associated with the level of inclusion, which reinforces the knowledge that seaweed dietary supplementation should not exceed the 5% level. Nevertheless, these results lead to question if the alterations observed in immune parameters and antioxidant levels were a positive outcome of seaweed supplementation or if they reflected an inhibitory effect of the seaweeds over such mechanisms.

To address this question, **Chapter IV** was designed to test whether dietary supplementation may have a protective effect against exposure to a bacterial challenge. To this aim meagre (*Argyrosomus regius*) were fed diets supplemented with two different seaweeds *Gracilaria* sp. (Rhodophyta) and *Alaria* sp. (Phaeophyta) at 5% dietary supplementation level and challenged with a selected pathogen, *Photobacterium damselae* subsp. p*iscicida* (Phdp). This bacteria was selected since it has been identified as triggering agent of severe disease outbreaks in aquaculture of several fish species, including meagre (Labella *et al.* 2011). In aquaculture ponds Phdp infects meagre through the gills, increasing the number of circulating leukocytes and humoral immune parameters within 24 hours (Galhano *et al.* 2016). The results of **Chapter IV** indicated that adding 5% seaweed in meagre diets did not affect dietary palatability, digestibility or nutritional balance. These results are in accordance with the results of Ribeiro *et al.* (2015) who concluded that meagre growth and digestion are not significantly altered by vegetable protein and oil substitutions. Taking that into consideration as well as the lack of differences in growth performance between dietary treatments, we evaluated the effects of seaweed supplementation on meagre metabolic rates. This experimental assay revealed a significant increase in the MMR of meagre fed supplemented diets suggesting that meagre fed seaweed supplemented diets might be able to allocate energy more efficiently in stressful conditions. After the *P*. damselae infection, several innate immunity markers (ACH50, lysozyme and peroxidase) were analyzed yet no significant alteration was observed due to high variability of the results. Variability in immune indicators has been previously described and associated with stress events which could be responsible for immunosuppression (Shailesh 2008). Considering that in this assay meagre were handled to test oxygen consumption, as well as for the intraperitoneal infection, these stressors may have caused immunosuppression of the complement titer and circulating

enzymes with immune activities. Such events have been previously described in several fish species, where stressors such as handling, changes in temperature or salinity and crowding triggered an acute response associated with an increase of acute phase proteins, the release of cytokines and hormones and peptides that are stored or produced in a short-term basis (Tort 2011). Hence, the pro-inflammatory milieu resulting from handling may culminate in immunosuppression and increase the susceptibility to pathogen infection (Mauri *et al.* 2011). However, in order to avoid the harmful consequences of invading pathogens the immune system uses nutrients to support their destruction (Tort 2011). These actions will involve activation or suppression pathways depending on stressor characteristics and orient specific energetic and metabolic pathways in order to support the above actions. Therefore, seaweed dietary supplementation may play a role in supplying bioactive molecules for this adaptive immune response. Simultaneously, in this chapter, other health indicators, such as cholesterol, glucose and triglycerides levels, were also assessed showing no significant changes associated with seaweed supplementation. The exception was the plasma lactate level that increased in meagre fed diet with 5% *Alaria* sp., which is in accordance with the maximum metabolic rate data. Biologically these results are associated since higher physical activity is naturally connected with higher lactate production in muscle tissue (Gibb and Dickson 2002). Such results were also in agreement with antioxidant biomarkers data, since meagre fed with diets containing seaweeds showed a decrease in lipid peroxidation, particularly in fish fed supplemented *Gracilaria* sp., reinforcing the red seaweeds antioxidant properties. Overall, important information about meagre was gathered in this chapter however, the results did not elucidate the role of dietary seaweed supplementation on the response mechanisms to infection. Although increasing the belief that dietary seaweed supplementation positively influences both immunity and antioxidant stasis, there is still a gap in our understanding of how supplementation interacts in infection scenarios.

In an attempt to further explore the mechanisms of how diets supplemented with seaweeds influence fish susceptibility to diseases, I hypothesized that seaweed extracts, containing concentrated compounds, when supplemented in fish diets, would elicit a more tangible response. In aquaculture, the dietary administration of plant extracts has been previously applied to potentiate their immunostimulant properties (Christybapita *et al.* 2007). Thus, in **Chapter V** I performed a methanolic extraction of *Gracilaria* sp. and obtained two fractions: an extract fraction, corresponding to 10% of the raw seaweed composition, and the residue fraction, which represented 90% of the raw seaweed yield. These two fractions were supplemented separately in two experimental diets at 0.5 and 4.5%. The two levels were chosen since they replicate their concentration in 5% crude seaweed, the ideal level selected from the previous chapters. The assumption behind the use of extracts was that the

compounds with bioactivity, namely immunostimulants and antioxidants, under these conditions are more concentrated and may influence fish health more profoundly (Guardiola *et al.* 2016). In this work seabass were fed the 0.5 and 4.5% *Gracilaria* sp. supplemented diets for 42 days and growth performance as well as digestive enzymes activities were monitored revealing similar results as those observed in **Chapters II** and **III**. Additionally, innate immune parameters and oxidative stress biomarkers were also evaluated. Moreover, assuming that compounds such as pigments and vitamins would be more concentrated in a particular fraction of the extract, this could impact fillet quality as previously described by Ramalho Ribeiro *et al.* (2015). This entailed analyzing fillet color, pH and skin color after a seven-day period of storage. Outcoming results from this experiment showed that supplementation of *Gracilaria* sp. extracts to seabass diets was indeed proven possible without negative consequences for growth parameters or digestive function. Likewise, the analysis of innate immune parameters revealed no differences between dietary treatments. The reason why, despite concentrated, *Gracilaria* sp. supplementation did not elicit alterations in these parameters could be related with the extraction processes where the compounds chemical structure could be altered. A different physical structure, caused for example by molecule cleavage, alters affinity and could therefore be sufficient to bypass seabass immune system, faulting to trigger an immune response. Additionally, the assumption that pigments, namely chlorophylls a and d, phycoerythrin, phycocyanin α and β-carotene, would be more available for incorporation in seabass muscle was not confirmed, while fillet color analysis depicted no differences between dietary treatments. Conversely, skin color after the 7-days storage period did reveal differences in coloration, with both supplemented diets displaying lighter coloration. Consumers could interpret this alteration as a freshness sign, increasing acceptance of aquaculture seabass. The measurements performed on the oxidative stress parameters, which alterations are broadly associated with the presence of molecules with antioxidant capacity, also lacked modulation from dietary treatments. Nor the potential concentrated pigments or seaweed mineral composition altered antioxidant enzymes activity level or lipid peroxidation of seabass. To summarize, this work supports the possibility of using by-products from seaweeds extraction processes for application as supplements in aquafeeds, for example after agar extraction for food industries.

Nonetheless, questions regarding the use of seaweed extracts remained unanswered and more concrete evidences about seaweeds immunostimulant properties needed to be gathered. To this aim, another experiment using *Gracilaria* sp. aqueous extract supplementation in seabass diets was designed (**Chapter VI**). In this experiment, similarly to the analysis using meagre (**Chapter IV),** the modulatory capacity of seaweeds over seabass immune system was tested against a biotic stressor. Again, in this work *P. damselae* was

selected as pathogen to challenge seabass defense mechanisms. Moreover, in addition to the parameters studied before, the expression levels of genes related with immunity and antioxidant systems were also analyzed in target organs. With these analyses, I aimed to deepen the understanding of how seaweed supplementation affects fish health. Seabass were fed the experimental diets, control diet and 5% *Gracilaria* sp. supplemented diet, for 80 days and then infected intraperitoneally with Phdp. After infection, fish were monitored revealing that the groups fed *Gracilaria* sp. supplemented diet registered less deaths, indicating higher resistance to infection. Similar results have been described by Yeh *et al.* (2008) when feeding orange-spotted grouper (*Epinephelus coioides*) with diets supplemented with sodium alginate and challenged with *Streptococcus* sp.. These authors reported increased growth, survival rates, complement activity, lysozyme and phagocytic activity in groups fed the supplemented diets. In **Chapter VI,** as well as in rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with 5% *Porphyra dioica* (Soler-Vila *et al.* 2009a), no differences in growth performance between dietary groups were observed. Immune parameter evaluation revealed interesting results since in one hand peroxidase levels were lower in fish fed seaweed-supplemented diet, in both placebo and Phdp infected groups. On the contrary, when lysozyme was analyzed *Gracilaria* sp. groups demonstrated higher levels in both infection groups. A similar pattern between peroxidase and lysozyme was observed in seabream (*Sparus aurata*) in response to dietary supplementation with cell wall-modified yeast (*Saccharomyces cerevisiae*) (Rodríguez *et al.* 2003) The antioxidant parameters analysis in **Chapter VI** demonstrated higher glutathione transferase (GST) activity levels in supplemented groups. This difference was present in both placebo and infected groups, suggesting a dietary influence, but not a correlation with seabass defensive capacity against pathogens. The lipid peroxidation levels however revealed a significant influence of *Gracilaria* sp. in the defenses against pathogen infections, which was associated with the increased antioxidant load in fish fed supplemented diets. These results are consistent with those described for black seabream (*Acanthopagrus schlegeli*) fed diets supplemented with Vitamin E where lower peroxidation in tissues was associated with decreased damage from pro-oxidative stress (Peng *et al.* 2009). The plasma bioindicators in this work evidenced that *Gracilaria* sp. influences the level of triglycerides, exclusively in the placebo groups. The lower TGA levels found in GR within placebo groups may be an indication of shifted energetic metabolism, in which fatty acids are not used as the main energy source. This may be associated with the polysaccharide content in seaweeds, which by being readily available becomes the primary energy source. It has been described that in tilapia (*Oreochromis niloticus*) diets containing high carbohydrate levels stimulate glycolysis, providing the necessary substrates for lipogenesis (He *et al.* 2015). Moreover, in **Chapter VI** I intended to demonstrate, at the transcription level, the underlying changes associated with *Gracilaria* sp. supplementation. The altered expression levels of key genes

involved in the immune and antioxidant systems determined the influence of the *Gracilaria* sp. supplemented diet when seabass were compared to the placebo group fed CTRL diet. Cytokines are secreted in response to immune stimuli inducing the expression of immune related genes through multiple signaling pathways, contributing to the initiation of an immune response (Reyes-Cerpa *et al.* 2012). In **Chapter VI** the up-regulation of chemokines and lymphokines, as well as immunoglobulins observed in head-kidney clearly indicates a contribution of *Gracilaria* sp. on mounting an effective immune response. In liver, the analysis of the expression levels of antioxidant related genes was also used to determine the role of *Gracilaria* supplementation in infected seabass. In an apparent contradiction to head kidney and immune associated genes, in liver the expression levels of the most contributing genes reflect a clear down-regulation in seabass fed GR and infected with Phdp. A similar result was observed in immune related studies with gilthead seabream (*Sparus aurata* L.) fed yeast supplemented diets (Reyes-Becerril *et al.* 2008). The results obtained in this work showed that in liver, genes encoding for chaperones and heat-shock proteins are among the genes exhibiting more pronounced expression variations, which are key in response to stress, as well as glutathione related genes, reflecting antioxidant and scavenging capacity against the ROS increase caused by infection and the inflammatory process. Since *Gracilaria* sp. was reported to have high antioxidant content (Souza *et al.* 2012), the import of antioxidants and scavenging molecules from the diet, and their consequent availability in fish systems may be responsible for the immediate response to oxidative stress molecules, preventing a propagation of inflammation and ultimately managing damages caused by the increased metabolites that result from mounting an immune response. In overview, the transcription levels of key genes analyzed in seabass reflect a major contribution of *Gracilaria* sp. supplementation on this species capacity to defend against pathogens and other possible stressors associated with aquaculture scenarios, such as high densities, poor water quality or low oxygen availability.

Overall, the work presented here supports:

1) Use of seaweeds supplementation in aquafeeds for aquaculture species;

2) Seaweeds application as supplement in aquafeeds, at 5% level, without compromising growth performance or digestibility;

3) Dietary seaweed supplementation alters fish metabolism and blood biochemistry, allowing physiological adaptations, which may confer resistance to stressors;

4) Dietary seaweed supplementation modulates the expression of genes associated with immune and antioxidant responses, evidencing its use as a tool to strength fish wellbeing.

By interpreting the results presented in this thesis, we fill some gaps in the understanding of how dietary seaweed supplementation modulates nutrient metabolism and stress responsiveness in aquaculture fish species. We expect to contribute to the development of the field through the application of marine bioactives as functional aquafeed ingredients.

Further research should address how dietary seaweeds supplementation modulates specific pathways involved in fish defense mechanisms, antioxidant responses and energetic metabolism. Such knowledge would allow the application specific amounts of functional diets, for short-periods before stressful events, enhancing fish resistance to bacterial infections and diminishing the recovery time that often results in decreased growth.

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