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CIÊNCIA ANIMAL – ESPECIALIDADE EM NUTRIÇÃO

Effects of rearing conditions on the composition and bioactive content of seaweed: Potential applications as dietary supplements in the gilthead seabream (*Sparus aurata*)

Francisca Isabel da Silva Brito

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Host Institutions:

No cumprimento do disposto no Decreto-Lei nº 204/2018 de 23 de Outubro, declara-se que a autora desta Tese participou na conceção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

Nesta tese incluem-se ainda três artigos científicos publicados e um submetido em revistas internacionais resultantes de uma parte dos resultados obtidos no trabalho experimental, referenciados como:

Silva-Brito F, Guardiola F, Cavalheri T, Pereira R, Abreu H, Kijjoa A, and Magnoni L (2020) Dietary supplementation with *Gracilaria* sp. by-products modulates stress response, antioxidant and immune systems of gilthead seabream (*Sparus aurata*) exposed to crowding. Journal of Applied Phycology 32: 1-13.

Silva-Brito F, Alexandrino DAM, Jia Z, Mo Y, Kijjoa A, Abreu H, Carvalho MF, Ozório R, and Magnoni L (2021) Fish performance, intestinal bacterial community, digestive function and skin and fillet attributes during cold storage of gilthead seabream (*Sparus aurata*) fed diets supplemented with *Gracilaria* by-products. Aquaculture 541: 736808.

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Silva-Brito F; Cardoso A, Machado M, Ramos-Pinto L, Hinzmann M; Abreu H; Costas B, Magnoni L. Dietary supplementation with *Gracilaria gracilis* by-products modulates the immune status and oxidative stress response of gilthead seabream (*Sparus aurata*) stimulated with *Photobacterium damselae* subsp. *piscicida* (submitted ID: FSIM-D-21-00257).

In compliance with what is stated in Decree-Law no. 204/2018 of 23 October 23, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts.

This thesis includes three published and one submitted articles in peer-reviewed international scientific journals from part of the results obtained in the experimental work, referenced as:

Silva-Brito F, Guardiola F, Cavalheri T, Pereira R, Abreu H, Kijjoa A, and Magnoni L (2020) Dietary supplementation with *Gracilaria* sp. by-products modulates stress response, antioxidant and immune systems of gilthead seabream (*Sparus aurata*) exposed to crowding. Journal of Applied Phycology 32: 1-13.

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Abbreviations List

a*	Redness value
$ABTS^{\bullet+}$	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical
ACH	Haemolytic alternative complement
ACH_{50}	50% haemolysis of RaRBC
ADF	Acid detergent fibre
ADL	Acid detergent lignin
AI	Anterior intestine
ANOVA	Analysis of variance
ARRIVE	*Animal Research*: *Reporting* of *In Vivo Experiments*
AW	Agar waste
b*	Yellowness value
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BL	Blue light
Cat	Catalase
CTR	Control
D	Diet
DGI	Daily growth index
DM	Dry matter
DPPH	1,1-diphenyl-2-picrylhydrazyl
E	Extract
EE	Ethanolic extract
EEA	Essential amino acids
EEW	Ethanolic extraction waste
FAO/WHO Organization	Food and Agriculture Organization and World Health
FCR	Feed conversion ratio
FTIR	Fourier Transform Infrared Spectroscopy
FW	Final weight
GIALT	Gill-associated lymphoid tissues
GIT	Gastrointestinal tract

GPx	Glutathione peroxidase
Gr	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HPI	Hypothalamic-pituitary-interrenal
HPLC	High performance liquid chromatography
Ht	Haematocrit
IC_{50}	Inhibitory concentration
Ig	Immunoglobulin
IMTA	Integrated Multi Trophic Aquaculture
L*	Lightness
LAB	Lactic acid bacteria
LPO	Lipid peroxidation
LPS	Lipopolysaccharides
MAAS	Mycosporin-like amino acids
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCS	Mast cells
MCV	Mean corpuscular volume
MDA	Malondialdehyde
NALT	Nasopharynx-associated lymphoid tissue
NCCs	Non-specific cytotoxic cells
NDF	Neutral detergent fibre
NOS	Reactive nitrogen species
NT	Neutral light
$O_2^{-\bullet}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
OS	Oxidative stress
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PER	Protein efficiency ratio

Phdp	*Photobacterium damselae* subsp. piscicida
PI	Posterior intestine
QIIME	Quantitative Insights Into Microbial Ecology
RaRBC	Rabbit red blood cells
RAS	Water recirculation system
RBC	Red blood cells
RD	Red light
RGR	Relative growth rate
RM-ANOVA	Repeated-measures ANOVA
ROS	Reactive oxygen species
SEM	Standard error of the mean
SGR	Specific growth rate
SOD	Superoxide dismutase
SW	Seaweed
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TD	Total days
TFC	Total flavonoid content
T_g	Gelling temperature
TG	Total glutathione
T_m	Melting temperature
TPC	Total phenol content
VFI	Voluntary feed intake
WBC	White blood cells
WD	Wild
WG	Weight gain

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Abstract

The concept of environmentally-friendly aquaculture in the context of the global economy is in the spotlight of the global guidelines established by the United Nations to be achieved by 2030. The introduction of sustainable and functional ingredients into the aquafeeds sector is an opportunity to improve the economic and ecological sustainability of this activity. Seaweed (SW) are recognized as one of the most promising ingredients to be included in fish diets due to their antioxidant and immune-stimulant properties. Particularly *Gracilaria gracilis*, an important red SW species produced sustainably in an Integrated Multi-Trophic Aquaculture (IMTA) system at the ALGAplus company in Portugal, emerged as a potential supplement to be added into fish diets. *Gracilaria sp.* is the main raw material used for agar extraction by collecting in the majority of wild SW. Increasing demand by the agar industry could drive into the overexploitation of this SW, limiting the potential applications by other industries, including aquafeed supplementation. As the agar industry produces large amounts of by-products (agar extraction residue or agar waste, AW), the main goal of this thesis was to test if AW can effectively be supplemented in diets, resulting in beneficial effects in cultured gilthead seabream (*Sparus aurata*) by inducing changed in the physiology, welfare and performance in this fish. This practical approach may contribute to the development of environmentally-friendly aquaculture, promoting the circular economy. An additional goal of this thesis was to test if SW composition can be modulated by changing the culture conditions, which opens an opportunity to explore forms to tailor the agar (yield and quality) and the bioactive compounds produced.

As a result, this thesis aimed to evaluate:

- ✓ The antioxidant activity of the residue from AW obtained from *G. gracilis* cultured in an IMTA system (**Chapter 2**);
- ✓ The effects that dietary AW supplementation has on the performance, intestinal bacterial community, digestive function, skin and fillet attributes during cold storage of gilthead seabream (**Chapter 3**);
- ✓ The effects that dietary AW supplementation has on the acute stress response, antioxidant and immune systems of gilthead seabream exposed to crowding (**Chapter 4**);

- ✓ The effects that filtered sunlight has on the agar properties and antioxidant activity of *G. gracilis* cultured in an IMTA system (**Chapter 5**);
- ✓ The effects that dietary AW supplementation, extracted from *G. gracilis* cultured under different filtered sunlight, has on the inflammatory responses, immune status, and oxidative stress response of gilthead seabream stimulated with an inactivated bacterial pathogen, *Photobacterium damselae* subsp. piscicida (*Phdp*) (**Chapter 6**).

In **Chapter 2** the antioxidant potential of extracts obtained from SW and AW was assessed. The results showed that the AW have similar to the antioxidant activity of those displayed by the different ethanolic extracts (E) obtained from the raw *G. gracilis*. This result provides support for the potential use of AW obtained from *G. gracilis* as a natural and sustainable source of antioxidant compounds to be supplemented in fish diets. Thus, **Chapter 3** evaluated the use of *G. gracilis* by-products, AW, and ethanolic extraction waste (EEW), as dietary supplements in gilthead seabream in a long-term feeding trial to modulate intestinal bacterial community, physiological responses as well as skin and fillet attributes during cold storage. The results pointed to a dietary modulatory effect on the relative abundance of some bacterial groups. Specifically, the dietary supplementation with 5% AW increases the relative abundance of *Clostridium* genus compared to those fed the 5% EEW diet, which may positively impact fish health in the long term. Despite these differences, no major differences were detected in the richness and diversity of the intestinal bacterial community of gilthead seabream fed the experimental diets. The lack of negative effects on the intestinal bacterial community diversity together with the absence of differences in fish growth performance were considered desired features. In addition, fish fed the 2.5% AW and 5% EEW displayed the highest yellowness value (b*) for the skin colour of all fish, which could be relevant to market acceptance. On the other hand, AW and EEW had no impacts on lipid peroxidation progress during cold storage of fish fillets, at least during a short period of storage (6 days). The results provided in **Chapter 3** showed evidence of the potential application of AW and EEW as a novel and eco-friendly dietary supplement to be included in future aquaculture feed formulations. Accordingly, **Chapter 4** evaluated the effects that those experimental diets could have on the response to an acute stressor (crowding), by investigating changes in stress biomarkers, antioxidant and immune systems of gilthead seabream. The results showed a decrease of stress markers after crowding in fish fed supplemented with SW by-products

(5% EEW, 5% AW, and 2.5% AW) compared to those fed the control (CTR) diet, which could be advantageous in aquaculture. In addition, the results also indicated that dietary AW supplementation may reinforce the antioxidant response in seabream as suggested by the lower glutathione peroxidase and glutathione reductase activities measured in the liver, without change in the LPO level. Although no differences for antiprotease, protease, and haemolytic complement activities were detected in the plasma of fish fed the different experimental diets, the peroxidase activity was increased in the plasma of fish fed 2.5% AW diet, which suggested an improvement of the immune response. Thus, altogether, these results suggest that dietary supplementation with natural by-products, particularly with AW, could improve the response of cultured fish to stressors as well as be a sustainable alternative to the use of antioxidant and immune-stimulant currently being supplemented into their commercial diets.

Chapter 5 investigated the effect of different spectral lights (neutral (NT), red (RD), and blue (BL)) applied to *G. gracilis* in an IMTA-system for 4 weeks. The study revealed higher agar yield and gel strength in SW cultured under BL condition, a promising result as the agar yield and gel strength are both key attributes for this product. In addition, the light conditions did not induce changes in pigment content or the antioxidant activity in the SW, although growth was decreased in the BL condition when compared to the NT condition. Despite this limitation, the BL condition could be useful if applied at later stages in the SW production cycle in IMTA-systems, as a means of modulating agar properties when biomass production reaches target levels.

Since the BL condition increased the polysaccharides content of SW, **Chapter 6** investigated the use of AW obtained from the SW cultured under the BL and NT conditions as a supplement at 5% in seabream diet for 15 days by evaluating the modulatory effects on the antioxidant and immune response in gilthead seabream stimulated with an inactivated bacterial pathogen (*Phdp*). Haematological parameters suggested a higher carrying-oxygen capacity of gilthead seabream when fed both BL and NT supplemented diets. The inflammatory response was enhanced in fish fed the NT diet, as showed by the higher neutrophil counts compared to the CTR group. Fish fed both AW supplemented diets exhibited an improvement on the humoral innate immune response, displayed by the enhancement of plasma protease activity. Finally, the antioxidant response was differently modulated in the liver and the intestine of fish fed the experimental diets.

This work provided evidence that the AW from *G. gracilis* cultured under NT and BL conditions might be used to supplement diets in cultured gilthead seabream as a sustainable prophylactic strategy to increase the immune and antioxidant response of gilthead seabream, which ultimately will benefit the fish health and welfare.

Lastly, the findings from **Chapters 2** to **6** were discussed and integrated in **Chapter 7**. Overall, the results presented in this thesis validated the potential use of AW by the aquaculture industry as a sustainable dietary supplement, increasing the economic and ecological sustainability of this activity. This study also indicated that dietary AW supplementation modulated the antioxidant and immune systems of fish without compromising the fish growth or digestibility. Also, no major alteration on the intestinal bacteria community was detected following the addition of the AW to the fish diet up to 5%. Finally, this thesis showed that by using a BL sunlight filter is possible to increase the agar yield and quality, opening the door for upscaling *G. gracilis* biomass production towards polysaccharide extraction under a biorefinery approach.

Resumo

O conceito de aquacultura sustentável no contexto da economia global representa uma das metas estabelecidas pelas Nações Unidas para atingir em 2030. A introdução de ingredientes funcionais no sector da ração para peixes representa uma oportunidade para melhorar a sustentabilidade económica e ecológica desta atividade. As algas (SW), devido às suas propriedades antioxidantes e imunoestimulantes, são reconhecidas como um dos ingredientes promissores para incluir nas deitas dos peixes. Em particular, a alga *Gracilaria gracilis*, uma importante alga vermelha produzida de forma sustentável num sistema integrado multitrófico (IMTA) na empresa ALGAplus em Portugal, emergiu como um potencial suplemento para as dietas dos peixes. Além disso, a *Gracilaria* sp. é a principal matéria-prima usada para a extração de agar, sendo que a maioria da biomassa é obtida a partir de populações selvagens. Desta feita, com o aumento da procura desta alga pela indústria do agar é expectável que se atinja a sobre-exploração deste recurso, limitando a sua aplicação para outros fins, incluindo a da suplementação da ração para peixes. Como a indústria do agar produz grandes quantidades de subprodutos (resíduo da extração do agar, AW), o principal objetivo desta tese foi testar se o AW pode efetivamente ser suplementado nas dietas para peixes para beneficiar a produção de dourada, melhorando a resposta fisiológica, o bem-estar e o crescimento dos peixes. Esta abordagem prática poderá contribuir para o desenvolvimento de uma aquacultura mais sustentável, promovendo a economia circular. Um outro objetivo deste trabalho visou testar se a composição da alga poderá ser modulada por diferentes condições de cultura, o que poderá abrir uma oportunidade para melhorar o rendimento e qualidade do agar extraídos da alga bem como aumentar o conteúdo em compostos bioativos.

Assim este trabalho visou avaliar:

- ✓ A atividade antioxidante do AW obtido a partir da *Gracilaria gracilis* cultivada num sistema IMTA (**Capítulo 2**);
- ✓ O efeito que a suplementação com AW tem no crescimento, comunidade bacteriana intestinal, função digestiva e atributos da pele e filete da dourada durante um período de armazenamento no frio (**Capítulo 3**);
- ✓ O efeito que a suplementação com AW tem na resposta aguda ao stress, sistema antioxidante e sistema imune da dourada exposta a um stress (**Capítulo 4**);

- ✓ O efeito que diferentes espetros de luz têm nas propriedades do agar e atividade antioxidante da *Gracilaria gracilis* cultivada num sistema IMTA (**Capítulo 5**);
- ✓ O efeito que a suplementação com AW, extraído a partir da *Gracilaria gracilis* cultivada sob diferentes condições de luz solar, tem sobre a resposta inflamatória, sistema imune e resposta do stress oxidativo em dourada estimulada com uma bactéria patogénica inativada (*Photobacterium damselae* subsp. piscicida (*Phdp*) (**Capítulo 6**).

No **Capítulo 2** o potencial antioxidante dos extratos obtidos a partir da alga e do AW foram avaliados. Os resultados demonstraram que o AW teve uma atividade antioxidante semelhante ou maior àquele revelada pelos extratos etanólicos extraídos diretamente da alga. Estes resultados suportam o potencial na utilização do AW como uma fonte sustentável de compostos antioxidantes a ser utilizado nas dietas dos peixes. Assim, o **Capítulo 3** avaliou a utilização de subprodutos da alga *G. gracilis*, AW e resíduos da extração etanólica (EEW), como suplementos na dieta de dourada avaliando os seus impactos na comunidade bacteriana intestinal, na resposta fisiológica bem como nos atributos da pele e filete da dourada armazenada no frio. Os resultados apontaram para um efeito modulador da dieta na abundância relativa de alguns grupos de bactérias. Mais concretamente, a suplementação com 5% AW aumentou a abundância relativa do género *Clostridium* comparado com a dieta 5% EEW, o que a longo prazo poderá ter impactos positivos na saúde do peixe. Apesar destas diferenças, não foram detetadas diferenças na diversidade e riqueza da comunidade bacteriana intestinal da dourada. A ausência de diferenças significativas na diversidade da comunidade bacteriana do intestino dos peixes juntamente com a ausência de diferenças no crescimento dos peixes representa um aspeto positivo para a aquacultura. Além disso, os peixes alimentados com a dieta 2.5% AW e 5% EEW revelaram um aumento da cor amareladas da pele o que é um atributo apreciado pelo consumidor final. Por outro lado, o **Capítulo 3** não revelou evidências de que a suplementação com AW ou EEW tem impactos na progressão da peroxidação lipídica no filete dos peixes, pelo menos durante um curto período de tempo (6 dias). Os resultados disponibilizados no **Capítulo 3** evidenciaram a potencial aplicabilidade do AW e EWW como uma estratégia nova e amiga do ambiente a ser implementada na formulação de rações para a aquacultura. O **Capítulo 4** avaliou os efeitos que as dietas experimentais poderão ter na resposta a um stress agudo (confinamento), através da investigação de alterações na resposta de biomarcadores de stress e dos sistemas antioxidante e imune da dourada. Os resultados revelaram que os indicadores de stress diminuíram após o

confinamento nos peixes alimentados com as dietas suplementadas com os subprodutos de algas (5% EEW, 5% AW e 2.5% AW) comparado com aqueles que comeram a dieta controlo (CTR), o que poderá ser considerado um resultado promissor no contexto da aquacultura. Além disso, os resultados também indicaram que a suplementação com AW pode reforçar a resposta antioxidante das douradas, tal como sugerido pela diminuição das atividades da GPx e GR quantificadas no fígado dos peixes, sem que os níveis de LPO tivessem sido alterados. Apesar de não terem sido detetadas diferenças nas atividades da antiprotease, protease e complemento, a atividade da peroxidase aumentou no plasma dos peixes alimentados com a dieta 2.5% AW, indicando uma melhoria da resposta do sistema imune. Assim, os resultados sugerem que a suplementação com subprodutos naturais, particularmente o AW, pode melhorar a resposta da dourada a stressores bem como ser uma alternativa sustentável aos atuais antioxidantes e imunoestimulantes utilizados nas dietas comerciais.

O **Capítulo 5** investigou os efeitos de diferentes espetros de luz (neutro (NT), vermelho (RD) e azul (BL)) na alga *G. gracilis* cultivada durante 4 semanas num sistema IMTA. O estudo demonstrou que a luz azul levou a um aumento do rendimento do agar bem como da força do gel. Este é um resultado promissor, uma vez que estes são atributos-chave para este produto. Além disso, as condições de luz não induziram alterações no conteúdo de pigmentos nem na atividade antioxidante da alga, apesar do crescimento ter diminuído na condição BL quando comparado com a NT. Apesar desta limitação, a condição azul pode ser útil se aplicada numa fase tardia do ciclo de produção da alga, de forma a modular as propriedades do agar após atingirem-se níveis da biomassa da alga desejáveis.

Uma vez que a condição BL aumentou o conteúdo em polissacarídeos da alga, no último ensaio desta tese (**Capítulo 6)** utilizou-se o AW obtido da alga cultivada nas condições BL e NT para suplementar as dietas de dourada. Neste capítulo os peixes foram alimentados durante 15 dias com as dietas experimentais de forma a avaliar os seus efeitos moduladores na resposta imune e antioxidante da dourada após estimulação com uma bactéria patogénica inativada (*Phdp*). Os parâmetros hematológicos sugeriram uma maior capacidade de transporte de oxigénio da dourada quando alimentada com a dieta BL e NT. A resposta inflamatória foi maior nos peixes alimentados com a dieta NT, tal como sugerido pelo aumento do número de neutrófilos neste grupo em relação ao CTR. Os peixes alimentados com as dietas suplementadas (BL e NT) revelaram uma melhoria da reposta imune inata tal como demonstra o aumento da atividade da protease no plasma.

Por fim, a resposta antioxidante foi modulada de forma diferente no fígado e no intestino dos peixes alimentados com as dietas experimentais. Este trabalho demonstrou que o AW da *G. gracilis* cultivada sob as condições NT e BL podem ser utilizadas como suplementado nas deitas para a dourada como uma estratégia profilática sustentável para aumentar a resposta imune e antioxidante da dourada, o que poderá ser benéfico para a saúde e bem-estar dos peixes.

Por último, os resultados revelados nos **Capítulos 2** a **6** foram discutidos no **Capítulo 7**. No geral, os resultados apresentados nesta tese validaram o potencial do AW para serem utilizados na indústria da aquacultura como um suplemento sustentável, aumentando a sustentabilidade ecológica e económica desta atividade. Este estudo também indicou que o AW modulou os sistemas antioxidante e imune sem comprometer o crescimento e digestibilidade da dourada. Também não foram detetadas diferenças na comunidade bacteriana do intestino dos peixes após a suplementação das dietas dos peixes com AW até 5% de suplementação. Por fim, esta tese demonstrou que a utilização da luz azul aumentou o rendimento e a qualidade do agar, abrindo uma oportunidade para aumentar a produção da *G. gracilis* para a extração de polissacarídeos.

CHAPTER I

GENERAL INTRODUCTION

1.1. Seaweed

Seaweed (SW) are part of Plantae kingdom and represent one of the basis of the food chain in aquatic ecosystems (Ferdouse et al. 2018). SW grow in the intertidal and sub-tidal zones of the sea and contains photosynthetic pigments, which allows them to photosynthesize. SW are classified under three practical groups based on their pigmentation: brown SW, red SW and green SW (Ahmed et al. 2017). For green seaweed (Chlorophyta, about 2200 species), their colours are related mainly to the presence of chlorophylls (Morais et al. 2020) even though they also possess other auxiliary pigments. As for red SW (Rhodophyta, about 6100 species), besides chlorophylls they also contain phycobiliproteins (water-soluble pigment-proteins) which are divided into three groups: allophycocyanin, phycocyanin, phycoerythrin (Rowan 1989), as well as other auxiliary pigments such as β-carotene, lutein, zeaxanthin (Morais et al. 2020). For brown seaweed (Ochrophyta, Phaeophyceae, about 1800 species), the predominant pigments are carotenoid fucoxanthin, β-carotene, violaxanthin, diatoxanthin and in less extent chlorophyll (Rowan 1989; Morais et al. 2020).

1.1.1. Seaweed Aquaculture: Global Overview

The global annual production of SW does not stop growing in recent decades, reaching in 2018, 32.4 million tonnes fresh weight (FAO 2020). SW production takes place in about 50 countries, but the global production is dominated by a relatively smaller number of countries: China, Indonesia, and other Asian countries encompassing 47.9, 38.7, and 12.8% of the global production in 2016, respectively (Morais et al. 2020). The world production of SW has more than tripled, up from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018. Despite the slowdown in global production rates in recent years, the rapid growth in the farming of tropical seaweed species (*Kappaphycus alvarezii* and *Eucheuma* sp.) in Indonesia as raw material for carrageenan extraction has been the major driver in the increase of farmed seaweed production in the past decade (FAO 2020). Europe contributed in 2015 with 230,000 tonnes (fresh weight) of SW. Most of this production originated from Norway (65%), while France, Ireland, Iceland, and the Russian Federation accounted for 33% (Camia et al. 2018). The potential production of

SW at the global scale has been suggested to be 1000–100,000 million tonnes (Lehahn et al. 2016), although, except for Asia, the dominant practice is still to harvest natural stocks (Buschmann et al. 2017).

The global seaweed industry value is more than USD 6 billion per year of which about 85% are for human consumption. Seaweed-derived extracts (carrageenan, agar and alginates) make up almost 40% of the world's hydrocolloid market in terms of foods (FAO 2018).

1.1.2. Seaweed Aquaculture in Major Cultivated Species

According to FAO (2018), there are 221 SW species with a worldwide commercial use, of which about ten genera are intensively cultivated, including the brown seaweed *Saccharina* and *Undaria*; the red seaweed *Porphyra* (=*Pyropia*), *Eucheuma/Kappaphycus* and *Gracilaria*; and the green seaweed *Monostroma* and *Ulva/Enteromorpha*. The most important genera, in terms of volume of production are: *Eucheuma* (10.2 million tonnes in 2015), Japanese Kelp (8 million tonnes), *Gracilaria* sp. (3.9 million tonnes), *Undaria pinnatifid* (2.3 million tonnes), *Kappaphycus* (1.8 million tonnes), and *Porphyra* sp. (1.2 million tonnes) (FAO 2018), Fig 1.

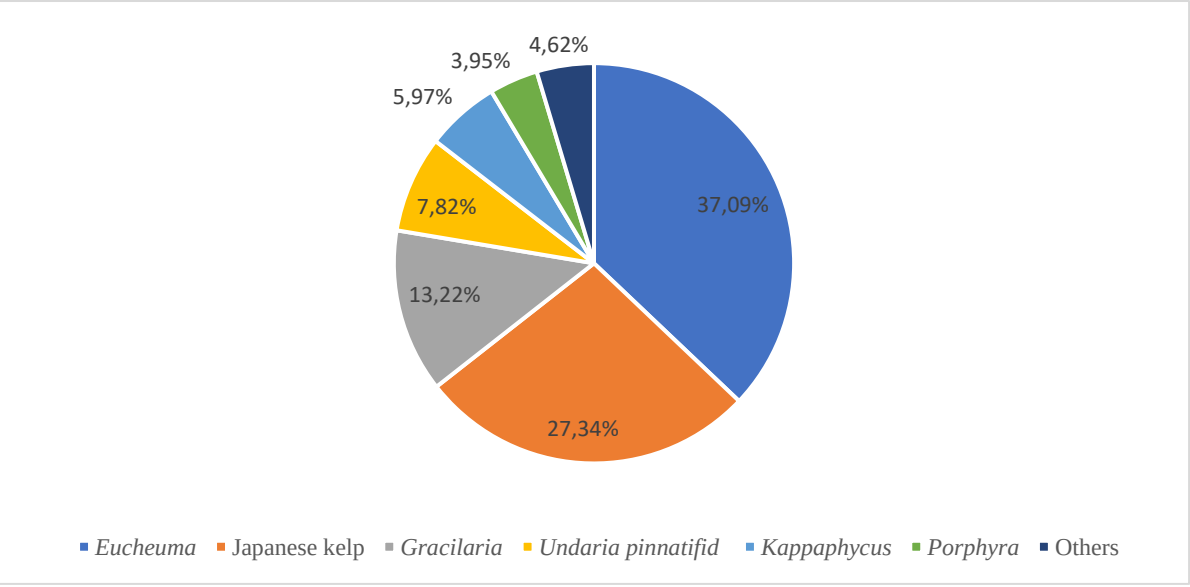

Figure 1 The main seaweed species cultured globally.

Adapted from FAO (2018)

1.1.3. The current state of seaweed production and demand

The interest in SW and their products is increasing globally and so has the interest in their production (García-Poza et al. 2020), although several strategies have been employed in different countries. In China and Indonesia, most of the SW demand is for direct human consumption as food and additives (47.9, 38.7% of the global production in 2016, respectively). Low-grade SW products and scraps from processing factories are used for other purposes, including for example their use as feed for abalone culture (FAO 2020). In contrast, in Norway, the main producer of SW in Europe, the brown SW were traditionally used for feed and as an agricultural fertilizer (Stévant et al. 2017), although nowadays SW are mainly demanded by the polysaccharide industry (García-Poza et al. 2020; Morais et al. 2020). The most important species for human consumption are kelp (including both *Saccharina japonica* and *Undaria pinnatifida*) and the genera *Porphyra* and *Gracilaria* (FAO, 2016), mainly produced by China. In Indonesia, *Kappaphycus* and *Eucheuma* genera are produced mainly for the carrageenan industry (FAO, 2016). Taken together, these five genera – *Saccharina*, *Undaria*, *Porphyra*, *Eucheuma*/*Kappaphycus* and *Gracilaria* – represent ~98% of the world's farmed SW production (FAO 2016). In contrast, Europe, Canada and Latin America still rely on harvesting natural SW to supply the polysaccharide industry (Rebours et al. 2014; Buschmann et al. 2017). In Chile, most of the production is based on the brown SW *Lessonia* and *Macrocystis*, and the carrageenophytes *Sarcothalia crispata*, *Gigartina skottsbergii* and *Chondracanthus chamisoii* (Buschmann et al. 2017). In Portugal, the Integrated Multi-Trophic Aquaculture (IMTA) concept has been employed by the ALGAplus company that grows *Gracilaria*, *Porphyra* and *Ulva* in proximity to other aquaculture species from different trophic levels, allowing the reduction of the waste generated by the activity (Pereira et al. 2006; Abreu et al. 2011; Neto et al. 2018).

There are concerns if the increasing demand by the hydrocolloid industry can be matched by the supply of natural existing SW, which may suggest that SW cultivation could play a leading role in the future to supply those required amounts. Therefore, the overexploitation of natural SW may drive important ecological, economic, and social losses at local, regional, and even global scales. For this reason, efforts towards

cultivation and replacement of wild harvest are even more relevant (Rebours et al. 2014; Buschmann et al. 2017).

1.1.4. IMTA- Seaweed cultivation

Integrated Multi-Trophic Aquaculture (IMTA) systems are gaining importance around the world as a productive and sustainable way to cultivate SW and other aquatic organisms. This kind of system is characterized by raising species, such as sea urchins or bivalves along with the SW, that utilize the co-products rich in nutrients (organic and inorganic wastes) excreted by other species from different trophic levels, including for example fish or shrimp (herbivores or carnivores species) (Buschmann et al. 2017; García-Poza et al. 2020). The production of aquatic organisms by IMTA systems may mitigate the ecological impacts of effluents generated by the aquaculture production, minimizing the eutrophication and stabilizing the pH, O_2 and CO_2 levels of the water. This form of aquaculture production can also bring financial benefits for the producers through the improvement by the diversification of products that they can commercialize (Huo et al. 2012; Ahmed et al. 2016b; Sukhdhane et al. 2018). IMTA systems have been established in several countries around the world. In China, for instance, the co-culture of *Saccharina sp.*, abalone, and sea cucumber has been employed. To do that, the suspended particulate matter (faeces and uneaten food) from the abalone are consumed by the sea cucumber and the dissolved inorganic nutrients are then assimilated by the kelp, which is reflected in increased productivity (Fang et al. 2009; Zhanhui et al. 2013). At the global level, the use of IMTA to produce fish, shellfish and SW is well developed. For example, the production of *Gracilaria verrucosa* has been integrated with yellow croaker (*Pseudosciaena crocea*), showing significant reductions in nutrients produced by the finfish (Cottier-Cook et al. 2016). In Brazil, combining shrimp cages with SW has resulted in improved economic returns for both species (Pellizzari et al. 2011). In Chile and France *Gracilaria* sp. has been used to remove the ammonium excreted by the salmon and oyster farms, respectively (FAO 2018). In South Africa, *Ulva* has been co-cultured on abalone farms (Bolton et al. 2009). In Israel, the same type of system using *Ulva* and abalone was adopted on a small scale (Neori et al. 2017). In Norway, SW exploitation of *Laminaria hyperborea* and *Ascophyllum nodosum* has been employed to minimize the

environmental impacts of salmon and trout farms generating economic profits (Stévant et al. 2017).

These studies have shown that SW cultivation represents a sustainable activity, having both economic and ecologic benefits, given its high productivity and contribution to mitigate threats to the environment (Arbit et al. 2019).

1.1.5. Seaweed bioactive compounds and their applications

SW are known to have several bioactive compounds with several beneficial effects on the health of animals and humans, most of them associated with anti-oxidant, anti-inflammatory, anti-viral, anti-tumoral, neuroprotective and hepatoprotective properties (Alkhalaf 2021; Bauer et al. 2021). Table 1 presents the main bioactive compounds that were described so far for SW, their commercial and biological applications. Molecules with structural and metabolic functions such as proteins, lipids and carbohydrates are synthetized by the SW, as well as other molecules (secondary metabolites) that are produced in less quantity having specific functions, including for example phenolic compounds (flavonoids and phlorotannins). Based on the production of these molecules, SW have great importance at the commercial level, in addition to its ecological importance. The phenolic compounds present in the SW, for instance, are being investigated for various production sectors, including the food, feed, agriculture, pharmaceutical and cosmetic industries, due to their high antioxidant activity (Sabeena Farvin et al. 2013; Farasat et al. 2014; Cotas et al. 2020). Other antioxidant compounds like sulfated agarans detected in *Gracilaria corticata* were described to have similar antioxidant activity to well-known antioxidants (ascorbic acid and butylated hydroxyanisole, BHA) (Seedevi et al. 2017). As photosynthetic organisms, SW can synthesize compounds with the capacity to absorb UV-rays, highly ionizing radiation, such as carotenoids and terpenes, mycosporin-like amino acids (MAAs) and phenolic compounds, used for the formulation of sunscreens (Gupta et al. 2011). Natural pigments extracted from SW such as carotenoids and fucoxanthin may be used as alternatives to replace synthetic pigments, as the latter is suspected of being promoters of carcinogenesis in the liver and produce renal toxicity (Fung et al. 2013). Although pigments are important in a large industrial range, other compounds are also highly relevant, such as the

hydrocolloids extracted from several SW (carrageenan, agar and alginate). These are the main constituents of red and brown SW cell walls and are widely used in the pharmaceutical, cosmetics and food industries as a gelling and/or stabilizing agent (Pereira et al. 2013; Leandro et al. 2020). A good example of these is carrageenan that has a function of fat substitute in food products including emulsified cheese spreads, meat balls and beef frankfurters, among others. The agar that is used as a gelling and stabilizing agent in water gels, canned meat and fish products, soups and sauces, can also be used as a thickening agent, coagulator, emulsifier, and stabilizer in the production of confectionaries such as gums, caramels, and marshmallows. Alginic acid and alginates, thickening and stabilizing agents, are used in salad dressings, sauces, syrups, milk shakes, ice cream toppings, etc..

SW are also used as fertilizer in agriculture and horticulture, as a source of essential macro and trace elements. For instance, the *Saccorhiza polyschides* extracts were described as being rich in macronutrients such as potassium, sodium, sulfur, calcium and magnesium as well as several micronutrients (zinc, boron, chloride, phosphorus, molybdenum, selenium, and iodine) (Soares et al. 2020). Other bioactive compounds such as chelating compounds (i.e., mannitol) present in the *Kappaphycus alvarezii* and *Gracilaria edulis* SW were associated with an improvement of growth and yield response of wheat by increasing the nutrient availability (Shah et al. 2013). Furthermore, SW can also be used as food supplements for animals, feed for aquaculture rich in useful metabolites (pigments, carotenoids, phlorotannins, polyunsaturated fatty acids, agar, alginate and carrageenan) and minerals (iodine, zinc, sodium, calcium, manganese, iron, selenium), (Morais et al. 2020). Thus, due to the bioactivity and potential applications of the SW and their extracts, their market value is increasing and will lead to an increased demand for these organisms' exploitation and their production (García-Poza et al. 2020). Current and future applications of SW and their products will require large quantities and improved qualities of the raw material that will exert great pressure on the existing natural SW resources (Dhargalkar et al. 2005).

Table 1 Seaweed polysaccharides, commercial applications and biological applications.

Seaweed/ Product	Example species	Sugar monomers	Bioactivity and/or applications	Reference
Chlorophyta				
Ulvan	*Ulva*	Sulfates, rhamnose, xylose, iduronic and glucuronic acid	Anti-tumoral, antioxidant, anticoagulant and immune stimulant activities.	(Lahaye et al. 2007)
Rhodophyta				
Carrageenan	*Chondrus crispus*, *Euchema*, *Kappaphycus*	D-galactose and 3,6-anhydro-galactose (3,6-AG)	Anti-tumoral, anticoagulant and immune stimulant activities.	(Cheng et al. 2008; Prajapati et al. 2014)
Porphyran	*Porphyra*	β-D-galactosyl and α-L-galactosyl 6-sulfate or 3,6-anydro-α-L-galactose	Antioxidant (scavenging free radical activity and reduction power), and immune stimulant (macrophage phagocytic) activities.	(Yoshizawa et al. 1995; Zhao et al. 2006)
Agar	*Gracilaria*, *Gelidium*	D-galactose and 3,6-anhydro L-galactose	Gelling and stabilising properties.	(Ramnani et al. 2012)
Phaeophyceae				

Laminarin	*Laminaria, Saccharina, Fucus,*	β (1→3)-glucan and β (1→6)-glucan	Anti-tumoral, anticoagulant, antioxidant, anti-inflammatory and immune stimulant activities.	(Rioux et al. 2010; Kadam et al. 2013)
Alginate	*Ascophyllum nodosum, Laminaria, Macrocystis*	α-L-guluronic acid and β-L-mannuronic acid	Immune stimulant.	(Cheng et al. 2008; Caipang et al. 2011)
Fucoidan	*Fucus, Saccharina, Laminaria, Sargassum*	(1→3)-α-I-fucopyranosyl, (1→4)- α-I-fucopyranosyl, sulfate sugar groups, fucose, fuco-oligosaccharide, uronic acid, xylose, mannose, galactose, glucose.	Anti-tumoral activity, anti-viral activity, antioxidant, anti-inflammatory, anticoagulant, and anti-thrombotic activity. immune-stimulant, Reduces plasma cholesterol, triglycerides and LDL-C. Increases HDL-C. Gastric protection, protection against urinary tract, kidney and liver diseases.	(Li et al. 2008; Caipang et al. 2011)

Adapted from Wan et al. (2019)

1.2. Hydrocolloid Industry

Hydrocolloids can be defined as substances that interact with water to form colloid systems either in the form of a gel or a sol system of solubilized particles (Rhein-Knudsen et al. 2015). They have multiple applications in the food, pharmaceutical, medicinal, and biotechnological industries.

Currently, the hydrocolloid polysaccharides can be obtained from several sources, including plant (pectin is, for example, extracted from apple pomace and citrus peel), microbial (xanthan gum is prepared by aerobic fermentation from *Xanthomonas campestris*), and SW (agar, alginates, and carrageenans are obtained from red and brown SW). Early industrialization in the use of SW began with the production of soda and potash from brown SW to obtain iodine and manufactured soap and glass (Jensen, 1979). The multipurpose-uses of SW phycocolloids include, for example, their use as an emulsifier in dairy products, treatment for leather and textiles, as well as by the pharmaceutical industries, including the treatment of arthritis, metal poisoning, bone grafting, immobilization of biological catalyst in the industrial processes, therapeutic health booster, beauty enhancer, for example, having a great value (Dhargalkar and Pereira, 2005). SW-derived hydrocolloids have a global value of approximately US$ 1.1 billion and expected to increase soon. SW, thus, constitute a unique source of high-value hydrocolloid polysaccharides: agar has the highest retail price per kg (18 US$/kg), whereas carrageenans currently have the highest commercial total production (60,000 tonnes/year) and contribute to the highest total value of US$ 626 million per year (Rhein-Knudsen et al. 2015). The hydrocolloids market is estimated to be valued at USD 9.7 billion in 2020 and is projected to reach USD 12.6 billion by 2025, at a compounds annual growth rate of 5.3% during the forecast period. The demand for hydrocolloids, increasing every year globally, is driven by the rise in demand for convenience food from the food industry (Abowei et al. 2011). As a consequence of this demand, in Southeast Asia, for example, SW is widely cultivated for its use primarily as raw material for the hydrocolloid industry (agar, alginate and carrageenan extracts). The phycocolloid market is estimated to be growing at 2-3% per year, mainly in Asia and the Pacific region (FAO 2018). SW supply could be affected by climatic conditions (e.g., the El Nino event that had destroyed *Ecklonia* sp. population in Northern and Southern Hemispheres), over exploitation,

pollution, or disease. Harvesting wild SW on an extensive and continuous basis may result in overexploitation and loss of ecological balance (Dhargalkar et al. 2009).

1.2.1. Agar structure and features

Agar is a linear polysaccharide composed of alternating residues of (1→3) linked D-galactose and (1→4) linked 3,6-anhydro-L-galactose. The basic disaccharide structural unit of all agar polysaccharides is agarobiose. Agar can be fractionated into two components, agarose and agaropectin (García-Poza et al. 2020). Agarose, a neutral polysaccharide, is the fraction that confers the greatest gelling capability. Agaropectin contains all the charged polysaccharide components and various hydroxyl groups that could be replaced by sulphate ester, methyl groups and pyruvic acid at various positions in the polysaccharide chain (Araki 1966; Labropoulos et al. 2002; García-Poza et al. 2020). Depending on the SW species and source, the agarose and agaropectin contents vary, affecting the physicochemical, mechanical and rheological properties of agar (Labropoulos et al. 2002).

Agar is extracted mainly from the cell walls of red SW belonging to the genera *Gelidium*, *Gracilaria*, and *Gelidiella*. Amongst cultivated SW, the genus *Gracilaria* is the largest raw material for agar extraction because of its rapid growth and high agar content. In 2016, global agar production reached 14,500 tonnes with a market value of US $246 million (Arbit et al. 2019). The main application of agar is as a gelling agent, due to its capacity to form gels even at very low concentrations (the threshold for gelation at around 0.1-0.2% w/v) in the absence of any cross-linking ions, in contrasts with carrageenan. Despite a low solubility of agar in water, the compound can be hydrated in hot water. The gelling phenomena happens when a water solution with agar is warmed up to a temperature between 76 and 95 ºC, occurring in the formation of random coils. Cooling the system to gelling temperature (33–45 ºC) results in the sol-gel transition that proceeds in two steps (Alba et al. 2018). Firstly, single agarose coils, randomly distributed, associate together due to the formation of hydrogen bonds, single and double helices, that stabilize the structure. Then, these left-handed threefold helices are stabilised by the presence of water molecules bound inside the double-helical cavity (Labropoulos et al. 2002). The gelation temperatures of agar are positively correlated with the degree of methoxylation, while higher ester sulphate content decreases the rate of “syneresis” of

gel, meaning the separation of the water from the system during the ageing of the gel (Alba et al. 2018).

1.2.2. Raw material for agar production

Agar could be extracted from SW belonging to the Rhodophyceae class (red SW), with species including the genera *Gelidium* and *Gracilaria* accounting for most of the raw material used for agar production. Although the extraction of *Gelidium* species gives the higher quality agar (gel strength), there is certain limitation in the supply as this SW is collected in the wild, therefore is not the main raw material for this product. Despite the efforts to grow *Gelidium* in tanks and ponds, its production has been proved to be economically unfeasible. On the other hand, *Gracilaria* species, formerly considered unsuitable for agar production due to their lower agar quality (lower gel strength), are currently the main source of agar extraction due to a pre-treatment of the seaweed with alkali before extraction, that increases the gel quality despite decreasing the yield. This process allowed the expansion of the agar industry, previously limited by the availability for *Gelidium* supply, and led to the harvesting of several *Gracilaria* species collected in the wild in several countries, for example, Indonesia. Nowadays, agar extracted from *Gelidium* represents only about 1.6% of the world's phycocolloid production (Porse et al. 2017; Santos et al. 2018).

1.2.3. Demand for raw material for phycocolloids industry

The demand of SW to supply the phycocolloid industry drove to an increase of SW harvesting over the years (Marinho-Soriano 2017; Santos et al. 2018). As a result, the main producing countries have been assisting to a decrease in the biomass of SW in natural beds. The major SW species exploited from natural beds were *Gracilaria verrucosa* (=*Gracilaria birdiae*), *G. caudala*, *G. cornea*, *G. cervicornis*, and *G. domingensis* (Marinho-Soriano 2017). Also, *Gelidium* is in decline in Morocco the actual main global supplier of this species, that massively harvested this natural resource. Consequently, the government implemented national enforcement of restrictive quotas

for the export of the raw material to slow down the harvesting of *Gelidium corneum* in the country (Santos et al. 2018). The exploitation of *Gelidium* to supply phycocolloids industry has been encouraged to be supported by management strategies to improve the sustainable harvesting of global *Gelidium* resources. In contrast, *Gracilaria* exploitation has been supported by the development of its production both in ponds and in the open waters of protected bays. Today the supply of *Gracilaria* in Indonesia is mainly derived from SW that has been cultivated. The country has become one of the most and best-positioned suppliers of agar to the food industry worldwide (Santos et al. 2018)

1.2.4. Current processing technologies of agar

The agar production is based on the extraction of agar from SW. The process is represented in Fig 2. The first step is to clean and wash the SW to remove any foreign material such as sand, salts, sticks and any debris, usually associated with the SW. After that, the SW is heated in water for several hours to dissolve the agar. The mixture is then filtered to remove the solid phase, the residual SW, and obtaining the liquid phase containing the gel. The hot filtrate is cooled down, which contains about one percent agar, which forms a gel that is broken into pieces and washed to remove all soluble salts. Following this step, the water is removed from the gel, either by a freeze-thaw process or by squeezing it under pressure (common procedure). Finally, the agar is milled to suitable and uniform particle size.

Depending on the type of SW, some differences in the treatment are employed. In the case of *Gelidium*, the process is simply washing with plain water or with a small quantity of acid to facilitate extraction. On the other hand, *Gracilaria* must be treated with alkali before extraction to obtain the optimal gel strength. To do that, *Gracilaria* is heated in 2–5% sodium hydroxide at 85–90 °C for one hour. After that, the SW is washed with water, and sometimes with weak acid to neutralize any residual alkali. Finally, extraction is performed with hot water at 105–110 °C for 2–4 hours in case of *Gelidium*, while in *Gracilaria* is extracted with water at 95–100°C for 2–4 hours, and followed by filtration to remove the SW residue (Alba et al. 2018).

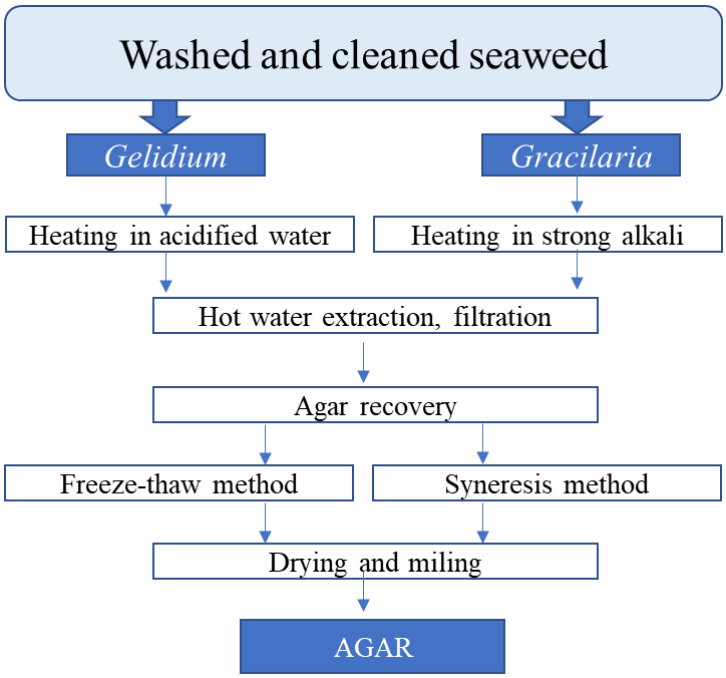

Figure 2 Agar extraction process from *Gelidium* and *Gracilaria* seaweed. Figure adapted from Alba et al. (2018).

1.2.5. Yield and quality of agar

Agar yield is the percentage of crude agar extracted from the SW (dry weight). Typically, the agar yields of seaweed range from 20 to 30%, although in some cases the agar yield could be as lower as 6% or as high as 71% (Lee et al. 2017). Agar yield is an important factor for SW farmers as the economic return on the crop is dependent on the yield of SW per dollar of production cost, the agar yield and the quality of the gel obtained (Cordover 2007). The quality of the agar is related to its physical characteristics (gel strength, gel syneresis, viscosity, gelling and melting temperatures) and chemical properties (the content of sulphate and 3,6-anhydrogalactose) (Santos 1990; Lee et al. 2017). Gel strength, the main indicator for agar quality, refers to compressive force (expressed in g cm^{-2}) required to fracture an agar gel of a standard concentration of 1.5% (w/v) (Santos 1990; Capillo et al. 2017). The agar gel syneresis, a natural phenomenon during which unbound excess water comes out from the formed gel matrix (Banerjee et al. 2011), is related to the aggregation of the double helices to form a network phase through which large molecules and particles could diffuse (water). These aggregate structures of the gels may contain 10 to 10^4 double helices, which makes agarose very useful in immunology,

biotechnology and genetic engineering. Many factors can affect gel synereses, such as agar concentration, storage time, sulphate content, agar gel strength and pressure. The gelling properties of agar are related to the time of gelation, gelation temperature and the minimum concentration of the dispersed phase required for gelation (Lee et al. 2017). Literature reported gelling and melting temperatures ranging from 30 °C to 50 °C and from 82 °C to 92 °C, respectively, depending on SW source, the method of preparation and the purity of the sample (Santos 1990). The chemical analysis for the agar examine possible replacements of the 3,6-anhydro-L-galactose residue by the galactose-6-sulphate, which drives to the kinks formations in the helix, an important characteristic to grade the quality of agar and linked to the strength of the gel (Lee et al. 2017).

1.2.5.1. Factors affecting yield and quality of agar

1.2.5.1.1. Algal species

According to the literature, *Gelidium* sp. and *Pterocladiella* sp. produce the best quality agar, while *Gracilaria* sp. yield low-quality agar (Dhargalkar et al. 2009; Lee et al. 2017; García-Poza et al. 2020). Despite that, *Gracilaria* sp. are one of the main SW used to produce agar due to their fast growth and large agar content (Arbit et al. 2019), being responsible for 80% of the global production of this phycocolloid (Ferdouse et al. 2018). The supply of *Gracilaria* is derived from the SW collected in the wild. For example, in South America, *Gracilaria* species are available along the coastline of Peru, Chile, Argentina, the south at Terra del Fuego and Falkland Islands (García-Poza et al. 2020). Cultivation in ponds and open coast started in Chile and other countries. Spain, Portugal, Korea, France, Morocco, USA, Mexico, Chile, New Zealand and Japan are the countries with the highest volumes for agar production with an approximate value of US $ 132 million annually (Dhargalkar et al. 2009). Presently no commercial cultivation of SW for agar production is carried out worldwide, mainly because the methodology to produce good quality agar is not available and also due to availability of several sources of SW collected from the wild (García-Poza et al. 2020).

1.2.5.1.2. Abiotic factors

The literature described seasonal variations in the yield and quality of agar obtained from wild SW. Most studies reported that the agar yield is higher in spring and/or summer than in winter (Chirapart et al. 1993; Freile-Pelegrín et al. 1995; Marinho-Soriano et al. 2003; Vergara-Rodarte et al. 2010; Martín et al. 2013; Nil et al. 2016), while the gel strength increases in winter compared to spring and/or summer (Chirapart et al. 1993; Freile-Pelegrín et al. 1995; Nil et al. 2016). However, other studies showed different results (Rodríguez-Montesinos et al. 2013). In addition, the rainy season positively impacted the agar yield whereas a reverse trend was observed for gel strength in SW obtained from tropical regions (Villanueva et al. 1999; Ganesan et al. 2008; Bezerra et al. 2010; Vallinayagam 2017), except for the studies by Marinho-Soriano et al. (2001) and Buriyo et al. (2003) which reported the opposite result.

A study carried out in natural populations of *Gracilaria verrucosa* described a negative correlation between the high salinity (range of salinity: 19.1-43.8 ‰) and the agar yield (Sasikumar et al. 1999). Under controlled laboratory experiment, a more extreme variation of salinity was tested and it was concluded that the agar yield extracted from *Gracilaria tenuistipitata* was higher when was exposed to fresh water 0‰ compared to 25 and 31‰, the salinity in the natural environments of most red SW (Bunsom et al. 2012). In another study, *Gracilaria changii* exposed to high salinity (50 ‰) produced an increase in the agar yield compared to the SW grown at 30 ‰ (Siow et al. 2012). As the agar yield increases at both low and high salinity, it is possible to speculate that agar yield is linked to the acclimatization process of SW to new environmental conditions, such as the adaptation of the SW to changes in salinity. In the case of SW exposed to low salinity, the agar between cell walls is increased to provide additional structural support for turgid cells, which led to the increased agar yield from the SW. The same procedure may occur when SW is acclimating to osmotic stress induced by long-term exposure to high salinity (Li-hong et al. 2002).

The effects of temperature on agar yield and agar strength are also conflicting. Some studies positively correlated the increase in water temperature with increased agar yield (Marinho-Soriano et al. 2003) while others demonstrated that higher temperature decreases the agar yield (Bird et al. 1990; Freile-Pelegrín et al. 1997). Similarly, inconsistency was observed for gel strength, as a study in *G. verrucosa* showed higher

gel strength when cultured under lower temperature (Daugherty et al. 1988), while other studies revealed the opposite effect (Bird et al. 1990).

The effects of pH on agar yield and gelling properties were documented for *G. tenuistipitata* var. liui (Israel et al. 1999), showing that pH had no significant impact on agar yield and gel strength. On the other hand, the agar content in *G. changii* increased at pH 6.61 and pH 9.30 compared to pH 8.04, where the gel strength was significantly lower in SW cultured at pH 9.30 compared to that cultured at pH 8.04 (Lee et al. 2019).

1.2.5.1.3. Light intensity and quality

The quality and intensity of light are two factors involved in SW metabolism that may influence both agar yield and gel strength (Lee et al. 2017). In this line, some studies associated an increase of agar yield with the low/dim light condition (Araño et al. 2000; Bunsom et al. 2012). This is mostly due to the increase in floridean starch content, the major carbon storage polymer in red algae, and floridoside content, the principal low molecular weight photoassimilate, which are accumulated during the photosynthesis (light phase) and utilized later during the dark phase for the synthesis of cell wall polysaccharides (Craigie et al. 1968; Rotem et al. 1986). Thus, it is expected that increasing the irradiance, the synthesis of starch rather than cell-wall materials occurs as a consequence of photosynthesis and growth, which may explain the results reported in the literature (Sousa-Pinto et al. 1999; Araño et al. 2000). Nevertheless, the effect of light quality on yield and gel strength of *agarophytes* is poorly understood. Carmona et al. (1998) studied the effects of white, blue, red and yellow lights on polysaccharide content of *Gelidium sesquipedale*, concluding that blue, red and yellow lights increased the polysaccharides compared to white light. Other studies reported that light quality may modulate growth in several SW species. For instance, green and blue lights were both shown to improve the growth of *Halymenia floresii*, *Gracilaria furcata, and Porphyra haitanensis* (Godínez-Ortega et al. 2008; Wu 2016; Zhang et al. 2020). Also, blue light appears to trigger the synthesis of the pigments such as phycobiliproteins in *Halymenia floresii*, *Porphyra umbilicalis*, and *Gelidium sesquipedal*e (Figueroa et al. 1995; Carmona et al. 1996; Godínez-Ortega et al. 2008), while red light improved the antioxidant response in *Porphyra haitanensis* (Wu 2016). However, the effect that light quality may have on the agar yield and gel strength as well as certain physiological properties of

Gracilaria, the main raw material for agar production at a global scale remains to be investigated.

1.2.5.1.4. Nutrients

Macronutrients such as phosphate, nitrate and sulphate are essential for the growth of SW and may affect agar production in agarophytes (Lee et al. 2017). The effects of nitrogen concentration on the growth and agar yield of agarophytes have been extensively explored (Christeller et al. 1989; Buschmann et al. 1994; Martínez et al. 1996). In general, high nitrogen levels in the water of the system negatively impact the yield of agar in *Gracilaria* and *Gelidium* but increase the gel strength (Hoyle 1978; Bird et al. 1981; Carter et al. 1986; Christeller et al. 1989; Li-hong et al. 2002; Marinho-Soriano et al. 2003). Such effects have been reported in several studies carried out in the IMTA system using fish ponds to grow the SW (Buschmann et al. 1994; Martínez et al. 1996; Troell et al. 1997). Studies showed that under high nitrogen availability, SW can assimilate and store excess nitrogen, leading to an increase of protein/carbohydrate ratio, which reduces the agar yield (Bird et al. 1981; Hanisak 1990). Thus, increased uptake of nitrogen by the SW will favour protein over the polysaccharide synthesis (e.g. agar) (Vergara et al. 1995; Korzen et al. 2015a). Despite the lower agar content, growing SW under higher nitrogen levels produce an increase in gel strength and melting temperature (Bird et al. 1981; Lewis et al. 1996; Araño et al. 2000; Marinho-Soriano et al. 2003). Although phosphorus and sulphur are generally not limiting factors in the marine environment, some studies reporting a relationship between changes in their concentration and the agar production of several species belonging to *Gracilaria* and *Gelidium* genera. In this regards, a study by Sousa-Pinto et al. (1999) showed that in a clonal culture of *Gelidium robustum*, sodium phosphate content displays positive and negative correlations with agar yield and gel strength, respectively. In contrast, Lewis et al. (1996) reported a lower agar yield in the *Gracilaria* strain G-16S when cultured in high-phosphate media (15–30 μM), compared to that cultured at lower phosphate media (0–5 μM). These authors also showed that agar gel strength increases with higher phosphate content. Sulphate deprivation in *Gracilaria conferta* tips showed to be a limiting factor for agar yield (mg $tank^{-1}$ day^{-1}) when cultured for 3 weeks in aerated indoor tanks at 25 °C (Friedlander 2001).

1.3. Seaweed potential in the aquafeed- Benefits and potential action model

SW can be used in animal feeds as a sustainable alternative to plant meal as its production does not involve the need for arable land, freshwater and expensive fertilisers associated with terrestrial crop production (Wan et al. 2019).

In terms of nutritional properties, SW are a source of essential trace elements like iron, manganese, copper, zinc, cobalt, selenium and iodine and macro elements such as potassium, sodium, calcium, magnesium and phosphorus (Kumar et al. 2015; Debbarma et al. 2016; Morais et al. 2020). They have a low lipid content (1–5%), however the majority of those are polyunsaturated fatty acids (PUFAs) (Morais et al. 2020). SW have a high level of essential amino acids (EEA), like valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine and tryptophan, that represent 50% of the total amino acids (Peng et al. 2012). Additionally, the ratio value of EAA/non-essential amino acids and the essential amino acid index reported in several SW species are classified as high-quality protein, according to FAO/WHO recommended standards of ideal protein (FAO 2011).

There are also an important source of vitamins such as vitamins A, B_l, B_{12}, C, D and E, riboflavin, niacin, pantothenic acid, folic acid (Dhargalkar et al. 2005; Wells et al. 2017) and some of them, may contain vitamins E and B_1, and β-carotene that may exceed the concentrations of those in conventional foods considered to be rich sources of these compounds (Wells et al. 2017). Additionally, SW are considered a source of long-chain polysaccharides and soluble and insoluble dietary fibers essential for the digestive system and cardiovascular health (Leandro et al. 2020). In addition, SW contain several bioactive compounds, characterized by a broad spectrum of antioxidant, immunostimulant, antiviral, anthelmintic, antifungal, and antibacterial activities (Balaraman et al. 2016; Gomez-Zavaglia et al. 2019).

1.3.1. Intestinal microbiota

Healthy gut microbiota is crucial to support host health and well-being, especially because the gastrointestinal tract is one of the foremost routes of infection in fish. It is known that oligosaccharides and polysaccharides derived from microalgae and

macroalgae are not degraded by the digestive enzymes, having the potential to modulate the intestinal metabolism, and potentiate the growth of beneficial bacteria to detriment of harmful microbiota (Vidanarachchi et al. 2009; Ramnani et al. 2012; Cian et al. 2015; Hindu et al. 2018). A recent study showed that the supplementation of 0.5% galactomannan oligosaccharides in the diet of European seabass (*Dicentrarchus labrax*) positively modulated its intestinal bacterial community by increasing the beneficial microbiota composition with butyrate producer taxa at the expense potentially pathogenic for fish such as coliforms and Vibrionales bacteria (Rimoldi et al. 2020). Similarly, in gilthead seabream, the dietary supplementation of *Gracilaria cornea* increased *Lactobacillus delbrueckii* subspecies, while other bacterial species including the *Vibrio* genus were reduced (Rico et al. 2016). *Lactobacillus delbrueckii* has demonstrated their ability to inhibit the growth of pathogenic bacteria (Vázquez et al. 2005; Rico et al. 2016), as well as to induce an increase in intestinal T-cells and granulocytes in European seabass larvae (Picchietti et al. 2009) and to trigger a humoral immunologic response in gilthead seabream (Salinas et al. 2005). In addition, the presence of *L. delbrueckii* has a positive effect on the integrity of the epithelial surface of the gilthead seabream´s intestine, by improving nutrition and digestion (Rico et al. 2016).

In addition, the dietary supplementation of *Ulva ohnoi* in Senegalese sole (*Solea senegalensis*) is positively correlated with the diversity (Shannon index) of the intestinal microbiota, indicating changes in relative abundance distribution of operational taxonomic unit (OTU) among taxa, although no new taxa were present in the microbiota (Tapia-Paniagua et al. 2019). Similarly, the intestinal microbiota diversity increased in rainbow trout (*Oncorhynchus mykiss*) fed a diet with 5% dietary microalgal meal (*Schizochytrium limacinum*), in particular to taxa assigned to members of lactic acid bacteria (LAB), including *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Weissella* (Lyons et al. 2017). LAB is a group of bacteria including rods; *Carnobacterium*, *Dolosigranulum*, and *Lactobacillus*, cocci; *Aerococcus*, *Alloiococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus*, and the coccoid or rod-shaped genus *Weissella*, known to have beneficial effects in their host (Ringø et al. 2018a; Mathur et al. 2020). In particular, LAB produce growth inhibition substances such as bacteriocins, hydrogen peroxide, diacyl, which prevent the proliferation of pathogenic bacteria (Alakomi et al. 2000; De Vuyst et al. 2007), decreasing the adherence and colonization of pathogens in

the digestive tract (Li et al. 2018), having as well antioxidant effects, as well as enhancing the bioavailability of vitamins/minerals (Mathur et al. 2020).

1.3.2. Intermediate metabolism

Blood biochemical parameters are important to monitor the physiological response as well as the general health status of fish. The dietary supplementation of SW has been associated with changes in several biochemical parameters in the blood of fishes. For instance, plasma glucose, an effective indicator of acute stress and metabolic status in fish, decreased in plasma of gilthead seabream juveniles fed a diet with *Gracilaria cornea* (at 15%) or *Ulva rigida* (at 15 or 25%) (Vizcaíno et al. 2016). In Asian Sea bass (*Lates calcarifer*), the dietary input of fiber content and n-3 fatty acids, naturally present in *Gracilaria pulvinata*, was associated with a decrease in the total cholesterol and triglyceride in the plasma of fish, suggesting modulation of lipid metabolism (Morshedi et al. 2018). Also, Japonese meagre (*Argyrosomus japonicus*) fed a diet containing *Ulva* sp. decreased both blood cholesterol and alkaline phosphatase (Madibana et al. 2017). In Atlantic salmon (*Salmo salar*) the dietary supplementation of *Palmaria palmata* at 15% enhanced liver function through a decrease in serum alanine transaminase activity (Wan et al. 2016).

1.3.3. Antioxidant activity

SW have been in the spotlight of the most recent studies as a natural source of antioxidants compounds that potentially could replace synthetic antioxidants, currently restricted due to safety concerns (Thorat et al. 2013). The bioactive substances with antioxidant activity found in SW include phenolic compounds, polysaccharides, α-tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids such as fucoxanthin and β-carotene, and other pigments like phycobiliproteins, chlorophyll-a and their derivates (Khalid et al. 2018; Gomez-Zavaglia et al. 2019). These compounds, which show scavenging activity, can neutralize reactive oxygen species (ROS) through their oxidation since their affinity to these oxidative compounds is very high (Gomez-Zavaglia et al. 2019). Table 2 summarize the results of several *in vitro* studies describing the bioactive actives compounds involved

on the scavenging activity of SW. To give an example, the green SW *Ulva sp* and its methanolic extracts showed high phenolic and flavonoid contents which were positively correlated to their radical scavenging activity (Farasat et al. 2014). In red and brown SW, their extracts containing sulfated polysaccharides have been described to show free radical scavenging activity (Zhang et al. 2003; de Souza et al. 2007; Zhao et al. 2008; Sachindra et al. 2010). In addition, organic extracts from 30 species of Hawaiian SW, including 27 different genera (belonging to Chlorophyta, Phaeophyta and Rhodophyta phyla) showed that the total antioxidant activity was associated with fucoxanthin content, a pigment present in SW (Kelman et al. 2012). In red SW, *Gracilaria changii*, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was positively corelated to total phenol content (Chan et al. 2015). Also, other red SW collected from the South Eastern coast of India, including *Hypnea musciformis*, *H. valentiae*, and *Jania rubens*, showed DPPH, $ABTS^{\cdot+}$, and H_2O_2 scavenging activities, Fe^{2+} chelating capacity, reducing potential, and lipid peroxidation inhibitory capacity (Chakraborty et al. 2015). In addition, other studies evaluated the antioxidant ability of SW as supplement in diet for fish (Table 3). In this line, a recent study in Atlantic salmon confirmed the antioxidant capacity of SW by reducing ROS accumulation in the tissues (Kamunde et al. 2019), confirming previous results obtained from *in vitro* studies (Table 2). For instance, the dietary supplementation of *Gracilaria gracilis* powder at 0.25 - 1% in zebrafish (*Danio rerio*) favoured genetic expressions of catalase (Cat) in the whole body of fish (Hoseinifar et al. 2018). Similarly, a diet supplemented with brown SW of the genus *Laminaria* improved total antioxidant capacity in the plasma of Atlantic salmon (Kamunde et al. 2019). The dietary supplementation of *Gracilaria pygmaea* at 9-12% in rainbow trout, led to a significant reduction of hepatic SOD, glutathione peroxidase (GPx) activities and lipid peroxidation (LPO) products in the liver, indicating a reduced need to scavenge ROS (Sotoudeh et al. 2018). Similarly, in the liver European seabass (*Dicentrarchus labrax*) was noted an increase in the glutathione-S-transferase (GST) and Cat activities, as well as a decrease in LPO levels in fish, fed a diet supplemented with 5% *Gracilaria* sp. aqueous extract (Peixoto et al. 2019a).

Thus, the *in vitro* studies confirm the antioxidant potential of SW while the *in vivo* study supports the effect of SW to directly increase the levels of antioxidant compounds in circulation/tissues and indirectly modulate the activities of antioxidant defense systems of fish (Kamunde et al. 2019).

Table 2 Bioactive compounds involved on the radical scavenging activity of seaweed extracts.

Seaweed	Extract type	Bioactive compounds	Radical scavenging activity	References
Chlorophyta				
Chaetomorpha sp.	Ethanolic	Flavonoid (189.14 mg QE g^{-1}), Phenol (21.92 mg GAE g^{-1}), and tannins (21.81 mg GAE g^{-1})	IC_{50} 9.41 mg mL^{-1}	(Haq et al. 2019)
Chaetomorpha sp.	Aqueous	Flavonoid (26.89 mg QE g^{-1}), phenol (2.15 mg GAE g^{-1}), and tannins (2.13 mg GAE g^{-1})	IC_{50} 15.44 mg mL^{-1}	(Haq et al. 2019)
Cladophora rupestris	Ethanolic	Phenol (15.83 - 21.72 mg GAE g^{-1})	IC_{50} 2.22 - 0.76 mg mL^{-1}	(Surget et al. 2017)
Ulva sp.	Methanolic	Phenol (1.26 – 5.08 mg GAE g^{-1}), flavonoid (8.05 – 33.09 mg RE g^{-1})	IC_{50} 1.92 - 2.07 mg mL^{-1}	(Farasat et al. 2014)
Rhodophyta				
Laurencia papillosa	Dichloromethane:methanol	Alcohol, phenols; ester and alkanes	IC_{50} 110.8 µg mL^{-1}	(Omar et al. 2018)
Amansia multifida	Methanolic	Phenol (18.46 mg GAE g^{-1})	IC_{50} 78.38 µg mL^{-1}	(Lima et al. 2016)
Cryptonemia crenulata	Methanolic	Phenol (11.61 mg GAE g^{-1})	IC_{50} 82.89 µg mL^{-1}	(Lima et al. 2016)
Eucheuma cottonii	Methanolic	Alcohol, phenols; carboxylic acids; aromatics alcohols; carboxylic acids, esters, ethers; alkenes	IC_{50} 32.74 – 31.44 mg mL^{-1}	(Foon et al. 2013)

Gracilaria verrucosa	Methanolic	Phenol (22.93 mg GAE g^{-1})	IC_{50} 168.03 µg mL^{-1}	(Lee et al. 2015)
Gracilaria changii		Flavonoid (52.76 mg RE g^{-1}); carotenoids (4569.33 µg BE g^{-1}); Phenols (10.83 mg PGE g^{-1})	IC_{50} 2.36 mg mL^{-1}	(Chan et al. 2015)
Gracilaria corticata	Methanolic	Phenol (5.8 mg CA g^{-1})	Peroxyl radical scavenging activity (2.1%); ABTS radical scavenging (16.1%); Hydroxyl radical scavenging (14%).	(Sachindra et al. 2010)
Porphyra haitanesis	Formaldehyde solution, and then extracted with hot water.	Sulphated polysaccharide	IC_{50} 0.06 - 1.6 mg mL^{-1}	(Zhang et al. 2003)
Gelidium amansii	Methanolic	Phenol (14.13 mg GAE g^{-1})	IC_{50} 150.98 µg mL^{-1}	(Lee et al. 2015)
Phaeophyceae				
Dictyota dichotoma	Methanolic	Phenol (19.94 mg GAE g^{-1})	IC_{50} 78.70 µg mL^{-1}	(Lima et al. 2016)
Sargassum vulgare	Methanolic	Phenol (17.97 mg GAE g^{-1})	IC_{50} 76.97 µg mL^{-1}	(Lima et al. 2016)
Fucus vesiculosus	Aqueous	Polysaccharide fucoidan	IC_{50} 0.058 mg mL^{-1}	(de Souza et al. 2007)
Padina sp.	Methanolic	Alcohol, phenols; carboxylic acids; aromatics alcohols; carboxylic acids, esters, ethers; alkenes	IC_{50} 29.64 – 32.45 mg mL^{-1}	(Foon et al. 2013)

Milligram quercetin equivalent g^{-1} dry extract (mg QE g^{-1}); mg gallic acid equivalents g^{-1} dry extract (mg GAE g^{-1}); mg rutin equivalent g^{-1} dry extract (mg RE g^{-1}); μg β-carotene equivalent g^{-1} dry extract (μg BE g^{-1}); mg phloroglucinol equivalent g^{-1} dry extract (mg PGE g^{-1}); mg catechol equivalent g^{-1} dry extract (mg CA g^{-1}). Half maximal inhibitory concentration (IC_{50}).

Table 3: Effects of dietary seaweed supplementation on the antioxidant status of fish.

Seaweed	Fish specie	Supplementation (%)	Duration (days)	Antioxidant status	References
Rhodophyta					
Gracilaria pygmaea	*Oncorhynchus mykiss*	3, 6, 9, 12% of *G. pygmaea* meal to the basal diet at the expense of wheat meal	49	Liver: ↔Cat; ↓SOD; ↓GPx at 6, 9, 12% inclusion	(Sotoudeh et al. 2018)
Gracilaria sp.	*Dicentrarchus labrax*	0.5% methanolic extract or 4.5% residue of methanolic extractions	42	Liver: ↔Cat; GPx, GR; GT and LPO levels	(Peixoto et al. 2019b)
Phaeophyceae					
Laminaria sp.	*Salmo salar*	3, 6, 10% seaweed flakes	30	Plasma: ↑Cat, SOD, GSH	(Kamunde et al. 2019)

↑ increase, ↔ no change, ↓ decrease compared to the control diet (P<0.05).

1.3.4. Immune system

The use of SW polysaccharide as an agent to strengthen the immune defense system have become the most promising method of controlling disease in aquatic animals. Some of the substances produced by the SW, mainly polysaccharides, have the potential to modify the immune system of aquatic animals and increase protection against various diseases caused by pathogens (Newaj-Fyzul et al. 2015; Hindu et al. 2018). The potential immune-stimulant effects of SW have been confirmed in olive flounder (*Paralichthys olivaceus*) fed a diet with an autoclaved water-soluble extract from *Porphyra yezoensis*. Fish fed supplemented diets showed higher survival during the *Streptococcus iniae* challenge test and an increase of lysozyme activities in the plasma and kidney, as well as higher levels of interleukin-4 and -6 proteins in plasma (Choi et al. 2015). Also, *Gracilaria lemaneiformis* improved some nonspecific immune parameters (lysozyme and alternative complement activity (ACH_{50})) in white-spotted rabbitfish (*Siganus canaliculatus*) (Xu et al. 2011). In Nile tilapia (*Oreochromis niloticus*) the ACH_{50} was higher in fish fed a diet with *Ulva* sp. (Valente et al. 2016). In rainbow trout, the dietary supplementation of *Gracilaria vermiculophylla* at 5% can enhance both lysozyme and ACH_{50} activities by over 60% when compared to the dietary control (Araújo et al. 2016). These immunological responses conferred by the dietary supplementation of SW may be due to the presence of complex carbohydrates (e.g. these usually included in alginates and agar). The use of carbohydrates and crude extracts obtained from SW in aquafeeds confirms this as previous studies have observed positive modulation of immune parameters in several fish species (Hindu et al. 2018). For instance, dietary supplementation of low molecular weight agar (0.2%) in Basa fish (*Pangasius bocourti*) has been associated with an increase in plasma lysozyme, phagocytosis, respiratory burst, alternative complement activities and survival rate post-challenge with a bacteria *Aeromonas hydrophila* (Van Doan et al. 2014). Similarly, Atlantic salmon fed diets supplemented with alginate at 5% extracted from *Ascophyllum nodosum*, showed enhanced plasma lysozyme activity (Gabrielsen et al. 1998). Other studies had also shown that crude extracts from SW produced immunostimulatory effects in fish. For example, feeding *Pangasianodon hypophthalmus* fingerlings fucoidan-rich extracts from *Sargassum wightii* showed the enhancement in a variety of immunological parameters (e.g. respiratory burst activity, lysozyme activity, phagocytic activity and total leucocyte count), including higher survival after having been

challenged with *Aeromonas hydrophila* (Prabu et al. 2016). Dietary supplementation in olive flounder with the brown SW *Ecklonia cava* at 1.0% improved serum lysozyme and myeloperoxidase activities Lee et al. (2016). These authors showed that both 1.0% raw *Ecklonia cava* and 0.1% or 0.5% ethanol extract of *Ecklonia cava* were effective in the reduction of mortality of olive flounder against pathogenic bacterial infections (*Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio harveyi*). In European seabass, the ACH_{50} activity in plasma was increased when fed a diet supplemented with a *Gracilaria* sp. methanolic extract at 5% (Peixoto et al. 2019b), while dietary supplementation *Gracilaria* sp. aqueous extract at 5% increased plasma lysozyme activity and delayed mortality after infection with *Photobacterium damselae* subsp. piscicida (Peixoto et al. 2019a).

1.3.5. Skin and flesh attributes

A beneficial effect of SW supplementation in the fish diets in terms of flesh and skin attributes has been explored by some authors (Ribeiro et al. 2015; Araújo et al. 2016; Valente et al. 2016; Peixoto et al. 2019b). The impact of dietary supplementation with SW has been demonstrated in the fish colouration, dependent on dietary pigments inclusion, as fish has no the ability for *de novo* synthesis (Maoka 2020). This topic is particularly relevant to the aquaculture sector as fish skin and flesh colours are taken as one of the most important quality attributes of fish, which is often evaluated by consumers and is therefore important to market acceptance (Hernández et al. 2009; Ünal Şengör et al. 2018). The supplementation of red and green SW has been proved to increase the carotenoids in fish blood (Adel et al. 2021), promoting their deposition in the flesh and skin, improving coloration (Araújo et al. 2016; Ebeneezar et al. 2020). One of the groups of SW used to attain this red SW *Gracilaria* sp. Indeed, dietary supplementation in electric yellow cichlid (*Labidochromis caeruleus*) with extracts from *Gracilaria persica* improved skin coloration compared to the inclusion of extracts from brown SW *Sargassum boveanum* and green SW *Entromorpha intestinalis* at 0.1% of supplementation levels (Pezeshk et al. 2019). In particular, these authors have shown that the inclusion of *G. persica* produced the highest b* value (yellowness) in the skin, an important feature in market demand for this species. In rainbow trout, the dietary supplementation of *Gracilaria vermiculophylla* produced a fillet more luminous (L*),

less yellowish (b*), and more reddish (a*) (Araújo et al. 2016). In this study, the decrease of b* values, associated with the yellow colour, was correlated with the lower deposition of lutein and zeaxanthin with the increased supplementation levels of this SW (Araújo et al. 2016). Other red SW, such as *Porphyra* sp. induced a change of colouration from pinkish-white to pinkish-orange or dark orange in the flesh of salmonids, improving its commercial value (Soler-Vila et al. 2009). These authors have shown that the increase of coloration in salmonids has been attributed to the yellow xanthophylls, especially lutein and zeaxanthin pigments as they are fat-soluble pigments. Similarly, the supplementation of a mixture of *Ulva* sp. (*Ulva rigida* and *U. lactuca*), rich in chlorophylls (a and b) and β-carotene, increased the lightness and yellowness of the muscle of Nile tilapia, although this effect was not related to carotenoids deposition in this tissue (Valente et al. 2016). On the other hand, these authors have shown that dietary *Ulva* sp. inclusion increased total carotenoid content in the skin of fish. In addition to the effects that some SW may have on fish coloration, the brown SW, *Sargassum siliquastrum* (at 30% supplementation levels) and *Laminaria digitata* (at 10% supplementation levels) resulted in improved flavour and nutritional properties by increasing the total bromophenol, which is associated with a desirable sea-like and fish-like flavour of fish (Ma et al. 2005), and iodine contents in the fish flesh of some fish species (Ribeiro et al. 2015). Despite the potential benefits of dietary SW supplementation in the attributes of the flesh and skin of fish, the response for a given SW seems to be dose-dependent, with potential differences in its digestibility among species (Araújo et al. 2016).

1.3.6. Seafood preservation

In recent years SW have been proposed as a natural and safe source of antioxidants to replace the synthetic ones to be applied in foods and cosmetics preservation, by delaying lipid oxidation in lipid-rich products, increasing their shelf-life (Farvin et al. 2015; Hermund et al. 2015; Hanjabam et al. 2017; Poyato et al. 2017). SW were shown to minimize lipid peroxidation progression in the seafood, including canned salmon fillet by using *Durvillaea antarctica*, *Ulva lactuca* and *Porphyra columbina* aqueous extracts (Ortiz et al. 2014), as well as in minced fillet of tilapia by adding Crassulaceae (*Sedum fusiforme*) and seaweed (*Porphyra yezoensis*) extracts (Ribeiro et al. 2014). These antioxidant properties have been attributed to several compounds present in the SW

extracts, including polyphenols, pigments, minerals, and polysaccharides (Vinayak et al. 2011). However, few studies have focused on the use of dietary SW supplementation in fish and the potential changes in lipid peroxidation of the fish and/or its fillet during conservation, or the antioxidant properties of SW into the diet itself, which could be important for aquafeed storage. A recent study in this area reports that dietary supplementation in European seabass with *Gracilaria* sp. methanolic extract and its insoluble residue changed the fillet colour after a period of 2 to 5 days of storage at 4 ºC (Peixoto et al. (2019b). This is an area that remains to be further explored, having in mind the potential beneficial effects that SW and their extracts may have by delaying the lipid peroxidation of fillets and aquafeeds.

1.3.7. Growth performance

One of the earliest studies on the dietary supplementation of SW in fish diets investigated the replacement of fish meal by dried *Ulva pertusa* in black seabream (*Acanthopagrus schlegeli*) (Nakagawa et al. (1984). This study showed that the SW addition at 10% into formulated diets increased the protein efficiency of fish, although growth performance along with other parameters remained unchanged. Since then, many studies have investigated the use of SW as ingredients in aquafeed, including different fish and SW species, and various dietary supplementation or replacement levels. Prior studies have shown that raw SW supplementation at low levels (up 5%) in general enhanced growth performance parameters (Table 4). For example, feeding European seabass fry a diet supplemented with *Pterocladia capillacea* at 5% was reflected in an increase in growth performance (Wassef et al. 2013). Similarly, growth performance was improved in Nile tilapia fed a diet supplemented with *Ulva* meal at 5% (Ergün et al. 2009). Also, in red seabream (*Pagrus major*) the dietary supplementation of *Porphyra yezoensi*s spheroplasts at 5% resulted in increasing growth performance, including weight gain (WG), percentage growth rate, specific growth rate (SGR), and protein efficiency ratio (PER), although feed conversion ratio (FCR) was decreased (Kalla et al. 2008). The benefits that dietary supplementation of raw SW at low levels (up to 5%) may have on fish growth performance have been attributed to several constituents, including protein/ amino acids, fatty acids, vitamins and minerals contents, as well as the bioactive compounds present (Mendonça et al. 2019). In contrast, other studies revealed that dietary supplementation

of raw SW at high levels (20% or above) may impair their growth performance due to the presence of soluble non-starch polysaccharides and high mineral content (19.7%) (Mendonça et al. 2019). For example, a study in African catfish (*Clarias gariepinus***)** reports that the dietary supplementation of *Ulva lactuca* at 10% did not produce changes in growth performance, while supplementation above 20 % has a negative effect on growth performance (Abdel-Warith et al. 2016). The dietary supplementation of *Gracilaria* sp. in rainbow trout is possible up to 5% without negative effects on growth performance (Valente et al. 2015; Araújo et al. 2016), while *Porphyra dioica* can be effectively included in diets for rainbow trout up to 10% without a decrease in weight gain and growth rate (Soler-Vila et al. 2009). In European seabass, the dietary supplementation of *Gracilaria bursa-pastoris* and *Ulva rigida* at 10% is feasible without negative consequences on growth performance, nutrient utilization or body composition, while dietary supplementation of *Gracilaria cornea* should be limited to no more than 5% in order to avoid an impairment on growth performance (Valente et al. 2006). A summary on the effects of the dietary supplementation of several raw SW and their crude extracts on growth performance and related parameters in some farmed finfish are presented in Tables 4 and 5.

1.3.8. Dosage of dietary seaweed supplementation

The choice of the levels for dietary supplementation with SW in fish feeds has an extreme relevance, as a low level may contain reduced bioactive compounds while high levels may display detrimental effects, i.e. by impairing fish growth performance. Several studies analysed the effects that different levels of dietary supplementation with raw SW or their extracts have on growth performance. Most of these studies revealed that dietary supplementation of raw SW at 5% had positive or neutral effects on the growth performance of several farmed finfish, while SW crude extracts could be supplemented up 0.5% without negative effects on growth performance (Tables 4 and 5).

However, caution should be taken when selecting the supplementation levels of raw SW or their products, as the effects that dietary SW supplementation has on fish depends not only on the species fed but also on the SW selected, including type and concentration of bioactive molecules in the SW as well as the level of supplementation (Mendonça et al. 2019).

Table 4: Effects of dietary raw seaweed supplementation in the growth performance of some farmed finfish.

Seaweed	Fish specie	Supplementation (%)	Duration (weeks)	Growth effect	References
Chlorophyta					
Ulva lactuca	Gilthead seabream	2.6, 7.8 (Exp 1); 14.6, 29.1 (Exp 2)	15.9 (Exp 1) 20.1 (Exp 2)	↔ No changes (Exp 1) ↓FW and SGR at 29.1% (Exp 2) ↔ FW, SGR, WG at 15.9% (Exp 2)	(Shpigel et al. 2017)
Ulva lactuca	European seabass	5, 10, 15	8	↑ FW, WG, protein productive value but ↓ when % inclusion ↑ ↑ FCR when percentage inclusion ↑	(Wassef et al. 2013)
Ulva lactuca	Nile tilapia	5; 10	8,6	↔FW, WG, SGR, RGR, FCR, PER,	(Natify et al. 2015)
Ulva rigida	Gilthead seabream	5 Ulva + different levels of lipid content (13, 16, 19, 22 % lipid)	7	↔ FW, SGR, FCR and PER at 5% Ulva + 13, 16 and 19% lipid ↑ FW, SGR and PER at 22% lipid + 5% Ulva ↓ FCR at 22% lipid + 5% Ulva	(Emre et al. 2013)
Ulva rigida	Gilthead seabream	5, 15, 25	10	↔FW and SGR at 5 and 15% ↔CF and FCR ↑ FW and SGR at 25%	(Vizcaíno et al. 2016)
Ulva rigida	Nile tilapia	5	7	↑FW, WG, SGR and PER ↓ FCR	(Ergün et al. 2009)

Ulva pertusa	Black seabream	10	20.4	↔ Growth rate, FE ↑ PER ↓ FW, Growth rate	(Nakagawa et al. 1984)
Rhodophyta					
Gracilaria Bursa-pastoris	European seabass	5, 10	10	↔FW, daily WG, FCR, PER, VFI	(Valente et al. 2006)
Gracilaria sp.	Meagre	5	10	↔FW, WG, DGI, FCR, PER, VFI	(Peixoto et al. 2016a)
Gracilaria cornea	European seabass	5, 10	10	↔ PER and VFI; ↓ FW and WG when inclusion ↑ ↑ FCR when inclusion ↑	(Valente et al. 2006)
Gracilaria cornea	Gilthead seabream	5, 15, 25	10	↔FW, SGR and FCR at 5 and 15% ↔FW at 25%; ↓SGE at 25%; ↑ FCR at 25%	(Vizcaíno et al. 2016)
Gracilaria persica	Persian sturgeon	0; 2.5, 5, 10	8	↔FW, WG, FCR, SGR	(Adel et al. 2021)
Gracilaria lemaneiformis	Black seabream	5, 10, 15	8	↔FW, WG and FE at 5, 10 and 15% ↓FW, WG and FE at 20%	(Xuan et al. 2013)
Gracilaria vermiculophylla	Rainbow trout	5;10	13	↑FW, FCR, VFI at 10%	(Valente et al. 2015) and (Araújo et al. 2016)
Kappaphycus alvarezii	Asian seabass	5	10	↔FW, WG, SGR, Total FI, Daily, FI, FCR, PER	(Shapawi et al. 2016)
Pterocladia capillacea	European seabass	5, 10, 15	8	↑FW, WG at 5%	(Wassef et al. 2013)

				↓FW, WG at 10 and 15% ↑FCR at 15% ↓PER at 10 and 15%	
Porphyra dioica	Rainbow trout	5, 10, 15	12.5	↓FW at 15% ↔FW, WG at 5 and 10%	(Soler-Vila et al. 2009)
Porphyra yezoensis	Red seabream	5	6	↑FW, WG, % growth, SGR and PER ↓FCR	(Kalla et al. 2008)
Phaeophyceae					
Ascophyllum nodosum	Red seabream	5, 10	10	↓FW at 10% ↓FE at 10% ↑FE at 5%	(Yone et al. 1986)
Sargassum polycystum	Asian seabass	5	8	↔FW, WG, SGR, FCR and PER ↑VFI	(Shapawi et al. 2016)
Alaria sp.	Meagre	5	10	↔FW, WG, DGI, FCR, PER, VFI	(Peixoto et al. 2016a)
Ecklonia cava	Olive flounder	6	6	↔FW, WG, FCR, PER and VFI	(Kim et al. 2014)

↑ increase, ↔ no change, ↓ decrease compared to the control diet (P<0.05).

FW, Final weight; SGR, specific growth rate; WG, weight gain; FCR, feed conversion ratio; RGR; relative growth rate; PER, protein efficiency ratio; FE, feed efficiency; FI, feed intake; VFI, voluntary feed intake; DGI, daily growth rate; SGR, specific growth rate; VFI, voluntary feed intake. Adapted from Wan et al. (2019).

Table 5: Effects of dietary extract seaweed supplementation in the growth performance of some farmed finfish

Seaweed	Fish specie	Type of extract	Supplementation (%)	Duration (weeks)	Growth effect	References
Chlorophyta						
Entromorpha intestinalis	Electric yellow cichlid	Chloroform (1:6 w/v) and methanolic (2:1 w/v)-crude	0.1	8	↑FW, SGR, ↓FCR	(Pezeshk et al. 2019)
Chaetomorpha antennina	Labeo rohita	Methanolic –(crude)	0.0025; 0.0075; 0.0125	4	↔FW ↑WG and SGR at 0.0075 and 0.0125% ↓FCR	(Sattanathan et al. 2020)
Rhodophyta						
Gracilaria sp.	European seabass	Methanolic (crude)	0.5	6	↔FW, WG, DGI, VFI, FCR	(Peixoto et al. 2019b)
Gracilaria sp.	European seabass	Residue of methanolic	4.5	6	↔FW, WG, DGI, VFI, FCR	
Gracilaria persica	Electric yellow cichlid	Chloroform (1:6 w/v) and methanolic (2:1 w/v)	0.1	8	↑FW, SGR, ↓FCR	(Pezeshk et al. 2019)
Phaeophyceae						
Sargassum boveanum	Electric yellow cichlid	Chloroform (1:6 w/v) and methanolic (2:1 w/v)-Crude	0.1	8	↑FW, SGR, ↓FCR	(Pezeshk et al. 2019)

Sargassum oligocystum	*Pangasius hypophthalmus*	Aqueous (hot, 1:1 w/v)	0.0001; 0.0005	0.0003;	12	↑WG and DGR at 0.0003 and 0.00005% ↔FCR	(Baleta et al. 2019)

↑ increase, ↔ no change, ↓ decrease compared to the control diet (P<0.05).

FW, Final weight; SGR, specific growth rate; FCR, feed conversion ratio; WG, weight gain; DGI, daily growth rate; VFI, voluntary feed intake; DGR, daily growth rate.

1.4. *Gracilaria gracilis*, the seaweed species studied in this thesis

Gracilaria gracilis is a Rhodophyte, a marine red macroalga belonging to Florideae, Gigartinales, Gracilariaceae. The species belonging to *Gracilaria* genus are euryhaline SW tolerating a wide range of salinities (about 10–40 ‰), even although they grow best in ranges of 25–33 ‰. They can tolerate temperature from 0 to 35 ºC but have an optimal range of 20–28 ºC (García-Poza et al. 2020). Like other red SW, it has a notable economic relevance for its agar content (Qi et al. 2010) and its usage as food or food supplement for humans and various species of fish and shellfish (Ingram et al. 2009; Peixoto et al. 2016a; Morais et al. 2020). *Gracilaria* sp. includes lipids (fatty acids and sterols), proteins (amino acids), and carbohydrates in their composition, in addition to several bioactive compounds including, for example, phycobiliproteins and phenols which may be used by the pharmaceutical and cosmetic sectors as a gelling and stabilising agent and as a source of bioactive compounds (Capillo et al. 2017; Pimentel et al. 2018). Unfortunately, in the last years the quality of wild *Gracilaria* sp. for these uses has been declining because of changes in environmental conditions along with an increased occurrence of diseases (García-Poza et al. 2020)].

Figure 3 *Gracilaria gracilis*

Source: https://alga4food.wixsite.com/.

1.5. Fish aquaculture and the main challenges faced by this sector

Aquaculture is currently one of the fastest-growing food sectors in the world. In particular, finfish production is rapidly expanding, as it is expected that the total world fish production would reach 109 million tonnes in 2030, surpassing the capture fisheries (FAO 2020). The advantages of aquaculture over other forms to secure the seafood supply for a growing population are irrefutable. However, the implementation of this activity also raises several concerns related to sustainability as well as the quality and safety of fish. One of the most relevant risks of aquaculture production is the high contagious diseases by keeping fish under high density (Leung et al. 2013). It is well known that rearing fish under crowded and stressful conditions increases its susceptibility to bacterial infection, causing major stock losses, being also a concern in terms of animal welfare. Thus, the prophylactic and therapeutic use of chemical disinfectants and antibiotics are a recurring practice within the aquaculture sector to control disease outbreaks (Cabello et al. 2013). Unfortunately, the use of these substances can result in negative consequences to consumers' health, with food safety hazards and environmental issues, possing as well other concerns related to the appearance of antibiotic-resistance bacteria (Rasul et al. 2017). Therefore, in recent years efforts have been made to find strategies to reduce the effect that pathogenic bacteria have as well as the animals' susceptibility to diseases, aiming to improve the health and growth of culture fish by implementing several nutritional tools. Most studies look for natural ingredients or groups of compounds with immune-stimulant and antioxidant properties that could be supplemented into the diet, aiming to increase fish resistance against pathogenic bacteria infections as well as reducing the negative impact of stressors.

1.5.1. The immune system of fish

The fish immune system is divided into 2 subsystems:

1) the innate immune system and,
2) the adaptive immune system.

The innate immune system is defined as the first line of defense to respond to initial infection and disease (Riera Romo et al. 2016; Smith et al. 2019). This defense mechanism may induce a response during primary infection and create inflammatory conditions (Newton et al. 2012; Riera Romo et al. 2016). The primary defense response can be activated due to conserved pathogen-associated molecular patterns (PAMPs), such as peptidoglycans and lipopolysaccharides (LPS) in the bacterial cell wall, fungal β1,3-glucan, viral double-stranded RNA and bacterial DNA, which are detected by the pattern-recognition receptors (PRRs) (Magnadóttir 2006; Iwasaki et al. 2015). The innate immune system can be categorized into three defense mechanisms:

1) physical barriers,
2) cellular components and,
3) humoral responses (Riera Romo et al. 2016; Smith et al. 2019).

In fish, the innate response has been considered a crucial mechanism in fighting pathogens due to limitations of the adaptive immune system, related to their poikilothermic nature, their limited repertoire of antibodies with slow proliferation, maturation and memory of their lymphocytes (Whyte 2007). The adaptive immune system is highly specific to a particular antigen and can provide long-lasting immunity (Secombes et al. 2012).

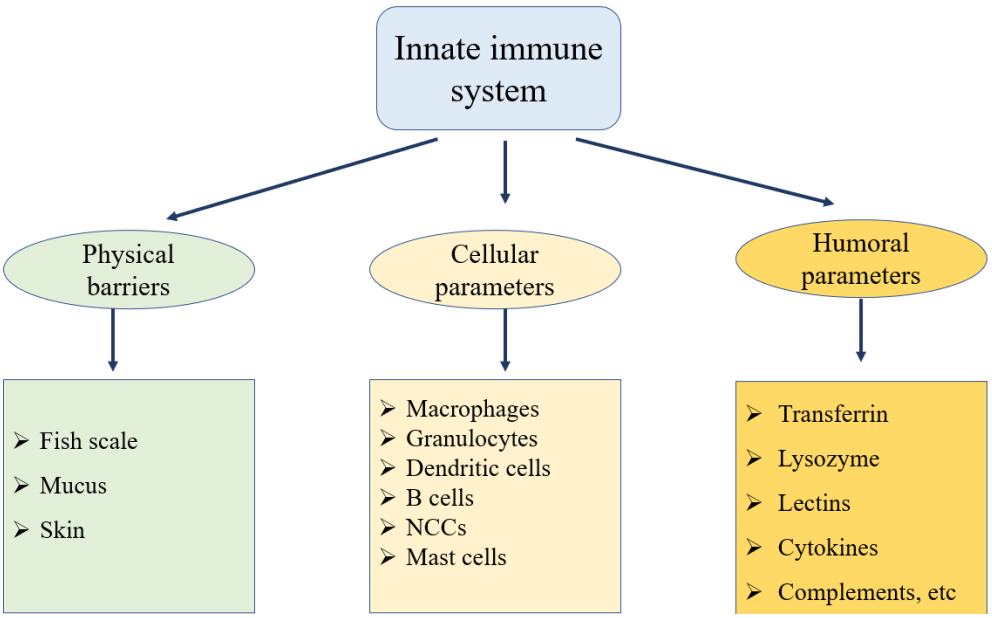

Figure 4 The components of the innate immune system of fish.

Adapted from Kordon et al. (2018)

1.5.1.1. The innate immune system - Physical barriers

The first lines of defense in the fish's innate immune system are physical barriers to isolate the internal environment from external factors, such as harmful substances and pathogenic microorganisms (Riera Romo et al. 2016). The physical barrier includes the skin (e.g., scales and mucus), gills, and the epithelial layer of the gastrointestinal tract (Uribe et al. 2011; Smith et al. 2019). Teleost skin has been shown to contain skin-associated lymphoid tissue that consists of multiple cell types including secretory cells (e.g., goblet cells), lymphocytes (B and T cells), granulocytes, macrophages, and Langerhans-like cells. In contrast with mammals, teleost skin is not keratinized (Yu et al. 2020), so the skin mucus is highly dynamic, acting as a natural, physical, biochemical, and semipermeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes (Esteban 2012). Concomitantly, mucus plays a critical role in acting as a biological barrier as it contains lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and immunoglobulin M (IgM) (Fast et al. 2002; Uribe et al. 2011; Esteban 2012; Smith et al. 2019). The gills, involved in balance and gas exchange, also are an important physical barrier, having both innate and adaptive immune components. The physical barrier of the gills consists of the gill epithelium, a glycocalyx layer, and a mucus layer. Immune cells, including macrophages, neutrophils and eosinophilic granulocytes have been observed in the gill-associated lymphoid tissues (GIALT) of teleost fish (Salinas 2015). The gastrointestinal tract (GIT) facilitates the absorption of nutrients while preventing pathogen invasion through its epithelium. If a pathogen is ingested, it will encounter the GIT, which, like the skin and gills, contains both innate and adaptive immune cellular components. The intestine of teleost fish, especially the posterior segment, contains both innate and adaptive immune cells including macrophages, mast/eosinophilic granule cells, dendritic cells, B cells, and T cells (Salinas 2015; Tafalla et al. 2016). Recently a nasopharynx-associated lymphoid tissue (NALT) was described in fish (Salinas 2015), containing diffuse lymphoid cells without organized structures, and IgT^{+}-B cells that play a crucial role in pathogenic invasion defense (Yu et al. 2020).

1.5.1.2. The innate immune system– Cellular components

The cellular components of the fish innate immune system consist of several different cell types with several functions:

1) The phagocytic cells: monocytes/macrophages, granulocytes such as mast/eosinophilic granule cells and neutrophils,
2) dendritic cells,
3) non-specific cytotoxic cells (NCCs) (Magnadóttir 2006; Smith et al. 2019).

These cells can be activated when encounters a pathogen, by recognizing a PAMP present on the pathogen. Once activated, the innate cell will participate in several responses depending on their cell subtype including, but not limited to, phagocytosis and subsequent destruction of the pathogen, production of various cytokines and activation of the adaptative immune system via antigen presentation along with cytokine stimulation (Smith et al. 2019).

1.5.1.2.1. Macrophages

Macrophages are large mononuclear cells present in animals including fish, specialized in phagocytosis. These cells possess a germline-encoded PRRs area which will recognize the PAMPs, such as LPS from the gram-negative bacterial cell wall, peptidoglycan, and lipoteichoic acid from the gram-positive bacterial cell wall (Kordon et al. 2018; Lu et al. 2019). During the recognition of pathogens via different PRRs, macrophages attach and ingest pathogens into vesicles known as phagosomes that fuse with lysosomes to form phagolysosomes. Macrophages produce antibacterial substances, such as reactive oxygen and nitrogen species (ROS and NOS, respectively) which kill and destroy the pathogen in the phagolysosomes (Esteban et al. 2015; Lu et al. 2019). Therefore, macrophages have a crucial role in innate immunity, by eliminating pathogens and by reducing the risks of an infection. In addition to innate immunity, macrophages are essential in the initiation of adaptive immunity (Kordon et al. 2018).

1.5.1.2.2. Granulocytes

Fish granulocytes, also known as polymorphonuclear leukocytes due to the distinct structure, contain a large number of granules in their cytoplasm; therefore, they are divided into three types of granulocytes:

1) neutrophils (heterophil),
2) eosinophils, and
3) basophils (Firdaus-Nawi et al. 2016).

Fish granulocytes are present in the different compartments of fish. They can be isolated from peripheral blood, lymphoid tissues, and peritoneal cavity (Kordon et al. 2018).

Neutrophils, being the most abundant granulocytes, have a low-affinity binding to acidic and basic dyes (Kordon et al. 2018). Neutrophils are phagocytes that mediate the acute inflammatory response in teleost fish and are the first leukocytes being recruited to the site of infection by chemokines (Havixbeck et al. 2015). Activated neutrophils ingest pathogens and produce ROS, release toxic substances from intracellular granules, thus becoming very potent killers (Flerova et al. 2013; Havixbeck et al. 2015). In addition, neutrophils secrete neutrophil extracellular traps which consist of antimicrobial granular proteins that capture and immobilize bacteria preventing the dissemination of pathogens (Zhao et al. 2017).

Eosinophils of teleosts are stained by acidic dyes, such as eosin, and have a role in allergic inflammatory reactions acting on the control of parasitic infections (Kordon et al. 2018).

Basophils can be stained with basic dyes (e.g., toluidine blue pH 9.0) and are very rarely found in the blood of teleost (Tavares-Dias 2006). Secreted IgD, a class of immunoglobulins, binds to the surface of basophils and triggers the production of the antimicrobial factors, such as cathelicidin, pentraxin-3, and defensin (Kordon et al. 2018).

1.5.1.2.3. Non-specific cytotoxic cells

Non-specific cytotoxic cells (NCCs) are unique to fish and are considered to be functionally similar to mammalian natural killer (NK) cells (Firdaus-Nawi et al. 2016). NCCs target various cells which include tumor cells, virus-infected cells, and some

protozoa, and can kill the affected cells through lysis. The binding of NCCs to the antigens of parasites occurs in the presence of Mg^{+2}, and the lysis of target cells requires Ca^{+2} (Kordon et al. 2018).

1.5.1.2.4. Mast cells

Mast cells (MCs) have an irregular shape, and their cytoplasm is filled with a wide range of electron-dense and membrane-bound granules (Dezfuli et al. 2011). They are involved in inflammation response during bacterial infection by releasing lipid mediators, proteases, biogenic amines, cytokines, and chemokines, which help the active recruitment of neutrophils. MCs are important players in adaptive immunity as well. In teleosts, the MCs can be found in connective tissues, skin, gill filaments, intestinal submucosa, and the brain (Dezfuli et al. 2011; Kordon et al. 2018).

1.5.1.3. The innate immune system– Humoral parameters

Humoral responses are mediated by macromolecules produced by cells and released into the extracellular fluids after infection by a pathogen. The most studied humoral components in fish include the complement system, lysozyme, antimicrobial peptides, and acute-phase proteins. These components have many different functions including the promotion of inflammation, toxin neutralisation, complement activation, opsonin promotion of phagocytosis and pathogen elimination (Firdaus-Nawi et al. 2016; Smith et al. 2019).

1.5.1.3.1. The haemolytic complement system

The complement is one of the major mechanisms of the humoral component of the immune system. It is involved in both initiations of the innate immune response and mounting of an adaptive immune response (Secombes et al. 2012; Smith et al. 2019). To do that, the complement system is composed of more than 35 soluble proteins (Holland et al. 2002) that act cooperatively against pathogens through a molecular cascade that

mediate defense mechanisms including the elimination of pathogens through opsonization and phagocytosis and the promotion of the inflammatory response (Riera Romo et al. 2016; Smith et al. 2019). This system is better characterized in mammals, where three activation pathways have been identified: the classical, the alternative and the lectin pathways (Holland et al. 2002; Smith et al. 2019), being these three pathways represented in the Fig. 5. The classical pathway is composed by a complex protein consisting of two molecules each of C1r and C1s bound to one molecule of C1q (Holland et al. 2002). This pathway is triggered by antibody binding to the cell surface (generally with immunoglobulin G (IgG) or immunoglobulin M (IgM)) making a bridge between innate and adaptive immunity systems (Holland et al. 2002; Uribe et al. 2011). Then, surface-bound IgM or IgG binds C1q resulting in the activation of C1s. The activated C1s drive to the activation of the C3 convertase which cleaves inactive C3 into C3a and C3b. The C3a acts as a chemotactic factor and aids in inflammation, while the C3b serves as an opsonin, i.e. increases phagocytosis by binding to specific receptors on the surface of phagocytic cells, and can form a complex with C4bC2a to give rise to C5 convertase. Upon cleavage of C5 by the C5 convertase, the fragments C5a and C5b are generated. The smaller fragment, C5a, contributes to the inflammation process by attracting phagocytic cells to the site of infection while the larger fragment, C5b, triggers a process of cell lysis and death of the pathogen through a selfassembly of a membrane attack complex by subsequent binding of C6, C7, C8 and C9 (Holland et al. 2002). In contrast to the classical pathway, the alternative pathway is independent of antibodies and activated directly by pathogens (Boshra et al. 2006). Activation occurs when C3b generated from the cleavage of C3 binds to carbohydrates or proteins on a foreign cell surface. Factor B binds to the deposited C3b, and is activated and cleaved into Bb and Ba resulting in a C3bBb complex that acts as the C3 convertase of the alternative pathway. The lectin pathway is triggered by the binding of the mannose-binding lectin (MBL) and the serine proteases, mannose-binding lectin associated proteases 1 and 2 (MASP-1 and -2), to mannose (or other sugar) residues present on the pathogen surface (Roca et al. 2006). The MBL/MASP complex acts similarly to C1r/C1s to form the C3 convertase acting similarly to the classical pathway (Holland et al. 2002).

Most of the mammalian complement components have homologs in various teleost species, including rainbow trout (Chondrou et al. 2006), zebrafish (Wang et al. 2008), channel catfish (Jenkins et al. 1993), European seabass (Mauri et al. 2011) and gilthead seabream (Najafpour et al. 2020).

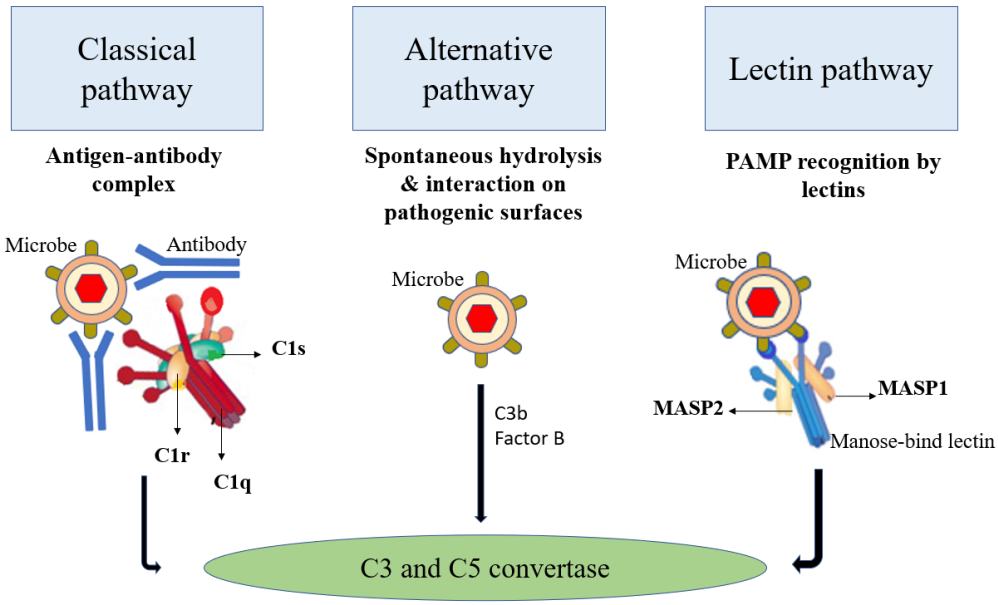

Figure 5 Activation of the three complement pathways and functions.

Based on (Smith et al. 2019) and (Holland et al. 2002)

1.5.1.3.2. Lysozyme

Lysozyme is a nonspecific bacteriolytic enzyme, important in mediating protection against microbial invasion (Saurabh et al. 2008). The enzyme acts on the peptidoglycan layer of Gram-positive bacterial cell walls by hydrolyzing 1–4 β-linked glycoside bonds (Magnadóttir 2006; Uribe et al. 2011; Smith et al. 2019). Gram-negative bacteria are not directly damaged by lysozyme. However, when the outer cell wall of Gram-negative bacteria is disrupted due to the action of complement or other enzymes exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective (Saurabh et al. 2008). This enzyme is known to be an opsonin and activates the complement system and phagocytes as well (Saurabh et al. 2008; Smith et al. 2019). Lysozymes are synthesized in both the liver and the extra-hepatic sites (Bayne & Gerwick 2001). High activities of the enzymes are found against *Micrococcus lysodeikticus* infection in the lymphomyeloid tissues of elasmobranchs and the plasma of teleosts. Lower activities were detected in the spleen of fish, and low or no activity was detected in the Atlantic hagfish and cod (Saurabh et al. 2008). Two types of lysozyme have been described in several teleost species, c-type and g-type. The enzyme is found in neutrophils, monocytes and to a lesser extent in macrophages associated with several tissues including liver, kidney, spleen, and

gills (Uribe et al. 2011; Smith et al. 2019) plasma (Magnadóttir 2006) and skin mucus (Saurabh et al. 2008; Smith et al. 2019).

1.5.1.3.3. Antiproteases

Antiproteases play crucial roles in acute phase reactions, acting as defense mechanisms against pathogens, as many of them secrete a variety of proteases to penetrate the host and invade new tissues (Potempa et al. 2009). Some proteases are non-specific and powerful enzymes that degrade many proteins involved in innate immunity, and others are extremely precise and specific in their mode of action. (Gonzalez-Silvera et al. 2018). Antiproteases inhibit proteases either by binding to their active sites or by 'trapping' the enzyme to prevent protein hydrolysis (Gonzalez-Silvera et al. 2018), preventing bacteria to invade and grow in fish (Agbowuro et al. 2018). Antiproteases are divided into four classes based on their protease active site, which includes serine, cysteine, aspartic and metalloprotease (Leung et al. 2000; Kumaresan et al. 2015).

1.5.1.3.4. Proteases

Proteases act as a catalyst in the cleavage of peptide bond of bacteria (Nigam et al. 2012; Kumaresan et al. 2015), improving the natural resistance of fish to bacterial infection (Ingram 1980). They also activate and enhance the production of complement components, immunoglobulin and antibacterial peptides (Cho et al. 2002; Nigam et al. 2012). The action of proteases is tightly regulated with endogenous inhibitors. The interaction between proteases and their inhibitors is important to homeostasis (Kumaresan et al. 2015). Based on the catalytic mechanism, they are categorized into serine, cysteine, aspartic and metalloproteases (Verma et al. 2016).

1.5.2. Oxidative stress in fish

Oxygen (O_2) is the major biological acceptor of electrons which serves a vital role in cellular functions. Although, the dependence on oxygen can represent a danger for

aerobic life since oxygen could carry oxidative stress risk (Hoseinifar et al. 2020). Oxygen could contribute to the undesirable formation of reactive oxygen species (ROS), being the most important the superoxide anion ($O_2^{-}\bullet$), the hydroxyl radical (OH●), and a non-radical compound, the hydrogen peroxide (H_2O_2) (Chowdhury et al. 2020). The first ROS formed is typically the $O_2^{-}\bullet$ and it is produced mostly by mitochondria leading to several chemical reactions altering several biological functions (Hoseinifar et al. 2020). The OH● has a very short half-life and is considered the most reactive and harmful ROS. Since no antioxidant capable of preventing the OH● action is available, it is only possible to inhibit its formation or repair the damage caused by its action (Schieber et al. 2014). Differently, the non-radical ROS, the hydrogen peroxide (H_2O_2) is produced in abundance in the mitochondrial matrix during the O_2 reduction process (Hoseinifar et al. 2020).

Under normal physiological conditions, at low concentration levels, ROS plays important roles, including host defense (i.e., defense against pathogenic microorganisms), cellular cycle regulation, maintenance of the redox balance, and as a component of cellular signalling pathways (Martínez-Alvarez et al. 2005; Chowdhury et al. 2020). At low to moderate concentrations, ROS does not cause damages, but at high concentrations, they induce negative modifications on lipids, proteins, and nucleic acids within the cellular compartments which in turn can lead to activation of cell death processes such as apoptosis (Lushchak 2011; Redza-Dutordoir et al. 2016). Lipid peroxidation is a common example of ROS-induced damage where the fatty acids are prone to free radical attack leading to the disruption of the membrane lipid bilayer arrangement and the alteration of the membrane structure and permeability. Moreover, malondialdehyde (MDA) and unsaturated aldehydes, products of lipid peroxidation, may affect the activity of many proteins inducing the formation of protein cross-linkages function (Hematyar et al. 2019). ROS could potentially attack also DNA resulting distinct pattern of chemical modification (Chowdhury et al. 2020).

1.5.3. The antioxidant defense system in fish

To avoid the negative effects of naturally produced ROS, the living organisms developed an antioxidant defense system with two distinct classes: the enzymatic antioxidant system including different enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT); the non-enzyme antioxidants such

as glutathione, thioredoxin, vitamin C and vitamin E (Moussa et al. 2019; Hoseinifar et al. 2020).

This antioxidant defense system is able of providing the balance between production and removal of ROS under normal physiological conditions (Martínez-Alvarez et al. 2005). However, when ROS levels exceed the scavenging capability of the antioxidant defense system an imbalance could be generated, by impairing the homeostasis of the organism and inducing oxidative stress (Slaninova et al. 2009; Hoseinifar et al. 2020; Huang et al. 2020). Thus, the action of the antioxidant defense system is important, by protecting fish from the negative effects of ROS accumulation, playing as well a role by improving the defense response (Hoseinifar et al. 2020).

1.5.3.1. Superoxide dismutase

SOD consists of a family of metalloenzymes containing copper and zinc or manganese or iron, playing a crucial antioxidant role. SOD is the first enzyme to cope with the negative effects of $O_2^{-}\bullet$. This enzyme is found both in the mitochondria (Mn^{2+}-SOD) and in the cytosol (Cu^{2+}-SOD and Zn^{2+}-SOD) (Hoseinifar et al. 2020). SOD catalyses the dismutation of two molecules of $O_2^{-}\bullet$ into H_2O_2 and O_2, which can then be further broken down by CAT and GPx or it may be further released into the cytoplasm (Schieber et al. 2014; Moussa et al. 2019).

1.5.3.2. Catalase

CAT is an enzyme that is active both in mitochondria and in peroxisomes and facilitates the removal of H_2O_2, which is metabolized to O_2 and H_2O. Hydrogen peroxide, if not detoxified, can pass easily through membranes and may be further released into the cell cytoplasm. In the presence of metal ions with changeable valence, H_2O_2 can be converted to highly reactive $OH\bullet$ (Dong et al. 2017; Hoseinifar et al. 2020). The H_2O_2 detoxification is mediated by an irreversible inactivated CAT step, forming CAT-H_2O_2 that goes through an NADPH-dependent process to regenerate active CAT. This process is essential for proper cellular function. Although CAT seems to be more important in catalyzing the decomposition of H_2O_2, it shares this role with GPx (Grissinger 2017).

1.5.3.3. Glutathione peroxidase, glutathione reductase, and glutathione S-transferase

GPx includes two different forms, a selenium-dependent and a selenium-independent. The selenium-dependent form reacts with hydroperoxides, including H_2O_2 and organic peroxides, while the selenium-independent form reacts only with organic hydroperoxides (Ursini et al. 2013). Unlike CAT, GPx requires co-factors, including GR and NADPH. GR activity is required for the regeneration of reduced glutathione (GSH), previously oxidized (glutathione disulfide, GSSG) by the action of GPx (Ursini et al. 2013).

GST is a group of multifunctional isoenzymes, which play an important role in detoxification of toxic electrophiles by catalyzing the conjugation of the reduced glutathione (GSH) to electrophilic centers of many endogenous or exogenous toxic compounds, involving chemical carcinogens, oxidative stress products, insecticides, herbicides, and cancer chemotherapeutic agents (Nimmo 1987; Özaslan et al. 2018)

1.5.3.4. Glutathione: reduced and oxidized forms

Glutathione exists in reduced (GSH) and oxidized form (GSSG). The GSH is involved in the scavenging of H_2O_2, free radicals, and other peroxides through SH groups. When GSH reacts with oxidants, it becomes converted into the oxidized form, glutathione disulphide. The GSSG is then reduced by the action of the GR that is responsible to maintain the GSH form. Thus the elevation of GSSG is considered as one of the oxidative stress biomarkers (Srikanth et al. 2013).
Figure 6 summarizes the action mode of the antioxidant defense system of fish.

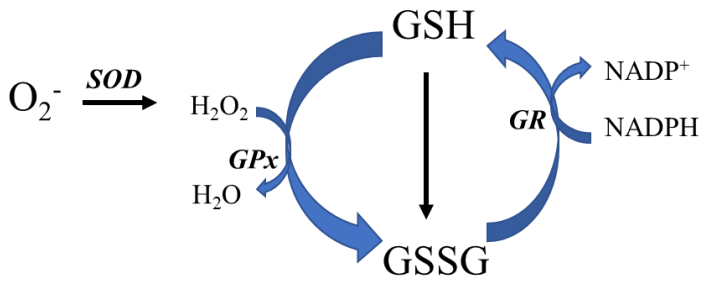

Figure 6 Schematic representation of the functions of the glutathione-related antioxidant system of fish.

SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Adapted from Srikanth et al. (2013)

1.5.4. *Sparus aurata*: Biology

Gilthead seabream (*Sparus aurata*), a member of the family Sparidae, is a eurythermic (5-32°C) and euryhaline fish. This species can be found in the Mediterranean Sea and extends into the Atlantic Ocean from the British Isles south to Senegal (FAO 2021).

The gilthead seabream is the main species in aquaculture in the Mediterranean region by volume production, with Greece (41.7% of total production), followed by Turkey (23.2%) and Spain (9.3%) as the main producers, accounting for over 70% of the total production (Manchado et al. 2016). The important development of seabream aquaculture has been directly associated with the robustness and plasticity of this species, able to adapt to different environments, together with the strong market demand (Borrego et al. 2017). However, the rapid development of gilthead seabream farming has not been paralleled by adequate progress in the veterinary aspects of its culture. In Mediterranean areas the annual mortality rates around 7-10% of the fish stock, although in some cases they may be as high as 80%, resulting in severe economic losses (Ibarz et al. 2010). The major disease problems affecting gilthead seabream are related to bacteria (*Photobacterium damselae* subsp. *piscicida* and subsp. *damselae, Vibrio alginolyticus, V. anguillarum, Pseudomonas anguilliseptica*), virus (*Iridoviridae*, Aquareovirus, Virus-like particle) and endoparasites (*Myxidium leei*) (Assefa et al. 2018; FAO 2021)

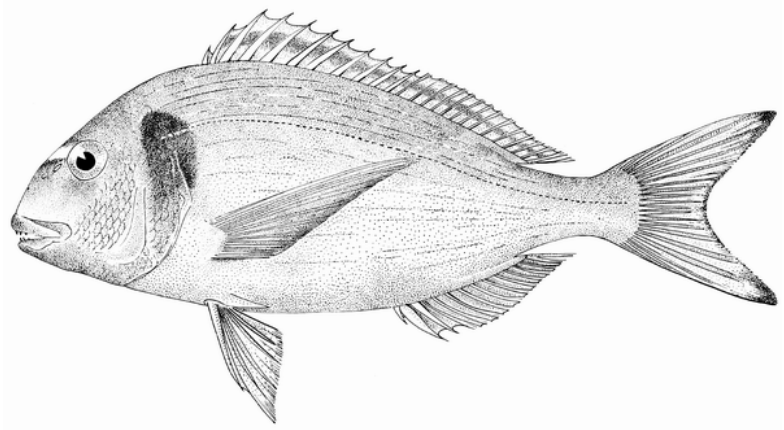

Figure 7 Phenotypical anatomy of *Sparus aurata*.
Source: http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en

1.6. Objectives of this thesis

The concept of environmentally-friendly aquaculture in the context of the global economy is in the spotlight of the global guidelines established by the United Nations to be achieved by 2030. The introduction of sustainable and functional ingredients into the aquafeeds sector is an opportunity to improve the economic and ecological sustainability of this activity.

One of the most promising ingredients to be included in the fish diets is SW, which are recognized to have several benefits antioxidant and immune-stimulants effects by enhancing the physiologic status and welfare of some farmed fish species. Red SW including *Gracilaria sp.* has been recognized to induce beneficial effects in several farmed fish species. In particular, *Gracilaria gracilis* is a SW produced sustainably in an IMTA system at the ALGAplus company, Portugal. Therefore, this SW could be particularly relevant when included in the diet of cultured gilthead seabream, as this species consumes SW, including *Gracilaria sp.* in natural environments. However, the supplementation of *Gracilaria sp.* in aquafeeds competes with the agar industry, the main industrial sector for utilization of this resource. As the agar industry produces large amounts of by-products (agar extraction residue or agar waste) it is suggested that the supplementation of agar waste in seabream diets may contribute to the development of environmentally-friendly aquaculture, promoting the circular economy. In addition, the composition of SW could be modulated by the culture conditions, which opens an

opportunity to explore forms to tailor the agar (yield and quality) and the bioactive compounds produced.

As a result, this thesis aimed to evaluate:

- ✓ The antioxidant activity of the residue from agar extraction (agar waste, AW) obtained from *Gracilaria gracilis* cultured in an IMTA system (**Chapter 2**);
- ✓ The effects that dietary supplementation with AW has on performance, intestinal bacterial community, digestive function, skin and fillet attributes during cold storage of gilthead seabream (**Chapter 3**);
- ✓ The effects that dietary supplementation with AW has on the stress response, antioxidant and immune systems of gilthead seabream exposed to crowding (**Chapter 4**);
- ✓ The effects that filtered sunlight has on the agar properties and the antioxidant activity of *Gracilaria gracilis* when cultured in an IMTA system (**Chapter 5**);
- ✓ The effects that dietary supplementation with AW obtained from cultured *Gracilaria gracilis* under filtered sunlight has on immune status and oxidative stress response of gilthead seabream stimulated with inactivated *Photobacterium damselae* subsp. piscicida (**Chapter 6**).

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CHAPTER 2

Antioxidant activity of agar waste obtained from *Gracilaria gracilis* cultured in an Integrated Multi-Trophic Aquaculture (IMTA) system

Francisca Silva-Brito[1,2], Anake Kijjoa[1,2], Rui Pereira[3], Leonardo Magnoni[1*] -

[1] *CIIMAR- Interdisciplinary Centre of Marine and Environmental* Research - University of Porto
Address: Terminal de Cruzeiros de Leixões. Av. General Norton de Matos s/n 4450-208 Matosinhos, Portugal

[2] ICBAS-Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Address: Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] ALGAplus - Production & commercialization of high-quality seaweed and derived products. Travessa Alexandre da Conceição s/n 3830-196 – Ílhavo, Aveiro, Portugal

Abstract

Gracilaria gracilis is used for agar extraction, generating significant amounts of agar waste (AW) which may contain compounds with antioxidant properties. This study investigated the antioxidant activity of AW extracted with ethanol at 95 ºC (AW_{95}) or 25 ºC (AW_{25}) in comparison to ethanolic extracts of raw *G. gracilis* (E) obtained at 25 ºC or 60 ºC for 3h or 6h ($E_{25/3}$, $E_{25/6}$, $E_{60/3}$, and $E_{60/6}$, respectively). The total phenolic content of AW was similar to that of $E_{25/3}$, but higher than those in $E_{25/6}$, $E_{60/3}$, and $E_{60/6}$. The highest free radical scavenging activity was detected in both $E_{25/3}$ and AW_{95}. A high capacity for the inhibition of lipid peroxidation was observed in AW_{95}, which was similar to that in Vitamin E. AW presented a similar antioxidant activity to that quantified in ethanolic extracts, suggesting that AW from *G. gracilis* represents a sustainable source of antioxidant compounds for feed production.

2.1. Introduction

Reactive oxygen species (ROS) are produced in all organisms as a result of oxidative metabolic processes and have important roles in cell signaling and homeostasis (Schieber et al. 2014). However, certain environmental/physiological conditions may be linked to a burst in ROS formation in organisms, resulting in oxidative stress (OS) with subsequent cellular and tissular damage. ROS are a heterogeneous group of highly reactive compounds such as hydroxyl radical $^{\bullet}OH$, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$). In biological systems, ROS can react with macromolecules including lipids, proteins, RNA and DNA, ensuing OS. To achieve a balance between prooxidants and antioxidants, organisms are protected from the risks of OS by several enzymatic and non-enzymatic antioxidant mechanisms (Birben et al. 2012).

In particular, the use of antioxidants in foods and feeds could decrease the negative outcome of OS, as well as improve food and feed preservation (Finley et al. 2011). Synthetic antioxidant compounds like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are added in foods/feeds for preservation, but recently some health concerns have been raised (Shahidi et al. 2005). From the consumer's point of view, natural antioxidants are preferred over synthetic compounds, as they are seen as being healthy, ecological, and safe products. Consequently, finding new and safe natural antioxidants from natural sources is of great interest to industrial food and/or feed producers (Brewer 2011), particularly for those formulating aquafeeds.

Seaweeds (SW) are a renewable resource, which contains compounds with antioxidant and immune-modulating capacities (Pal et al. 2014). In particular, the high antioxidant activities of SW have been associated with the exposure of these organisms to harsh environmental conditions, including elevated UV radiation, high temperatures, and desiccation (Maharana et al. 2015). Therefore, the success of SW to adapt to these kinds of harsh conditions may rely on an efficient system to protect themselves from OS, as cellular and tissue damage in SW could increase under severe environmental conditions (Maharana et al. 2015). Indeed, protective mechanisms may be achieved by the production in SW of secondary metabolites with antioxidant properties such as polysaccharides, sterols, polyphenols, pigments, and vitamins (MacArtain et al. 2007; Lordan et al. 2011). The production of secondary metabolites with antioxidant properties can be improved by culturing SW under controlled conditions that are capable of stimulating the production of secondary metabolites.

Unfortunately, the application of SW, particularly red SW, as an antioxidant for aquafeed formulation competes with well-established industries that use SW as a raw material. One of the most important industries that use red SW as a raw material is the phycocolloid industry which has greatly increased over the last century boosting the demand for red SW. As result, SW cultivation has grown substantially. This sector now supplies more than 50% of the global SW demand (West et al. 2016). Because SW demand is still increasing, new and sustainable practices should be implemented to promote SW utilization for aquafeed formulation. By-products generated by industries that utilize SW as a raw material can be reused, and a good example of that is the agar waste from the phycocolloid industry (Al-Khatib et al. 2007). The global production of agar is around 14500 tonnes per year (Bixler et al. 2011), 30% of which is agar waste (AW), suggesting that approximately 2880 tonnes of AW are generated annually around the world (Meinita et al. 2017). However, only 40% of the AW is recycled by the industry to produce paper, bioethanol, and fertilizers (Ennouali et al. 2006; Meinita et al. 2017). Bearing this in mind, a huge amount of by-products could be reused by the aquaculture industry since AW may still contain large amounts of bioactive compounds which could have potential commercial applications (Pal et al. 2014).

The potential for recycling AW for aquafeed formulation could be further enhanced by adopting sustainable and controlled SW production. To this end, the Integrated Multi-Trophic Aquaculture (IMTA) system appears as a sustainable alternative method of SW cultivation since it involves recycling by-products from fish farming (such as uneaten food and faeces) to enhance SW production, a practice that has beneficial impacts on economic, environmental, and social sustainability (Troell et al. 2009; Sasikumar et al. 2015). Additionally, under the IMTA system, SW production conditions can be optimized to promote the production of secondary metabolites adding value to the final product (Korzen et al. 2015b).

Recent studies have focused on the impact of the SW extraction process on the polyphenol content (e.g. catechins and flavonoids) and the antioxidant activity of products (Chan et al. 2015; Topuz et al. 2016; Boi et al. 2017). In particular, a previous study has shown that the conditions applied during the SW extraction process influence the antioxidant activity and phenolic content in the extraction products of the red SW *Laurencia obtuse* (Topuz et al. 2016). These authors found a positive correlation between phenolic content in extracts with increasing time, temperature, and the solvent to SW ratio in the extraction.

In addition, ethanolic extracts demonstrated higher antioxidant activity than that of aqueous extracts of the red SW *Gracilaria bursa-pastoris* (Ramdani et al. 2017), indicating ethanol as the best solvent to extract antioxidant compounds from red SW. However, the information available on how the conditions of agar extraction may affect the content of key compounds related to the antioxidant activity itself is still incomplete. The objective of this study was to investigate whether AW obtained from the extraction process of the red SW *Gracilaria gracilis* may display antioxidant activity. *G. gracilis* was selected for this study because this species is one of the most important SW species of hydrocolloids industry (McHugh 2003) and is known to have a high content of antioxidant compounds (Pal et al. 2014). In addition, *G. gracilis* used in this study was produced in Portugal under controlled conditions in a land-based industrial-scale *IMTA* system (Abreu et al. 2009; Abreu et al. 2011). The potential antioxidant activity of ethanolic extracts derived from AW of *G. gracilis* was compared to that determined in ethanolic extracts from raw *G. gracilis* (E) prepared by using different temperatures and extraction times. Subsequently, the antioxidant activities of AW and E were compared with those of synthetic compounds (BHT, BHA) and vitamins (Vit. E and C).

2.2. Materials and methods

2.2.1. Seaweed origin and processing

G. gracilis was commercially farmed at ALGAPlus, Lda., Ílhavo, Portugal (40.604547, -8.667092). It was harvested after two weeks of cultivation in May 2017. The SW was cleaned on-site under running water and dried at 25 ºC for 24h. The dried SW was stored in sealed bags and transported to the Biomedical Sciences Institute of Abel Salazar (ICBAS) of the University of Porto. The samples were subsequently dried in an oven for 24h at 65 ºC and ground using a hammer mill (Retsch GmbH, Haan, Germany) to pass through a 4 mm (pore size) sieve. The resulting powder was stored in sealed bags at room temperature and protected from light until extraction. The proximate composition and mineral composition of *G. gracilis* are presented in Table 6.

Table 6 Proximate composition and mineral content of *Gracilaria gracilis* produced in an IMTA system.

Proximate composition	
Protein	23.92±0.09
Fat	0.82±0.05
Ash	31.13±0.05
Neutral detergent fibre	27.80±0.06
Acid detergent fibre	8.00±0.04
Acid detergent lignin	2.20±0.03
Energy	12.63±0.06
Mineral content	
Calcium	0.68±0.03
Phosphorus	1.81±0.03
Iron	2.96±0.05
Magnesium	0.30±0.00
Potassium	46.8±0.04
Zinc	21.5±0.69

*Protein, fat, ash content, neutral detergent fibre, acid detergent fibre, and acid detergent lignin are expressed as % dry matter content (% DM). The energy is expressed as kilojoules per gram of SW (Kj g^{-1}).

Mineral content is expressed as g kg^{-1} of dry SW, except for zinc which is expressed as µg kg^{-1}. Values are expressed as mean±standard error.

2.2.2. Chemical analysis

SW was lyophilized before further analyses. Analyses were carried out in duplicate following the methodology described by AOAC (2006). Ash was analyzed by combustion (500 °C 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 °C) 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). The neutral detergent fibre (NDF, with α-amylase and without

sodium sulphite), acid detergent fibre (ADF; 1 h boiling in acid detergent solution), and acid detergent lignin (ADL; 3h in sulfuric acid at 72%) were determined following the procedure described by Maia et al. (2019). Mineral composition was determined by analysis of atomic absorption spectrophotometer.

2.2.3. Agar extraction and preparation of ethanolic extracts from AW

The agar extraction followed the industrial procedure described by Pangestuti et al. (2015) that reported the main steps in processing agar from agarophytes as follows: extraction with hot water; filtration to eliminate the weed residues; concentration and purification; and drying (Pangestuti et al. 2015). *G. gracilis* powder was weighed (50 g), placed inside conical flasks (2000 mL), and protected from light. The agar extraction was performed by adding 1000 mL of distilled water at 95 ºC to SW. In parallel, another agar extraction was performed at 25 ºC. This treatment was included as a control for the agar extraction process at high temperatures. The AW from both treatments was obtained by filtration using a three-ply cheesecloth (Kumar et al. 2009). The AW obtained at 95 ºC (AW_{95}) and 25 ºC (AW_{25}) were dried in the oven at 50 ºC for 48h. After which the AW_{95} and AW_{25} were extracted by adding 500 mL of 96% ethanol in a 1:10 ratio (w/v) and extracted for 3h at 60 ºC, using an agitation plate (1500 rpm). The process was repeated twice and the obtained extracts were pooled. Then, the combined extract was filtered through Whatman No.1 filter paper (Sigma-Aldrich). Finally, the solvent was evaporated using a rotatory vapor (Buchi Rotavapor^{TM}, Flawil, Switzerland) and the dry extracts were stored at room temperature, protected from light, until further analysis. Each extraction was performed once. Ethanolic extracts from raw *G. gracilis* and AW were used to measure antioxidant capacity.

2.2.4. Preparation of ethanolic extracts from raw *G. gracilis*

A second experiment was performed to evaluate the effect of the time and the temperature on the antioxidant property of ethanolic extracts from raw *G. gracilis* (E). Thus, G*racilaria gracilis* powder was weighed (100 g) and placed inside conical flasks (2000 mL), protected from light, and 1000 mL of ethanol 96% was added. Then, the extraction was carried out at two different temperatures (60 ºC or 25 ºC) and two different extraction lengths (3h or 6h) to obtain the extracts $E_{60/6}$, $E_{60/3}$, $E_{25/6,}$ or $E_{25/3}$. Agitation plates at

controlled temperature were used to ensure adequate extraction conditions (stirring at 1 500 rpm). SW was re-extracted twice repeating the conditions previously described. The consecutive extracts were pooled and the supernatants were filtered through Whatman No.1 filter paper (Sigma-Aldrich). Finally, the solvent was evaporated by the rotary evaporator under reduced pressure at 50 ºC and the dry extracts were stored in a sealed flask at room temperature in the dark until analysis. Each extraction was performed once.

2.2.4.1. The yield of the extraction process

The yield in raw *G. gracilis* was calculated as follows:

$$\text{Yield (\%)} = (W_{ext} \times 100)/W_{SW}$$

Where W_{SW} is the dry weight of the SW (g) and W_{ext} is the dry weight of the extract (g). The yields of each process are presented in Table 7.

Table 7 Yields for the *Gracilaria gracilis* extractions.
Extractions performed in ethanol at 60 ºC or 25 ºC for 6 h or 3h (E), and in water at 95 ºC or 25 ºC (agar waste, AW).

	E				**AW**	
Extracts	$E_{60/6}$	$E_{60/3}$	$E_{25/6}$	$E_{25/3}$	AW_{95}	AW_{25}
Yield (%)	11.93	10.38	6.05	3.53	5.50	3.05

2.2.4.2. Total phenolic content

Total phenolic content (TPC) in AW and E was measured using Folin Ciocalteu's method as described by Chan et al. (2015). Extracts were diluted in ethanol at 5 mg mL^{-1}. TPC content of each extract was quantified in triplicates using phloroglucinol as a standard (0.01-0.5 mg mL^{-1}). The absorbance of the solution was measured at 765 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek® Instruments, Inc., Winooski, Vermont, USA). The results were expressed as mg phloroglucinol equivalent g^{-1} dry extract (mg PGE g^{-1} E).

2.2.4.3. Total flavonoid content

The total flavonoid content (TFC) in AW and E was determined using the $AlCl_3$ colorimetric method as described by Chan et al. (2015). Extracts were diluted in ethanol at concentrations between 0.13-4.66 mg mL^{-1}. TFC was quantified in triplicates using rutin (Santa Cruz Biotechnology, Inc., Shandon, USA) as a standard (2-70 µg mL^{-1}). The absorbance of the solution was measured at 415 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek® Instruments, Inc., Vermont, USA). The samples were measured against the blank and those samples that were similar to the blank were considered undetected. The results were expressed in mg rutin equivalent g^{-1} dry extract (mg RE g^{-1} E).

2.2.4.4. Free radical scavenging activity

The α, α-diphenyl-β-picrylhydrazyl (DPPH) assay was performed for AW, E, and compounds with well-known antioxidant activity (BHT, BHA, Vit. E, and Vit. C), according to Chan et al. (2015) with slight modifications. Serial dilutions of extracts (0.20-52 mg mL^{-1}) or reference compounds (2.50 µg mL^{-1} - 0.32 mg mL^{-1}) were incubated with DPPH at a final concentration of 0.1 mM. A sample blank was performed in the same manner but without DPPH. An equal amount of ethanol and DPPH was used as a control. The DPPH curve standard was performed to determine DPPH concentration in each well. The scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = 100 - (100 \times [A_{517\ \text{sample}} - A_{517\ \text{blank}} / (A_{517\ \text{DPPH}} - A_{517\ \text{solvent}})])$$

The half-maximal inhibitory concentration (IC_{50}) was determined by linear regression analysis of the dose-response curve plotted between scavenging activity (%) against extract concentration. The IC_{50} is defined as the concentration of sample scavenging 50% of the initial DPPH radical, thus the higher DPPH value means the higher concentration of extract required to reduce 50% DPPH free radical (Chan et al. 2015).

2.2.4.5. Lipid peroxidation inhibitory assay

Thiobarbituric acid-reactive substances formation inhibition assay (TBARS) was performed in the presence of AW, E and with compounds with well-known antioxidant activity (BHT, BHA, Vit. E and Vit. C) based on the method described by Zeb et al. (2016) with slight modifications. The lipid substrate used in the TBARS assay was a homogenate of fish liver (*Dicentrarchus labrax*). Extracts were diluted in ethanol at 50 mg mL^{-1}. Reference compounds were diluted at a concentration of 0.75 mg mL^{-1} with ethanol (BHT, BHA, and Vit. E) or distilled water (Vit. C). Five microliters of extracts or reference compound were added to the liver homogenate (150 µl, diluted 1:10) and incubated for 16 h at 50 ºC. TBARS concentration was calculated using a standard curve based on MDA (200 µM – 6.25 µM). The results were expressed as mM of TBARS by g of liver (mM TBARS g^{-1} tissue).

2.2.5. Statistics Analysis

Data were expressed as mean±standard error of the mean (SEM) of six to nine replicates. All statistical analyses were carried out using Sigma Stat 3.1 applying an $\alpha = 0.05$. Data were checked for normality and homogeneity of variances (Shapiro-Wilk test and Brown-Forsyth test, respectively). One-way analysis of variance (ANOVA) and Tukey's post hoc test was applied to determine the significant differences between experimental conditions. *P*-values<0.05 were regarded as significant. The graphs were built by using GraphPad Prism 5 for Windows.

2.3. Results

2.3.1. Total phenolic content (TPC)

TPC content in raw *G. gracilis* (E) and agar waste (AW) are presented in Fig. 8. Results revealed that TPC in AW_{95} was 1.5 times higher than that in AW_{25} (31.76±5.16 and 20.53±1.47 mg PGE g^{-1} E, respectively, *P*=0.000). Higher extraction temperature negatively affected the TPC of raw *G. gracilis*, as this parameter was four times lower in $E_{60/6}$ and $E_{60/3}$ (7.08±3.20 and 7.52±3.32 mg PGE g^{-1} E) than those detected in $E_{25/6}$ and $E_{25/3}$ (25.02±7.21 and 33.07±4.04 mg PGE g^{-1} E, *P*=0.000). The length of the extraction process also had a negative impact on the TPC at a low temperature, as shown by the

higher content in $E_{25/3}$ when compared to $E_{25/6}$ (P=0.005). However, at 60 ºC no significant differences in TPC were detected between both extraction times ($E_{60/3}$ and $E_{60/6}$, P=1.000).

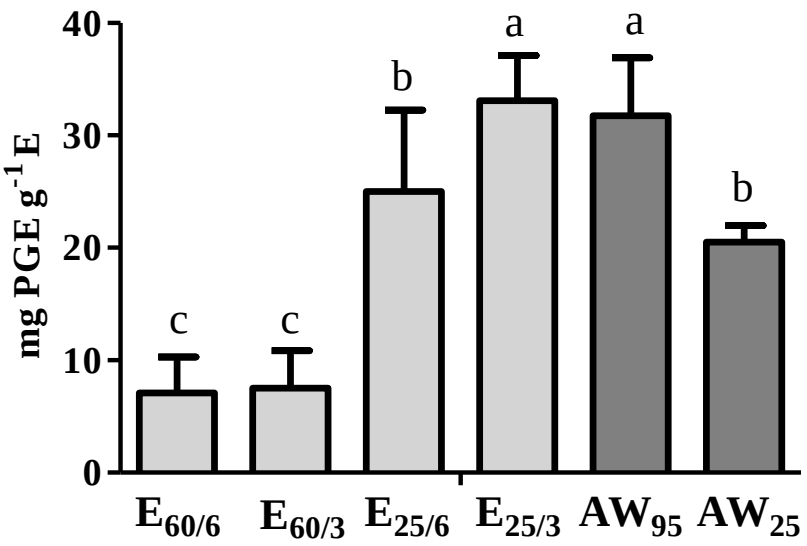

Figure 8 The total phenolic content (TPC) of *Gracilaria gracilis* extracts. Raw SW was extracted in ethanol at 60 ºC or 25 ºC for 6 h or 3h (E), and in water at 95 ºC or 25 ºC (agar waste, AW). Values are expressed as mean+SEM of 8-9 replicates. TPC is expressed as mg phloroglucinol equivalent g^{-1} dry extract (mg PGE g^{-1} E). Different letters indicate significant differences detected between the different experimental conditions (One-way ANOVA, P<0.05)

2.3.2. Total flavonoids content (TFC)

Total flavonoids content (TFC) in E and AW are shown in Table 8. Results show that TFC was not significantly different among $E_{60/3}$, $E_{25/6,}$ and $E_{60/6}$ (62.00±21.16, 98.06±45.48, and 107.73±17.84 mg RE g^{-1} E, respectively). Conversely, TFC remained undetected for $E_{25/3}$, $AW_{95,}$ and AW_{25}.

Table 8 Total flavonoid content (TFC) of *Gracilaria gracilis* extracts.
Raw SW was extracted in ethanol at 60 ºC or 25 ºC for 6 h or 3h (E), and in water at 95 ºC or 25 ºC (agar waste, AW).

Extract	TFC
$E_{60/6}$	62.00±21.16
$E_{60/3}$	107.73±17.84
$E_{25/6}$	98.06±45.48
$E_{25/3}$	ND
AW_{95}	ND
AW_{25}	ND

Values are expressed as mean±SEM of 3-5 replicates. TFC is expressed as mg rutin equivalent g^{-1} extract (mg RE g^{-1} E). No significant differences were detected between the different experimental conditions (One-way ANOVA). ND= Not detected.

2.3.3. Free radical scavenging activity

The free radical scavenging activity of E and AW were expressed as IC_{50} and the results are presented in Fig. 9. A lower IC_{50} value means a better free radical scavenging activity of E and AW. Thus, the free radical scavenging activity of AW_{25} was *ca*. 2 times higher than that exhibited by AW_{95} (IC_{50} of 838.49±95.57 and 1670.04±180.47 µg mL^{-1}, respectively, *P*=0.001). Considering the effect of temperature on *G. gracilis* extraction, results showed a lower free radical scavenging activity (1.5-2.5 times) of $E_{60/6}$ and $E_{60/3}$ in comparison with $E_{25/6}$ and $E_{25/3}$ (*P*=0.000). These results indicated a negative effect of high temperature (60 ºC) on the free radical scavenging activity. However, similar IC_{50} values between $E_{25/6}$ (1560.23±286.38 µg mL^{-1}) and $E_{25/3}$ (1212.52±114.42 µg mL^{-1}), and between $E_{60/6}$ (2497.22±486.57 µg mL^{-1}) and $E_{60/3}$ (2920.66±837.92 µg mL^{-1}) are suggested that longer extraction with ethanol did not influence the free radical scavenging activity. No significant differences among the IC_{50} values of Vit. C (5.08±0.34 µg mL^{-1}), BHT (5.08±0.14 µg mL^{-1}), Vit. E (10.11±0.92 µg mL^{-1}) and, BHA (13.10±5.73 µg mL^{-1}) were detected, all of them displaying IC_{50} values between 62 and 585 times lower than those in AW and E.

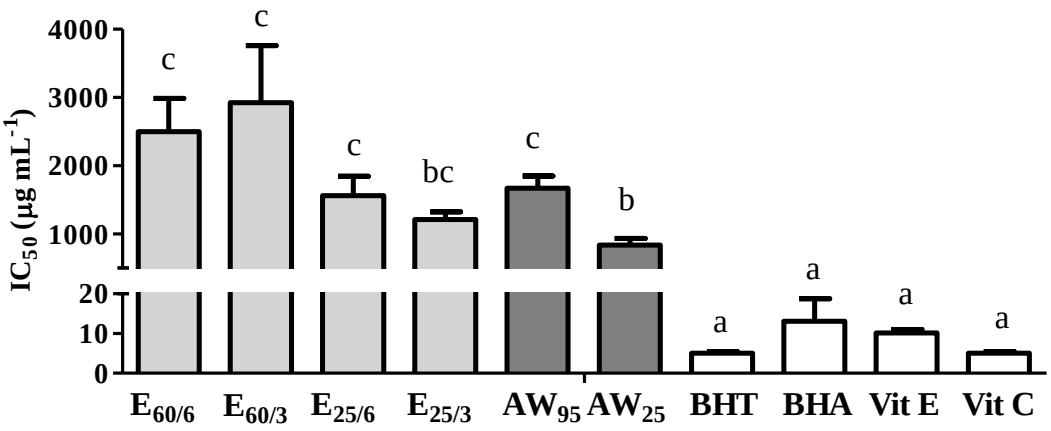

Figure 9 Free radical scavenging activity (IC_{50}) of *Gracilaria gracilis* extracts.
Raw SW was extracted in ethanol at 60 ºC or 25 ºC for 6 h or 3h (E), and in water at 95 ºC or 25 ºC (agar waste, AW). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), vitamin E (Vit. E), and vitamin C (Vit. C) were used as references. Values are expressed as mean+SEM of 4-6 replicate measurements. Free radical scavenging activity is expressed as the half-maximal inhibitory concentration (IC_{50}). Different letters indicate significant differences detected between the different experimental conditions (One-way ANOVA, $P<0.05$).

2.3.4. Lipid peroxidation inhibiting potential (TBARS)

The lipid peroxidation inhibiting potential of AW and E, as well as compounds with well-known antioxidant activity, are presented in Fig. 10. Agar extraction temperature did not influence the capacity of AW to inhibit lipid peroxidation, as both extracts, W_{95} and AW_{25}, possessed similar values (6.98±0.22 and 7.01±0.18 mM TBARS g^{-1} tissue, respectively, $P=1.000$). Both agar wastes (AW_{95} and AW_{25}) displayed a higher capacity to inhibit lipid peroxidation than those exhibited by raw *G. gracilis* ($E_{60/3}$, $E_{60/6}$, $E_{25/3}$, and $E_{25/6}$, $P=0.000$).

Regarding raw *G. gracilis*, $E_{60/6}$ (8.03±0.27 mM TBARS g^{-1} tissue) exhibited the highest lipid peroxidation inhibitory capacity, followed by $E_{60/3}$ (8.06±0.17 mM TBARS g^{-1} tissue), $E_{25/3}$ (8.10±0.21 mM TBARS g^{-1} tissue), and lastly $E_{25/6}$ (8.53±0.11 mM TBARS g^{-1} tissue).

Results showed that Vit. E (0.75 mg mL^{-1}) had a similar inhibitory capacity for lipid peroxidation to those displayed by AW_{95} and AW_{25} at a higher concentration (50 mg mL^{-1}). Also, the capacity to inhibit lipid peroxidation of Vit. C (0.75 mg mL^{-1}) was similar to

that displayed by $E_{60/3}$, $E_{60/6}$, $E_{25/3}$, and $E_{25/6}$ when tested at 50 mg mL^{-1}. Therefore, Vit. E and Vit. C showed approximately 7 times higher inhibitory capacity for lipid peroxidation than AW or E, for an equivalent concentration of reference compounds or extracts.

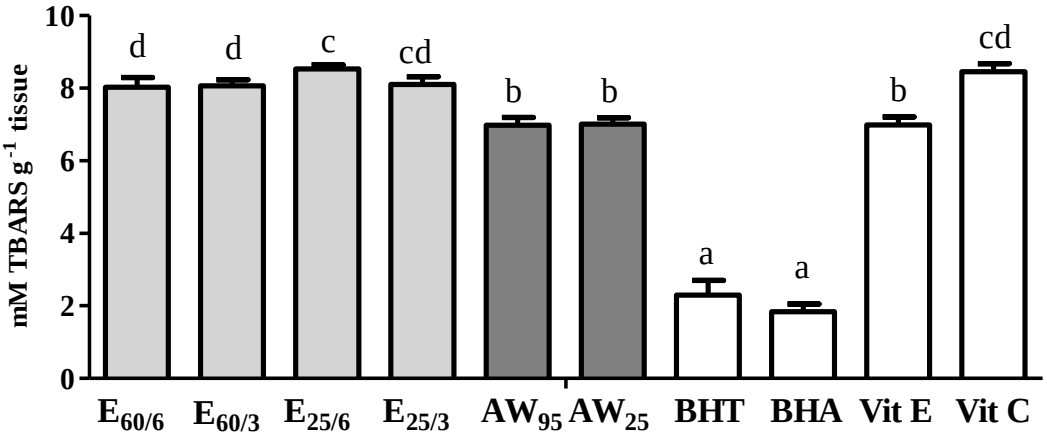

Figure 10 Inhibitory capacity for lipid peroxidation of *Gracilaria gracilis* extracts.
Raw SW was extracted in ethanol at 60 ºC or 25 ºC for 6 h or 3h (E), and in water at 95 ºC or 25 ºC (agar waste, AW). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), vitamin E (Vit. E), and vitamin C (Vit. C) were used as references. Values are expressed as mean+SEM of 5-6 replicates. Inhibitory capacity for lipid peroxidation is expressed as mM of TBARS by g^{-1} tissue. Different letters indicate significant differences detected between the different experimental conditions (One-way ANOVA, $P<0.05$).

2.4.Discussion

Gracilaria gracilis is a red SW of great commercial interest which is principally used for agar extraction (Sousa et al. 2010). The extraction of agar is generally performed with hot water at 95 ºC to maximize the extraction of polysaccharides from the cell wall (Arvizu-Higuera et al. 2008). This process, however, generates a significant amount (around 30% w/w dry weight) of agar waste (AW) (Meinita et al. 2017). The recycling of AW has stimulated much interest as a means of improving the sustainability of industries. Specifically, this kind of by-product may contain compounds of low solubility in water with antioxidant capacities. To test this assumption, we analyzed several parameters related to the antioxidant activity in AW generated from *G. gracilis* at two different

temperatures, 95 ºC (AW_{95}) and 25 ºC (AW_{25}), and compared them to raw *G. gracilis* at two temperatures (25 or 60 ºC) and two extraction lengths (3h and 6h).

Higher total phenolic content (TPC) may be related to the higher antioxidant potential of SW extracts. In this study, the TPC from AW_{95} (31.76 mg PGE g^{-1}) was comparable with those obtained by $E_{25/3}$ (33.07 mg PGE g^{-1}) and $E_{25/6}$ (25.02 mg PGE g^{-1}), suggesting that large amounts of phenolic compounds remained in the AW after agar extraction. This is in line with the previous studies showing low solubility of phenolic compounds in water, even when high temperatures were applied during the agar extraction process (Chan et al. 2015). In addition, the TPC detected in extracts from AW and raw *G. gracilis* were similar to those determined in *Gracilaria changii* extracts which ranged between 10.83 and 21.57 mg PGE g^{-1}, depending on the type of organic solvent. In contrast, $E_{60/6}$ and $E_{60/3}$ (7.08±3.20 and 7.52±3.32 mg PGE g^{-1}, respectively) showed the lowest TPC and these findings are consistent with DPPH assay results' since $E_{60/6}$ and $E_{60/3}$ exhibited the lowest free radical scavenging activity among the experimental extracts. This indicated that a high extraction temperature (60 ºC) negatively influenced both the TPC and the free radical scavenging activity of raw *G. gracilis*. High temperatures could increase the oxidation and degradation of phenolic compounds (Dahmoune et al. 2015; Boi et al. 2017), which may explain the decrease in the free radical scavenging activity of $E_{60/6}$ and $E_{60/3}$. Similarly, Li et al. (2017) and Boi et al. (2017) showed that the TPC in two different species of *Sargassum* dropped at higher extraction temperatures (55 ºC, *ca*. 57 mg PGE g^{-1} and 80 ºC, *ca*. 1 mg PGE g^{-1}, respectively).

TPC and free radical scavenging activity in AW_{95} were higher than those in $E_{60/6}$ or $E_{60/3}$, implying that differences in the efficiency of diffusion of ethanol and solubilization of phenolic compounds may occur (Pérez-Jiménez et al. 2011), as ethanol was added at a later step. This may be explained because the cell walls of SW are composed of complex molecules such as hemicellulose, lignin, and polysaccharides (Synytsya et al. 2015) which are known to be a barrier to solvent diffusion, making difficult the extraction of bioactive compounds from SW. On the other hand, agar extraction comprises the dissolution of polysaccharides from the cell wall which can facilitate further solvent diffusion throughout the AW, which may increase the extraction of water-insoluble compounds. This theory could explain the greater TPC and free radical scavenging activity in AW_{95} when compared to $E_{60/6}$ or $E_{60/3}$. Thus, these results suggested the utility of AW as a source of compounds with antioxidant activity for potential applications.

Another condition that is likely to have affected the TPC and antioxidant activity of raw *G. gracilis* extracts is the extraction time. Previous studies have shown that the time required for the solvent to interact with the SW is critical for solute recovery (Pinelo et al. 2006). However, extended extraction time may lead to alteration of compounds present in SW, which may lead to a decrease in antioxidant activity due to a combination of unfavorable conditions during the extraction process (Li et al. 2017). The impact of the time allowed for extraction was confirmed as the prolongation of this process by 3h decreased (1.5-times) both the TPC and antioxidant activity in $E_{25/6}$ compared with $E_{25/3}$. Our findings confirm the results obtained by Li et al. (2017) indicating long extraction time had a negative impact on the antioxidant activity of SW extracts, particularly if SW extraction is performed at lower temperatures. On the other hand, Topuz et al. (2016) found a positive correlation between TPC and time, the temperature of the extraction, and the solvent to SW ratio during the extraction process. Therefore, it will be important in the future to attempt to optimize the SW extraction conditions depending on the intended use of the products, particularly regarding the antioxidant activity of extracts.

The TBARS assay was carried out to analyze the capacity of AW and raw *G. gracilis*, to inhibit lipid peroxidation. Higher TBARS formation is linked to a lower inhibitory capacity for lipid peroxidation. AW_{95} showed the lowest TBARS formation among all tested extracts. These results suggested the potential of AW_{95} to inhibit lipid peroxidation. The inhibition of lipid peroxidation has been related to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions (Karawita et al. 2005). A good example of this is flavonoids, which can act as antioxidants and inhibiting lipid peroxidation. They could act directly against free radicals by reducing them, resulting in more stable and less reactive compounds (Panche et al. 2016). The total flavonoid content (TFC) was quantified in $E_{60/6}$, $E_{60/3,}$ and $E_{25/6,}$ varying from 62.00 mg RE g^{-1} E to 107.73 mg RE g^{-1} E. These values were in agreement with Farasat et al. (2013) who reported TFC values varying between 10.18 mg RE g^{-1} and 45.58 mg RE g^{-1}. However, it was not possible to quantify the TFC in $E_{25/3}$, $AW_{95,}$ and AW_{25}. The absence of TFC in AW could indicate that flavonoids were extracted during the agar extraction process. On the other hand, the absence of TFC in $E_{25/3}$ may indicate that higher extraction temperature (60 ºC) or/and long extraction length (6 h) are required to extract effectively the flavonoids from raw SW when employing ethanol. Spite of the lack of flavonoids in both in AW and $E_{25/3}$,

these fractions displayed similar or even higher antioxidant activity compared to other extracts, suggesting that other compounds may be linked to the antioxidant activity of the AW and E.

Despite the antioxidant activity detected in SW by-products, the free radical scavenging activity and lipid peroxidation inhibition of AW and E were 62 to 585-times lower than those of BHT, BHA, Vit. E and Vit. C, all compounds with a well-established antioxidant activity. This might suggest that higher concentrations of AW and E should be used to achieve a similar free radical scavenging ability to those exhibited by BHA, BHT, Vit. E and Vit. C.

2.5.Conclusions

The results showed that the residues obtained from the extraction of agar (agar waste, AW) have similar or twice as much high antioxidant activity as those displayed by the different ethanolic extracts (E) obtained from the raw *Gracilaria gracilis*. It was also found that the higher extraction temperature (60 ºC) had a negative impact on TPC and free radical scavenging activity in ethanolic extracts of raw *G. gracilis*. In addition, AW and E displayed similar antioxidant activity to those found in well-established compounds with antioxidant activity when they were used at higher concentrations (i.e. between 62-585 times higher). Flavonoids are not responsible for the free radical scavenging activity and lipid peroxidation inhibiting potential in AW and E from *G. gracilis*. Thus, the AW from *G. gracilis* represents a natural and sustainable source of compounds with antioxidant capacities. Future studies should be implemented to test the potential functionality of such SW by-products when supplemented into feeds.

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CHAPTER 3

Fish performance, intestinal bacterial community, digestive function and skin and fillet attributes during cold storage of gilthead seabream (*Sparus aurata*) fed diets supplemented with *Gracilaria* gracilis by-products.

Francisca Silva-Brito[1,2], Diogo A. M. Alexandrino[1,2], Zhongjun Jia[3], Yongliang Mo[3], Anake Kijjoa[1,2], Helena Abreu[4], Maria F. Carvalho,[1,2], Rodrigo Ozório[1], Leonardo Magnoni[1]*

[1] – CIIMAR Interdisciplinary Centre of Marine and Environmental Research, Terminal de cruzeiros de Leixões. AV. General Norton de Matos S/N, 4450-208 Matosinhos, Portugal

[2]- ICBAS Institute of Biomedical Sciences Abel Salazar, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

[3]- State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, Jiangsu Province, China

[4] – ALGAplus, Tv. Alexandre da Conceição, Ílhavo, Portugal

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Fish performance, intestinal bacterial community, digestive function and skin and fillet attributes during cold storage of gilthead seabream (*Sparus aurata*) fed diets supplemented with *Gracilaria* by-products

ABSTRACT

This study evaluated the use of the seaweed *Gracilaria gracilis* by-products in gilthead seabream (*Sparus aurata*) diet to modulate intestinal bacterial community, physiological responses as well as skin and fillet attributes during cold storage. Fish were fed for 52 days with six experimental diets: *i)* a diet containing a commercial antioxidant (CTR), *ii)* a diet without antioxidant (NO-AOX), *iii)* a NO-AOX with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX with 5% ethanolic waste (5% EEW), *v)* a NO-AOX with 5% agar waste (5% AW), *vi)* a NO-AOX with 2.5% agar waste (2.5% AW). Fish growth performance was not affected by the experimental diets. Proteobacteria (63-83%), Firmicutes (4-19%) and Actinobacteria (2-4%) were the predominant phyla in the intestine of fish fed all diets. The diet with 5% AW increased the relative abundance of Firmicutes phyla as compared to the other diets. Fish fed NO-AOX, 5% EEW, 5% AW and 2.5% AW showed a lower abundance of Oxalobacteraceae than those fed CTR diet. The *Clostridium* genera increased in the 5% AW compared to 5% EEW. Nevertheless, no major differences were detected in the richness and diversity of the intestinal bacterial communities of gilthead seabream fed the experimental diets. Fish fed 5% AW showed higher intestinal trypsin activity than those fed 0.5% EE. The skin yellowness was higher in the skin of fish fed 2.5% AW diet compared to those fed 0.5% EE diet. The results presented provided evidence of the potential application of AW and EEW as a novel and eco-friendly dietary supplement to be included in future aquaculture feed formulations.

3.1. INTRODUCTION

Seaweeds (SW) have been drawing the attention of the food and feed industries, including aquaculture, due to their foreseeable safety and wider acceptance by consumers (Brewer 2011; Mendes 2019). In particular, the red SW *Gracilaria* sp. has been highlighted by its antioxidant properties (Francavilla et al. 2013). In Portugal, *Gracilaria* sp. has been produced in an Integrated Multi-Trophic Aquaculture (IMTA) system, where they are used as "biofilters" to remove inorganic matter from fish farm effluents (Abreu et al. 2011). Most of *Gracilaria* sp. biomass is used as raw material for agar, food, and cosmetic industries (Kraan 2012; Pimentel et al. 2018). The global production of agar is around 9600 tonnes per year (Bixler et al. 2011), 30% of which is agar waste (AW), suggesting that approximately 288 tonnes of this waste are generated annually around the world (Meinita et al. 2017). The AW is a surplus non-value solid by-product generated from the agar industry that may still contain a large amount of polysaccharide material (Meinita et al. 2017). However, only 40% of the AW is recycled by the industry to produce paper, bioethanol, and fertilizers (Ennouali et al. 2006; Meinita et al. 2017). As a result, large amounts of by-products are generated, being indiscriminately discharged into the environment. Reusing SW by-products, such as agar waste (AW), from the agar industry or ethanolic waste (EEW) from the cosmetic industry, may represent an innovative strategy to improve the economic and ecological sustainability of the aquaculture industry. In particular, AW may contain bioactive compounds with antioxidant capacity, as most of them are water insoluble (Sindhi et al. 2013; Thorat et al. 2013; Pal et al. 2014), while EEW may be a source of polysaccharides which are not soluble in organic solvents. Polysaccharides are known to positively impact the intestinal bacterial community. The intestinal microbiota of fish plays an important role in host metabolism, nutrition, immunity, and health status of fish (Hindu et al. 2018). Therefore, the scientific and commercial interest in the effect of dietary SW supplementation on the intestinal bacterial community has been increased during the last years. Recent studies have described the modulatory effect of polysaccharides and oligosaccharides derived from SW on intestinal metabolism through a change of intestinal bacterial community (Ramnani et al. 2012; Rimoldi et al. 2020). In addition, modulation of the intestinal bacterial community by dietary supplementation of raw SW, such as *Ulva* sp. *and Gracilaria* sp., has been reported to enhance the abundance of beneficial bacteria, such as *Lactobacillus delbrueckii* subspecies, while reducing the abundance of potential deleterious bacteria,

such as *Vibrio* genus (Rico et al. 2016; Tapia-Paniagua et al. 2019). The dietary supplementation of SW can be used to achieve good animal growth and health, by promoting a well-balanced intestinal bacterial community, improving the digestive process and better preparing the host against pathogen outbreaks (Rico et al. 2016). For instance, previous studies reported an improvement in digestive capacity, immune response and stress resistance of fish-fed SW supplemented diets at supplementation levels ranging 2.5 and 5% (Peixoto et al. 2016b; Peixoto et al. 2019a). Also, the supplementation of extracts from SW showed beneficial effects on fish fillet quality and immune response when supplemented at 0.5% (Peixoto et al. 2019b). Nevertheless, current knowledge on the effects of the gut microbiome modulation on the physiology of fish is limited, and particularly how dietary seaweed supplementation may play a role.

In addition, the dietary SW supplementation can minimize lipid oxidation, thus improving the shelf life and freshness of farmed fish. The term "fresh" in fish is usually applied to food resulting from fisheries and aquaculture that have been kept in the cold (not frozen) or packed in a modified atmosphere (Mei et al. 2019). Freshness is intended as a high-quality product, very appreciated by most consumers (McManus et al. 2014). However, fish could take a week or more before being consumed. During that time the flesh quality may deteriorate due to endogenous enzyme activities and degradative effects of microbial growth (Ghaly et al. 2010). A large number of reactive compounds (e.g. hydroperoxides and peroxy radicals) and secondary compounds (e.g. aldehydes, ketones, alcohols, hydrocarbons) are generated post-mortem in fish, increasing oxidative rancidity, causing changes in nutritional characteristics, appearance, texture, and organoleptic properties of the flesh (Ahmed et al. 2016a; Secci et al. 2016). The antioxidant properties of some SW were shown to minimize lipid peroxidation progression in canned salmon fillet, using aqueous extracts from *Durvillaea antartica*, *Ulva lactuca* and *Porphyra columbina* (Ortiz et al. 2014) and in minced tilapia, after adding extracts from *Sedum fusiforme* and *Porphyra* yezoensis directly into the fish fillet (Ribeiro et al. 2014). These effects have been attributed to polyphenols, pigments, minerals and polysaccharides (Vinayak et al. 2011). However, few studies have focused on the study of dietary SW supplementation in fish and the potential changes in lipid peroxidation and fillet characteristics during conservation, as well as antioxidant capacity of aquafeeds during storage. A recent study conducted by Peixoto et al. (2019b) in European seabass (*Dicentrarchus labrax*) reported that dietary supplementation of *Gracilaria* sp. methanolic extract and its insoluble residue affected the fillet colour after a period of 2 to 5 days of storage at 4 ºC. However, it

remains to clarify if dietary *Gracilaria* supplementation could minimize lipid peroxidation, particularly during storage.

The gilthead seabream (*Sparus aurata*) is considered one of the most valued marine species produced in Mediterranean countries which are preferentially commercialized fresh (Mendes 2019). This species represents a valuable model to investigate the effects of dietary SW supplementation because it consumes a diversity of food items in the wild, including *Gracilaria* species (Pita et al. 2002). The dietary supplementation of *Gracilaria gracilis* by-products in this study was aimed to investigate their functional use toward eco-friendly production practices. It was hypothesized that dietary *Gracilaria gracilis* supplementation may modulate the intestinal bacterial community and improve the nutritional characteristics of both aquafeed and fish fillet, by slowing down lipid peroxidation. To test this hypothesis, the present study aimed to investigate the effects of *Gracilaria gracilis* products (ethanolic extracts (EE)) and by-products (i.e. ethanolic waste (EEW) and agar waste (AW)) supplementation in gilthead seabream diet on growth performance, intestinal bacterial community, intestinal digestive enzyme activities, physiological condition, as well as colour and lipid peroxidation progression during a cold storage.

3.2. MATERIALS AND METHODS

3.2.1. Seaweed origin and processing

Gracilaria gracilis were cultivated by ALGAplus, Lda., Ílhavo, Portugal, harvested, cleaned on-site under filtered water from the system, and dried (25 ºC for 24h) after two weeks of cultivation. *G. gracilis* was stored in sealed bags and transported to the Biomedical Sciences Institute of Abel Salazar (ICBAS) of the University of Porto, Portugal, for the extraction procedure. *G. gracilis* was cleaned one more time under running tap water and dried at 65 ºC for 48 h. Thereafter, *G. gracilis* was ground using a hammer mill (Retsch) to pass through 4 mm or 0.5 mm sieve before the ethanolic or the agar extraction, respectively. The resulting powder was stored in sealed bags at room temperature and protected from light before extraction.

3.2.2. Agar extraction

The agar extraction was performed by adding 1000 mL of distilled water at 95 ºC to ground raw *G. gracilis* in a 1:10 ratio (w/v). A three-ply cheesecloth (Kumar et al. 2009) was used to separate the agar and the agar waste (AW). The AW obtained was mixed with the ground diet as described later.

3.2.3. Preparation of ethanolic extracts

Ground raw *G. gracilis* was extracted by adding ethanol (purity of 96%) in a 1:20 ratio (w/v). The extraction was performed for 3h at 25 ºC on an agitation plate (1500 rpm) protected from light after three repetitions to warrant a complete SW extraction. The mixture was filtered through Whatman No.1 filter paper (Sigma-Aldrich) and the liquid obtained was evaporated under vacuum in a roto-evaporator (Büchi Labortechnik AG, Switzerland) at 50 ºC (Fu et al. 2016). The obtained dry extracts were stored in sealed flasks at 4 ºC in the dark until analysis. The insoluble portion of the SW, the ethanolic waste (EEW), was dried at 50 ºC for 48h, ground, and sieved (0.5 mm) before mixed into the basal diet.

3.2.4. Diets

Two isoproteic (44% crude protein) and isolipidic (18% crude lipid) diets were produced by SPAROS Lda. (Olhão, Portugal) to contain either a commercial antioxidant (Verdilox – Kemin) to be used as a positive control or a diet without the addition of an antioxidant to be used as a negative control. The diets were grounded below 500 µm in a hammer mill (Retsch GmbH, Haan, Germany) for the preparation of the six experimental diets: *i*) control diet (**CTR**) with commercial antioxidant; *ii*) **NO-AOX** diet, without antioxidant addition; *iii*) **EE** diet, NO-AOX diet with 0.5% ethanolic extract, *iv*) **EEW** diet, NO-AOX diet with 5% ethanolic waste, *v*) **5%AW** diet, NO-AOX diet with 5% agar waste and, *vi*) **2.5%AW** diet, NO-AOX diet with 2.5% agar waste. Each diet was mixed accordingly to the specified proportion in a paddle mixer (*Alexanderwerk* Inc.). For the EEW and AW diets, the mixture was humidified with 25% tap water. For the preparation of the EE diet, the extract was dissolved into 96% ethanol and pulverized on the ground diet. All six experimental diets were dry-pelleted in a laboratory pellet mill through a 5.0 mm die (California Pellet Mill, CPM Crawfordsville, IN, USA). Then, all the diets were dried

overnight in the oven at 45 ºC to remove any traces of water or ethanol. The diet formulation and approximate composition of all the diets are presented in Table 9.

Table 9 Ingredients and proximate composition of the diets.

i) a commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

	Diets					
Ingredients (%)	**CTR**	**NO-AOX**	**0.5% EE**	**5% EEW**	**5% AW**	**2.5% AW**
Fishmeal LT70	10.5	10.5	10.4	10.0	10.0	10.2
Fishmeal 60	15.0	15.0	14.9	14.3	14.3	14.6
Soy protein concentrate	12.0	12.0	11.9	11.4	11.4	11.7
Wheat gluten	6.0	6.0	6.0	5.7	5.7	5.9
Corn gluten	10.0	10.0	10.0	9.5	9.5	9.8
Soybean meal 48	12.0	12.0	11.9	11.4	11.4	11.7
Rapeseed meal	5.0	5.0	5.0	4.8	4.8	4.9
Wheat meal	12.7	12.9	12.8	12.3	12.3	12.6
Fish oil	4.4	4.4	4.4	4.2	4.2	4.3
Rapeseed oil	10.2	10.2	10.2	9.7	9.7	10.0
Vit. & Min. Premix	1.0	1.0	1.0	1.0	1.0	1.0
Antioxidant (Verdilox - Kemin)	0.2	0.0	0.0	0.0	0.0	0.0
MCP	0.6	0.6	0.6	0.6	0.6	0.6
DL-Methionine	0.2	0.2	0.2	0.2	0.2	0.2
L-Taurine	0.2	0.2	0.2	0.2	0.2	0.2
Gracilaria gracilis ethanolic extract			0.5			
Gracilaria gracilis ethanolic waste				5.0		

Gracilaria gracilis agar waste					5.0	2.5
Proximate composition (%DM)						
Dry matter	94.29±0.02	96.56±0.01	97.12±2.28	95.49±0.05	95.49±0.05	93.46±0.02
Crude protein	46.91±0.05	47.12±0.11	46.99±0.01	46.24±0.19	45.93±0.01	47.90±0.25
Crude fat	18.46±0.06	17.32±0.18	15.25±0.05	16.63±0.02	17.23±0.20	15.07±0.10
Ash	8.81±0.01	8.61±0.00	8.92±0.19	8.43±0.02	8.47±0.05	8.93±0.07

Proximate composition values calculated as mean±SEM of two analyses. No significant differences were detected in proximate composition between diets (One-way ANOVA, $P>0.05$).

3.2.5. Fish rearing conditions

This study was conducted under the supervision of accredited experts 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 fish trial took place at the aquaculture facilities of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR). The fish trial was designed to comply with the ARRIVE (*Animal Research*: *Reporting* of *In Vivo Experiments*) Guidelines for Reporting Animal Research.

Juvenile seabreams (average mean of 7 g) were produced by MARESA, Huelva, Spain, and transported to the quarantine facilities of CIIMAR, which were kept for 15 days after arrival. Thereafter, two hundred forty fish were randomly distributed in 20 tanks of 87 L each and fed twice a day with a commercial diet (SPAROS, Portugal) at a maintenance ration (2% body weight day^{-1}) for a week before starting the trial. Experimental tanks had a flow rate of 257 L h^{-1} and were connected to a water recirculation system under controlled conditions (temperature, 17±1 ºC; salinity, 35±1 ‰; pH, 8±1 and >95% air saturation). Water ammonium and nitrite levels were below the limits for the species (0.05 mg mL^{-1} and 0.5 mg mL^{-1}, respectively). The photoperiod regime was 14h:10h (day: night cycle). All rearing parameters were daily monitored periodically during the trial.

3.2.6. Feeding trial

Each diet was randomly assigned to triplicate tanks, except the control diet (CTR) that was assigned to quintuplicate tanks. Fish were hand-fed twice a day to apparent satiety at 9:00h and 16:00h for 52 days. At the end of the feeding trial, fish fasted for 24h before sampling. Six fish per tank were anesthetized with MS-222 (0.1 g L^{-1}), buffered with $NaHCO_3$ (0.2 g L^{-1}) before obtaining biometric data and blood samples. Blood was collected from the caudal vein using heparinized syringes (B Braun Medical, Lda) and plasma was obtained immediately after blood centrifugation at 10 000 x *g* for 5 min and stored at -80 ºC, pending analyses. Three fish per tank were used for the determination of fillet lipid peroxidation and digestive enzyme activities analyses. In addition, three other fish were sampled in each tank for the bacterial community analysis of the intestine, for skin and fillet colour determination. Then, the fish fillet was stored in individually sealed bags to record the colour measurement before (time 0) and after 3 and 6 days of storage

at 4 ºC. A sample of each diet (about 1 kg) was stored in plastic bags at 25 ºC (room temperature) for 90 days before thiobarbituric acid reactive substances (TBARS) analysis.

3.2.7. Fish performance

Growth performance was determined by calculation of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and, daily feed intake (DFI) using the tank as the experimental unit.

WG was calculated as: FBW – IBW;

SGR (%) was calculated as: 100 × [*Ln* FBW – *Ln* IBW] / TD;

FCR was calculated as: TFI / WG;

DFI was calculated as: TFI / (NF × TD);

where FBW and IBW are the final and initial average body weights (g), TD is the total time for the feeding trial (days), TFI is the total feed intake (g), WG is the weight gain (g) and, NF is the number of fish.

3.2.8. Body and diet composition analysis

Samples were freeze-dried before being analysed. Carcass and diets were analysed for dry matter in an oven (105 °C for 24h), ash by combustion in a muffle furnace (550 °C for 12 h), crude protein (Micro-Kjeldahl, Königswinter, Germany N × 6.25) after acid digestion, lipid content by petroleum ether extraction (Soxhlet 40 - 60 ºC) and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). Chemical analyses were run in duplicates.

3.2.9. DNA extraction and high-throughput sequencing of 16S rRNA amplicons

The intestinal wall was scrapped with a spatula, whereby samples included mucosa-associated bacterial community. A total of six samples per dietary treatment (2 fish per tank) were obtained and placed in Eppendorf tubes and immediately stored at -80 °C until DNA extraction. DNA was isolated from the intestinal matrix using the All prep Power viral DNA/RNA kit (Qiagen GmbH, Germany), according to the manufacturer's instructions. DNA quality and quantity were determined spectrophotometrically using a

Denovix DS-11 Spectrophotometer/Fluorometer, and the extracts were stored at -20 ºC until further analysis.

Before the amplification step, DNA templates were precipitated following a standard ethanolic precipitation procedure (Sambrook et al. 1989) and re-dissolved in sterilized distilled water to obtain a DNA concentration of ca. 2-15 ng μL^{-1} for all samples. Amplification of the 16S rRNA gene by PCR was done using the universal bacterial primer pair 515F/907R, which targets the V4-V5 hypervariable region of the bacterial 16S rRNA gene. The primer has a 12 bp barcode at the forward primer to identify different samples. The reaction (total volume, 50 µL) consisted of 2 µL of DNA template (DNA concentration: 2-8 ng μL^{-1}), 1 µL of each forward/reverse primer (each 10 µM), 25 µL of SYBR Premix Ex Taq™ (Tli RNaseH Plus, TaKaRa, Japan), and 21 µL of sterilized distilled water. The PCR thermal profile for amplifying the 16S rRNA gene consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s (Christner et al. 2001). PCR products were purified after 1.2% agarose gel electrophoresis to confirm amplicon size and specificity (single band) and subsequently mixed at equimolar concentrations for Illumina MiSeq sequencing. A sequencing library was constructed for each gene using the TruSeq Nano DNA LT Sample Prep Kit Set A, and the sequencing was performed with the MiSeq Reagent Kit v3 (600 cycles). All sequence data used in this study were deposited in the NCBI under accession numbers SRR12267836 to SRR12267871.

3.2.9.1. Bioinformatics analysis

16S rRNA gene sequences were analysed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline (Caporaso et al. 2010). The scripts and instructions of the QIIME pipeline are publicly available at http://qiime.org. Briefly, sequences were first joined by the command “join_paired_ends.py”, then barcodes were extracted by “extract_barcodes.py”, then trimmed and demultiplexed by ”split_libraries_fastq.py” at Phred quality score of 25. Chimera was identified and removed using the command “identify_chimeric_seqs.py” in usearch61. The 16S rRNA gene sequences were classified and clustered into OTUs at the 97% identity threshold, by using “pick_open_reference_otus.py.

The 16S rRNA dataset was further curated using the phyloseq package in the R environment (version 3.6.1) (McMurdie et al. 2013). Specifically, OTUs taxonomically

annotated as “Eukaryota”, “Chloroplast” and “Mitochondria” were removed from the dataset and a limit relative abundance threshold of 2% was applied. Additionally, alpha-diversity indexes, namely Observed OTUs, Chao1 estimator, and Shannon index were determined and Bray-Curtis distances (β-diversity) were calculated to estimate the divergence of the bacterial communities among the different diets.

3.2.10. Digestive enzyme activities

Activities were measured in samples from individual fish. The whole intestinal tract including pyloric caeca was homogenized with a Potter-Elvehjem homogeniser (Thomas Scientific) in buffer solution (1:5, w/v), made by 50 mM Tris-HCl and 200 mM NaCl at pH 8.0 (Sigma-Aldrich). Homogenates were centrifuged at 13 000 × *g* at 4 ºC for 30 min to separate the supernatant from the lipidic fraction.

The α-amylase (EC 3.2.1.1) assay was performed by mixing 50% homogenate, 15% NaCl (20 Mm), 25% sodium phosphate dibasic (0.2 M at pH 7.4) and, 10% starch solution (5%) in a total volume of 0.25 mL. The mixture was incubated at 37 ºC for 15 min and the reaction was stopped by adding 250 µL of 3,5-Dinitrosalicylic acid (DNS) 1%. The mixture was heated at 100 ºC for 5 min and cooled down by adding 2.5 ml of distilled water. The activity of α-amylase was determined by quantifying the maltose produced by the hydrolysis of α-D (1,4) glycosidic bond of starch (50 mg mL^{-1}) using 3.5-dinitrosalicylic acid as described by Bernfeld (1951). Enzyme activity was calculated as µmol of maltose released per min at 540 nm.

Lipase activity (EC 3.1.1.3) was performed by mixing 1% homogenate, 79% Sodium bicarbonate (0.1M at pH 9), and 20% p-nitrophenyl palmitate (0.01 M) in a total volume of 1.01 ml. The mixture was incubated at 30 ºC for 15 min. The reaction was stopped by adding 250 µL of Na_2CO_3 (1M) and centrifuged at 15 000 x *g* for 20 min at 4 ºC. Lipase activity was quantified by the enzymatic release of p-nitrophenol from p-nitrophenyl palmitate at 410 nm (Winkler et al. 1979) and the activity was calculated as µmol of p-nitrophenol produced per min.

Trypsin activity (EC 3.4.21.4) was performed by incubating 12.5% homogenate with 87.5% trypsin substrate (benzoyl-l-arginine-p-nitroanilide, BAPNA) at 25 ºC for 10 min. Then, the reaction was stopped by adding 30% acetic acid. The production of nitroaniline was measured spectrophotometrically at 410 nm, and compared with a nitroaniline standard curve (Torrissen et al. 1994). Chymotrypsin activity (EC 3.4.21.1) was

performed by incubating 12.5% homogenate with 87.5% trypsin substrate (succinyl-Ala-Ala-Ala-Pro-Phe-p-nitroanilide, SAAPFpNA) at 25 ºC for 10 min. Then, the reaction was stopped by adding 30% acetic acid. The production of nitroaniline was measured spectrophotometrically at 410 nm, and compared with a nitroaniline standard curve (Rungruangsak-Torrissen et al. 2000).

Total protein concentration in the supernatants was quantified by the Folin phenol method (Lowry et al. 1951) and the value was used to calculate specific enzyme activity. Protein concentration was determined by reference to a standard curve of bovine γ-globulin (Sigma-Aldrich).

3.2.11. Plasmatic parameters

Plasma parameters were measured using commercial kits following the manufacture's recommendation. Each sample was tested in triplicated using blanks and standards. Lactate measurements were performed using D/L-Lactic acid, UV method (NZYTech, Genes, and Enzymes). Cholesterol, glucose and triglyceride levels in plasma were determined using Cholesterol-LQ, Glucose-RTU, and Triglycerides–LQ, respectively (Spinreact, S.A./S.A.U.). Units were expressed as mmol L^{-1}.

3.2.12. *Fillet and skin colour*

Instrumental colour analysis was performed with a Chroma Meter CR-400 (Konica Minolta). The results were presented as lightness (L*), redness (a*), and yellowness (b*) as recommended by the Commission Internationale de L'Eclariage recommendations (l'Eclairage 1976). The a* represents the red/green length, with positive values for red and negative ones for green, and b* value represents the yellow/blue length, with positive values for yellow and negative ones for blue. The L* value (lightness) is performed as it is critical to the colour measurement and specular reflection changes the perceived colour, ranging from 100 (white) to 0 (black) (AMSA 2012). Skin measurements were taken along a lateral line at the level of the fore insertion of the dorsal fin and on the right side of the fish body. The fillet measurements were taken on the left side fish body after the skin was removed. The colour measurements in the eviscerated fish was carry out before (time 0) and 3 and 6 days after storage as described previously.

3.2.13. *TBARS measurements*

At the end of the feeding trial, diets and fillet were homogenized in a 1:10 (w/v) 0.1 M potassium phosphate buffer (pH 7.4) and using an Ultraturax (Heidolph RZR2020) for 1 min at 7 000 rpm. The samples were stored in aliquots of 150 µL with 5 µL of 4% butylated hydroxytoluene until analysis (24h). Lipid peroxidation (LPO) levels were measured using the thiobarbituric acid (TBA) reagent (Papastergiadis et al. 2012). The TBARS concentration was calculated using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and the results expressed as mM of TBARS per g of diet or per g of tissue.

3.2.14. Statistical analysis

Statistical analysis was performed using Sigma Stat 3.1 applying an $\alpha = 0.05$. Data were checked for normality and homogeneity of variances (Shapiro-Wilk test and Brown-Forsyth test, respectively). Data from fish performance, digestive enzyme activities, carcass composition, plasmatic parameters, and TBARS levels in diets were analysed by performing a one-way ANOVA (Analysis of variance) analysis. A Tukey post-hoc test was performed to assess the pairwise multiple comparisons among the diets.

A two-way repeated-measures ANOVA (RM-ANOVA) was performed to check the significant effect of the diets on the colour of skin and fillet of gilthead seabream during storage. Both factors (diets and storage) were analysed as fixed factors and including the interaction effect. When the effects of the factors were significant, the Holm-Sidak method was applied. A two-way ANOVA was performed to analyse the effect of the different diets and the time of storage on TBARS levels of the fillets. When the effects of the factors were significant, the Holm-Sidak method was applied.

Beta-diversity comparisons among the different dietary treatments were statistically validated through a one-way ANOSIM test based on the estimated Bray-Curtis distances.

3.3. RESULTS

3.3.1. Growth performance and body composition

Fish performance parameters and the crude protein and lipid contents in the carcass of juvenile gilthead seabream fed the different diets for 52 days are presented in Table 10. All the dietary groups displayed a similar increase in body weight at the end of the trial (~2.3-fold). The calculated growth performance was no different between groups as

indicated by the absence of significant differences in the SGR (1.38 – 1.72%, *P*=0.629). Additionally, both FCR (1.13–1.31, *P*=0.636) and DFI (0.29 – 0.33, *P*=0.792) remained similar among the dietary groups.

The protein (*P*=0.171) nor the lipid content (*P*=0.351) of the gilthead seabream carcasses were not significantly changed by the dietary treatment.

Table 10 Growth performance parameters and body composition of gilthead seabream.
Fish fed six experimental diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

	Diets					
Growth performance parameters	**CTR**	**NO-AOX**	**0.5% EE**	**5% EEW**	**5% AW**	**2.5% AW**
WG	14.38±1.03	13.76±2.19	13.16±1.32	12.03±4.44	13.80±2.59	14.92±1.98
SGR	1.70±0.03	1.65±0.06	1.60±0.05	1.38±0.36	1.63±0.09	1.72±0.06
FCR	1.14±0.02	1.24±0.06	1.16±0.03	1.31±0.28	1.18±0.07	1.13±0.04
DFI	0.31±0.01	0.33±0.02	0.29±0.02	0.32±0.03	0.31±0.02	0.32±0.02
Body composition (%)						
Crude protein	55.78±0.40	54.37±0.58	56.44±0.30	54.52±0.65	55.85±1.22	54.97±0.23
Crude lipid	30.50±0.42	32.03±1.37	30.09±0.29	31.30±0.88	29.91±1.08	31.66±0.41

WG, Weight gain (g); SGR, Specific growth rate (% weight gain day^{-1}); FCR, Feed conversion ratio; DFI, Daily feed intake (g day^{-1}).
Values for growth performance parameters are mean±SEM (n=3-5 tanks). Values for body composition are mean±SEM (n=6-10 fish). The absence of letters indicates no significant differences (One-way ANOVA, *P*>0.05).

3.3.2. Influence of the experimental diets on the structure and diversity of the intestinal bacterial communities

The effects of dietary treatments on the structure of the intestinal bacterial communities are presented in Figs. 11-14. At the phylum level, Proteobacteria was the dominant phylum (63-83% of the total bacterial community) in fish supplemented with all diets, followed by Firmicutes (4-19%) and Actinobacteria (2-4%) (Fig. 11A). The relative abundances of both Proteobacteria and Actinobacteria were similar among the different dietary treatments. On the other hand, the abundance of Firmicutes was five times higher in fish fed 5% AW, compared to those fed the EEW (*P*=0.001) and two times higher as compared to those fed the CTR, NO-AOX, EE, and 2.5% AW diets (*P*<0.05). At the

family level, *Enterobacteriaceae* (26-36%), Sphingomonadaceae (12-24%), and *Pseudomonadaceae* (6-15%) were the dominant taxa in the fish intestine (Fig. 11B). Dietary treatment had no significant effect on the relative abundance of the main identified families, namely Enterobacteriaceae, Sphingomonadaceae, and Pseudomonadaceae. However, the family Oxalobacteraceae was significantly more abundant in the intestine of fish fed CTR diet compared to the other diets (P<0.05). The NO-AOX diet showed the lowest abundance in Oxalobacteraceae which was significantly lower than that detected in the fish fed the EE diet (P=0.029). No statistically significant differences were resolved at the genus level among the different diets (P>0.05), except for the genus *Clostridium*, which was more abundant in the intestine of fish fed the 5% AW diet than in those fed the EEW diet (P=0.016) (Fig. 12).

Alpha-diversity indices of the intestinal bacterial communities of gilthead seabream fed the different diets are shown in Fig. 3. The shifts observed in the intestinal bacterial structure did not translate into significant differences in the bacterial richness and diversity (Fig. 13) of fish supplemented with the different dietary treatments (P>0.05). On the other hand, significantly lower Chao1 index values were detected in the 2.5% AW group, when compared to both CTR (P=0.023) and NO-AOX (P=0.004) groups (Fig. 3B). No evident clustering between the different dietary treatments was observed in the NMDS ordination based on Bray-Curtis distances (Fig. 14), which was further corroborated by the lack of statistical significance revealed by the ANOSIM test (R^2=0.063, P=0.209).

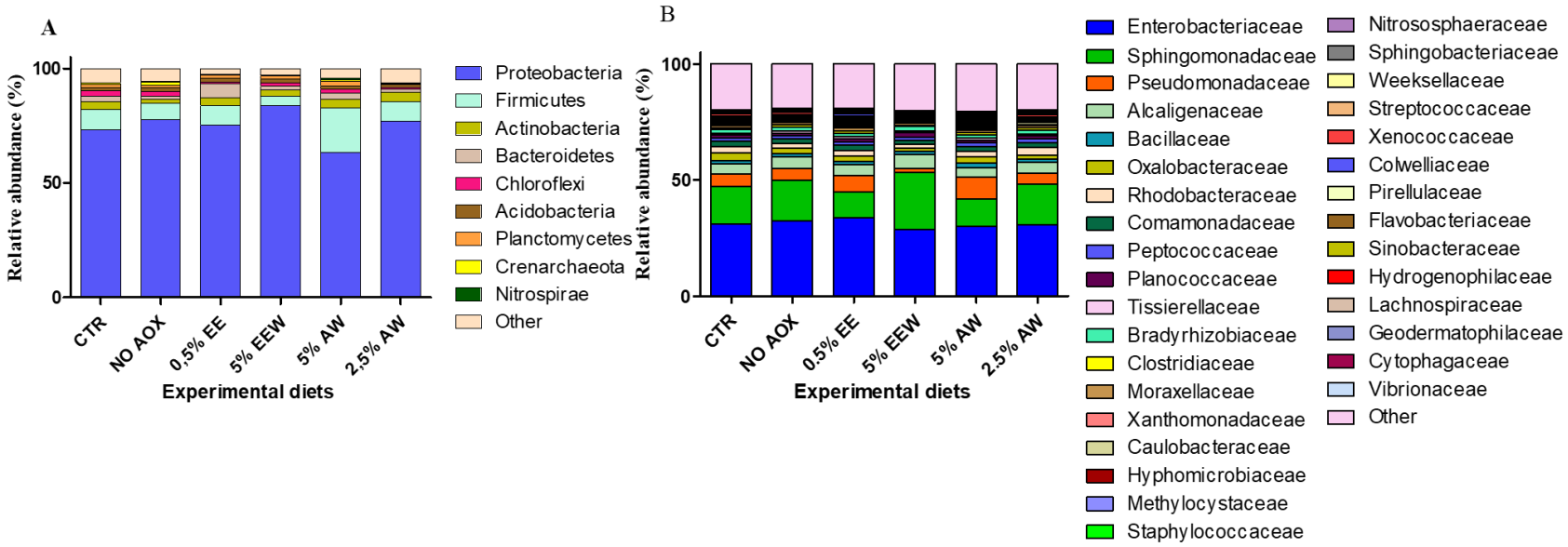

Figure 11 Relative abundance of the main (≥ 2% of relative abundance) phyla (A) and families (B) present in the intestinal bacterial communities of gilthead seabream fed the experimental diets.

Columns represent the mean relative abundance of each taxon (n=4). Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). The absence of letters indicates no significant differences among the experimental diets (one-way ANOVA, $P>0.05$).

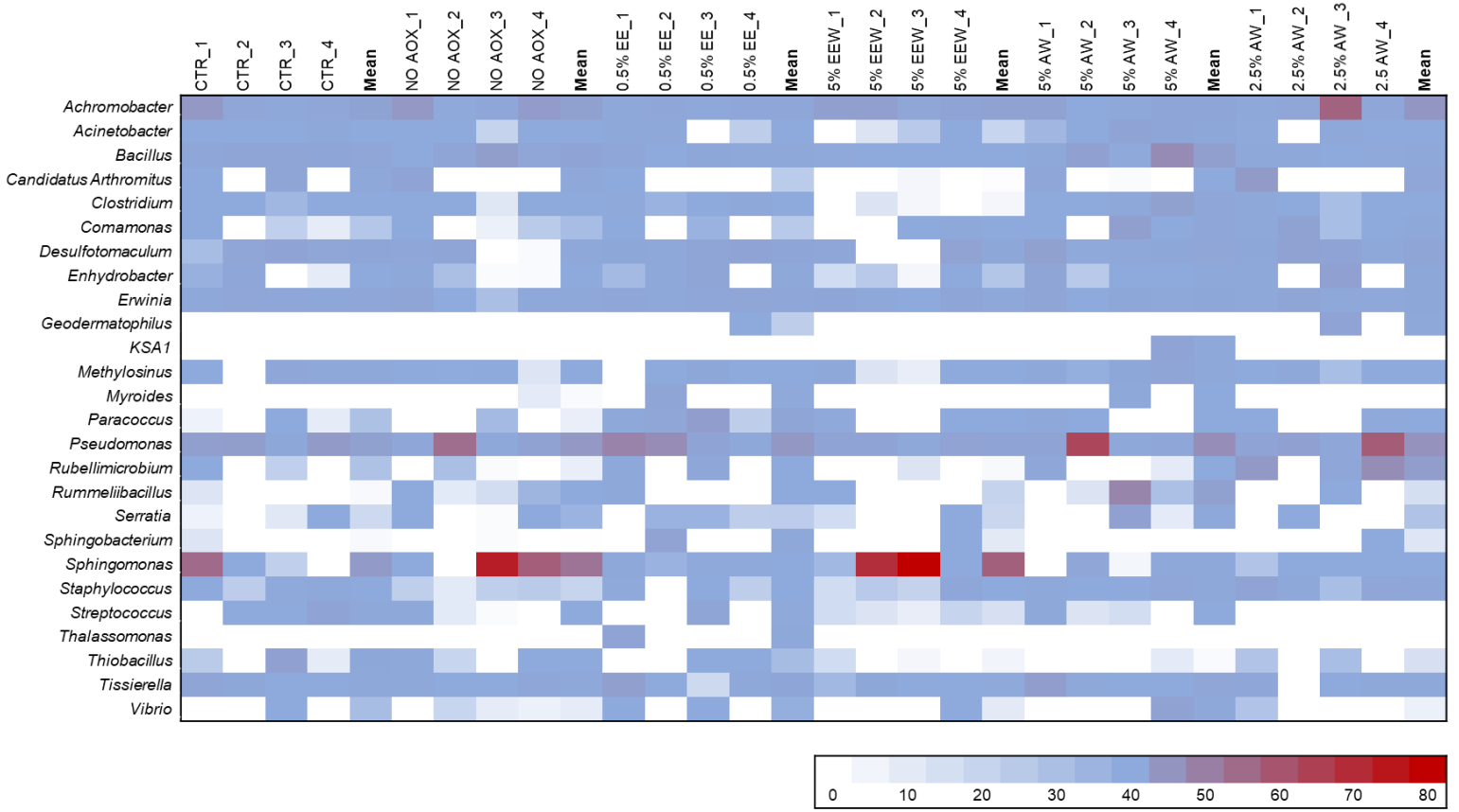

Figure 12 Heatmap patterns of the main (≥ 2% of relative abundance) genera present in the intestinal bacterial communities of gilthead seabream. Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

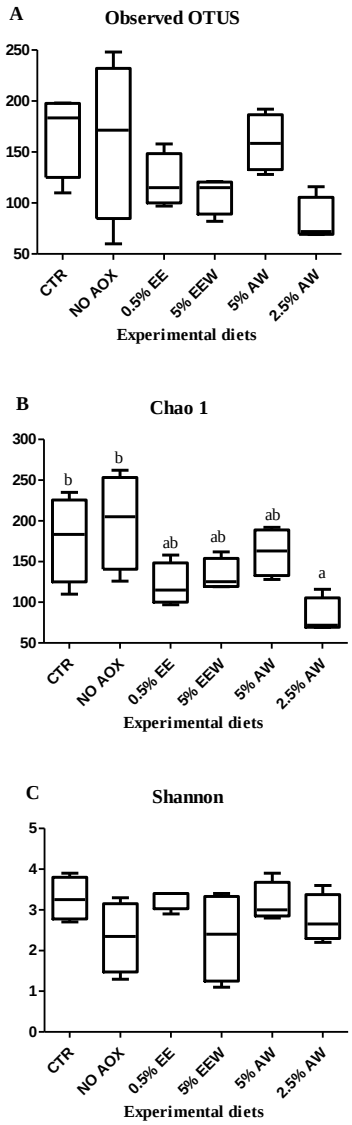

Figure 13 Alpha diversity metrics of autochthonous intestinal bacterial communities of gilthead seabream fed the experimental diets.

A) Observed OTUs; **B**) Chao 1 index; and **C**) Shannon-Wiener index (Shannon). Boxes represents mean±SEM (n = 4). Diets: *i*) a diet with a commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), vi) a NO-AOX diet with 2.5% of agar waste (2.5% AW). Different superscript letters indicate differences among the experimental diets (one-way ANOVA, $P<0.05$).

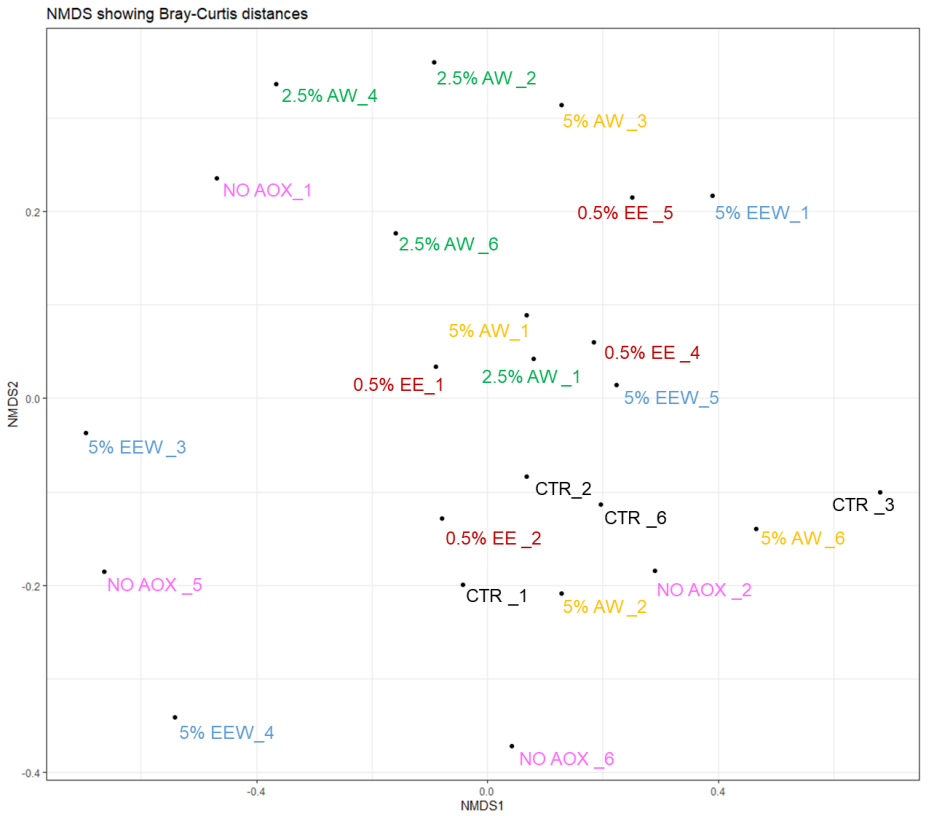

Figure 14 NMDS ordination based on Bray–Curtis distances of bacterial OTUs found in autochthonous intestinal bacterial communities of gilthead seabream fed the experimental diets

Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

3.3.3. Digestive enzymes activities

Intestinal digestive enzyme activities of gilthead seabream after 52 days of feeding the different diets are presented in Fig. 15. Results showed that seabream displayed a 2-time increase in trypsin activity when fed the 5% AW diet (25.84 mU mg^{-1} protein), compared to those fed EE (13.50 mU mg^{-1} protein) (*P*=0.003, Fig. 15A). On the other hand, trypsin activity did not differ among CTR, NO-AOX, EEW, or 2.5% AW groups. No significant differences were observed for intestinal chymotrypsin (*P*=0.622, Fig. 15B), lipase (*P*=0.065, Fig. 15C) or amylase (*P*=0.788, Fig. 15D) activities.

The activity ratio of trypsin to chymotrypsin (T/C *ratio*), used as an indicator of digestive efficiency and growth, was not different among the dietary groups, *P*> 0.05 (Table 11).

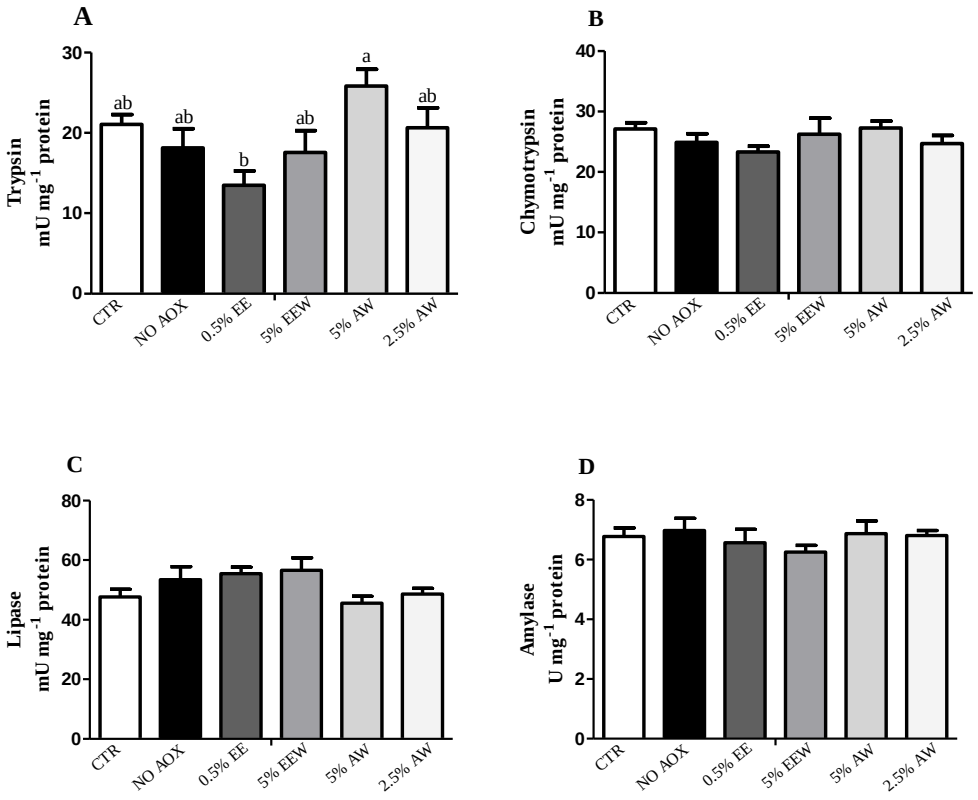

Figure 15 Intestinal digestive enzyme activities of gilthead seabream 24h after being fed the experimental diets.

A) Trypsin; **B**) chymotrypsin; **C**) lipase; and **D**) amylase activities. Columns represents mean+SEM (n = 8-15). Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). The absence of letters indicates

no differences among the experimental diets (one-way ANOVA, $P>0.05$). Different superscript letters indicate differences among the experimental diets (one-way ANOVA, $P<0.05$).

Table 11 Activity ratio of trypsin to chymotrypsin (T/C) of gilthead seabream.
Fish fed six diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). Values represent mean±SEM (n = 8-15). The absence of letters indicates no significant differences among the experimental diets (one-way ANOVA, $P<0.05$).

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	CTR	NO-AOX	0.5% EE	5% EEW	5% AW	2.5% AW
T/C ratio	1.72±0.14	1.50±0.18	1.37±0.11	1.47±0.20	1.64±0.13	1.45±0.11

3.3.4. Plasma metabolite levels

The effects of the experimental diets on several metabolic parameters in plasma of gilthead seabream are presented in Fig. 16. No significant differences in the cholesterol (P=0.778, Fig. 16A), triglycerides (P=0.935, Fig. 16B), glucose (P=0.141, Fig. 16C), or lactate levels (P=0.724, Fig. 16D) were shown between dietary groups.

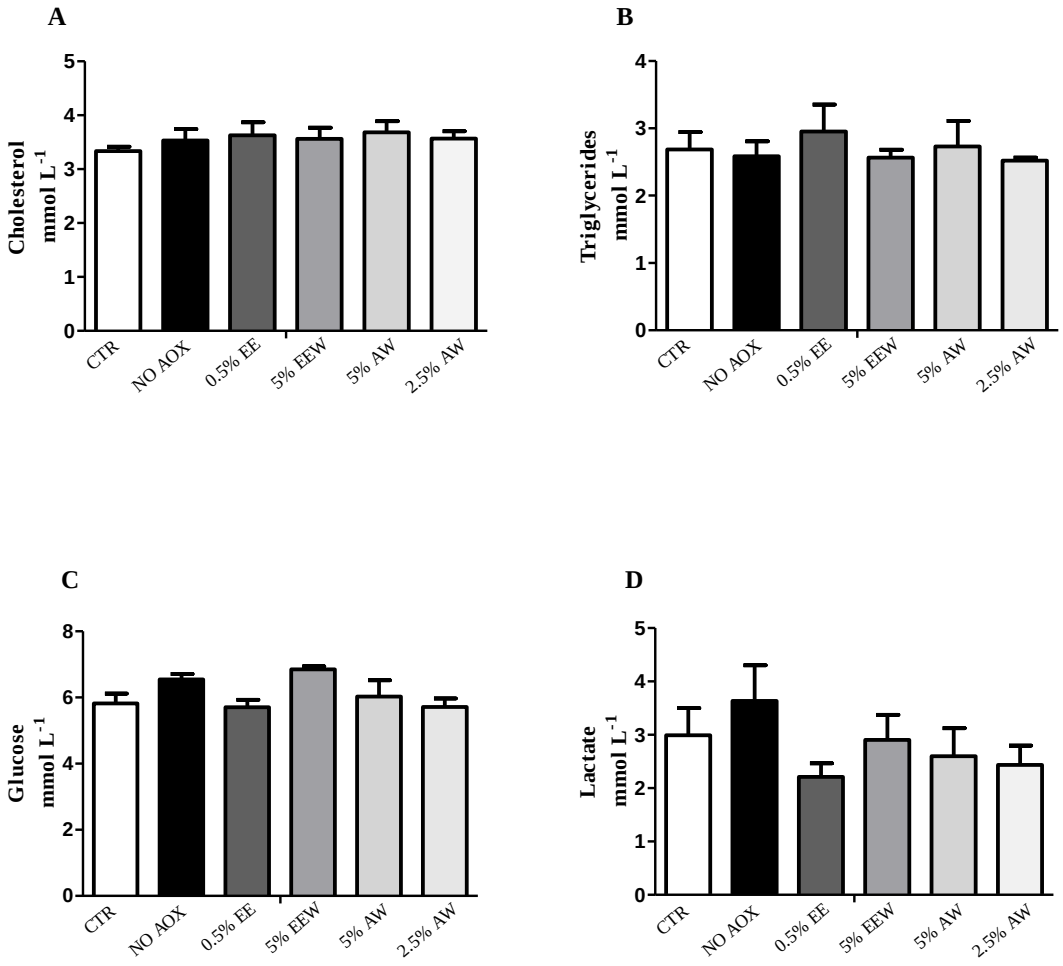

Figure 16 Plasma parameters of gilthead seabream 24h after being fed the experimental diets. **A**) cholesterol; **B**) triglycerides; **C**) glucose; and **D**) lactate levels. Columns represents mean+SEM (n=8-15). Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). The absence of letters indicates no differences among the experimental diets (one-way ANOVA, $P>0.05$).

3.3.5. Fillet colour

The outcome of the RM-ANOVA analysis on the effects of the diets (D) and storage (S) and their interaction (D*S), on the colour parameters lightness (L*), redness (a*), and yellowness (b*) of gilthead seabream fillet is presented in Table 12. The results showed that the diets had no significant effect on the L* of the gilthead seabream fillets ($P>0.05$). The a* measured in the fillets was similar between diets ($P>0.05$). In contrast, the b* was

higher in fish fed 5% AW compared to those fed the NO-AOX diet (*P*=0.003). No significant D*S interactions were detected (*P*>0.05). The L* decreased after 3 (*P*=0.017) and 6 (*P*=0.025) days of storage compared to the time 0. In contrast, the a* significantly increased after 3 and 6 days of storage compared to the initial values (time 0). On the other hand, b* was not significantly affected by the storage period (*P*=0.269).

Table 12 Fillet colour parameters of gilthead seabream.

Fish fed six diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). The colour parameters were measured at the beginning (time 0), and after 3 and 6 days of storage at 4 ºC.

	Colour parameters		
	L*	**a***	**b***
Diets			
CTR	49.72±0.91	-3.60±0.08	3.01±0.26^{ab}
NO-AOX	48.91±1.15	-3.61±0.11	2.09±0.34^{b}
0.5% EE	49.46±1.21	-3.62±0.11	2.78±0.34 ^{ab}
5% EEW	50.77±1.54	-3.51±0.11	2.62±0.36 ^{ab}
5% AW	48.96±1.21	-3.47±0.12	3.92±0.34^{a}
2.5% AW	50.29±1.21	-3.72±0.11	2.49±0.34 ^{ab}
Storage (days)			
0	52.73±0.29^{a}	-3.88±0.04^{a}	2.96±0.14
3	49.01±0.30^{c}	-3.15±0.05^{c}	2.65±0.14
6	47.31±0.31^{b}	-3.73±0.04^{b}	2.84±0.14
	Two-way ANOVA (*P* value)		
Diet (D)	0.842	0.671	**0.014**
Storage (S)	**<0.001**	**<0.001**	0.269
D*S	0.640	0.342	0.991

The independent effect of experimental diets and storage on L* (light/luminosity), a* (red/green), and b* (yellow/blue) are expressed as mean±SEM. The P-value of Two-way ANOVA is presented for diet (n=18), storage time (n=36), and interactions between both factors. Different letters represent significant differences between diets or storage time after two-way repeated-measures ANOVA analysis (*P*<0.05). The absence of letters indicates no significant differences (*P*>0.05).

3.3.6. Skin colour

The outcome of the RM-ANOVA analysis on the effects of the diets (D), storage (S) and their interaction (D × S), on the colour parameters lightness (L*), redness (a*), and yellowness (b*) of gilthead seabream skin is presented in Table 13. Results showed that both b* and a* remained similar between dietary treatments (P>0.05). On the other hand, the b* was higher in the skin of fish fed the 2.5% AW diet compared to those fed the 0.5% EE diet (P=0.003). However, no significant changes were detected in the b* among fish skin when fed the remaining diets (P>0.05). In addition, the diets had no significant effect on skin colour (L*, a*, and b* levels) during the storage at 4 ºC, as suggested by the absence of significant differences in the D × S interaction between both diet and storage (P>0.05). Both L* and a* increased significantly after 3 and 6 days of cold storage, when compared to values measured at time 0, regardless of the dietary treatment (P<0.05). Conversely, storage significantly decreased the b* value, particularly after 6 days, reaching the lowest value across of storage period (P<0.05). No statistical differences were noted between the initial measurement (time 0) and day 3 of storage for the b* value (P>0.05).

Table 13 Skin colour parameters of gilthead seabream.

Fish fed six diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

	Colour parameters		
	L*	**a***	**b***
Diets			
CTR	78.14±0.71	-5.56±0.12	9.77±0.35^{ab}
NO-AOX	78.35±0.89	-5.42±0.16	9.01±0.45 ^{ab}
0.5% EE	78.16±0.89	-5.64±0.16	8.36±0.50^{b}
5% EEW	77.65±1.09	-5.55±0.18	10.11±0.56 ^{ab}
5% AW	78.46±0.89	-5.24±0.15	9.63±0.47 ^{ab}
2.5% AW	78.53±1.11	-5.44±0.15	10.65±0.49^{a}
Storage (days)			
0	77.48±0.37^{b}	-6.00±0.09^{a}	10.56±0.19^{a}
3	81.88±0.35^{a}	-5.13±0.09^{b}	10.32±0.20^{a}
6	75.28±0.35^{c}	-5.29±0.10^{b}	7.88±0.20^{b}

	Two-way ANOVA (*P* value)		
Diet (D)	0.992	0.468	**0.032**
Storage (S)	**<0.001**	**<0.001**	**<0.001**
D*S	0.437	0.208	0.201

The independent effect of experimental diets and storage on L* (light/luminosity), a* (red/green), and b* (yellow/blue) are expressed as mean±SEM. The *P*-value of two-way ANOVA is presented for diet (n=18), storage time (n=36), and interactions between both factors. Different letters represent significant differences between diets or storage time after two-way repeated-measures ANOVA analysis ($P<0.05$). The absence of letters indicates no significant differences ($P>0.05$).

3.3.7. TBARS levels in fish fillet

The effect of the experimental diets on the lipid peroxidation (LPO) progression in the fillets of seabream during a period of storage is shown in Fig. 17. The two-way ANOVA analysis indicated no differences in LPO levels, regardless of the experimental diets and the storage time ($P=0.045$, Fig. 17A). In addition, the LPO was not affected by the diets, as shown by a lack of interactions between both factors (diet and time of storage) ($P=0.078$). On the other hand, the storage significantly increased 1.5-fold the LPO levels after 6 days of storage in sealed bags at 4 ºC stored ($P<0.001$, Fig. 17B)

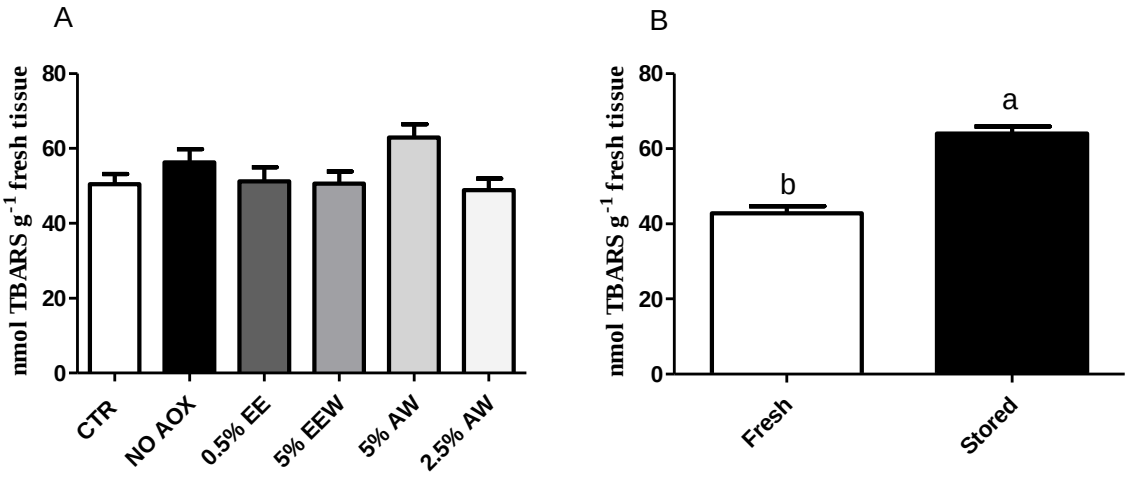

Figure 17 Lipid peroxidation levels in the fillet of gilthead seabream fed the experimental diets at the beginning (time 0), and after 6 days of storage at 4 ºC.

Columns are mean+SEM representing different dietary conditions independently of the storage time (**A**) (n=9-16), and storage time independently of the diet (**B**) (n=31-33). The absence of letters indicates no differences between diets *(P*>0.05), whereas different superscript letters indicate differences between storage time (two-way ANOVA, *P*<0.05). Fish fed six diets: *i*) a

commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW).

3.3.8. TBARS levels in fish diet

The lipid peroxidation levels in the different diets after 90 days of storage are presented in Fig. 18. Results indicated that diets supplemented with 0.5% EE (278.05 nmol TBARS g dry feed^{-1}) or 5% AW (272.06 nmol TBARS g dry feed^{-1}) presented significantly higher LPO levels compared to CTR (228.11 nmol TBARS g dry feed^{-1}). On the other hand, the LPO levels of NO-AOX (244.13 nmol TBARS g dry feed^{-1}), EEW (250.93 nmol TBARS g dry feed^{-1}), and 2.5% AW (242.08 nmol TBARS g dry feed^{-1}) diets were no significantly different from those observed in the CTR diet.

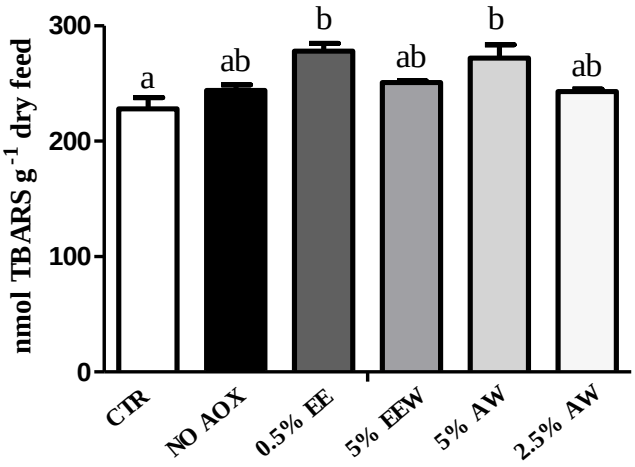

Figure 18 Lipid peroxidation levels in the diets after 90 days of storage at 25 ºC.
Columns represent the mean+SEM (n = 3-5). Diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). Different superscript letters indicate differences between diets (Tukey post-hoc test, $P<0.05$).

3.4. Discussion

This study investigated the effects of dietary supplementation with several products/ by-products (EE, EEW, and AW) obtained from the seaweed *Gracilaria gracilis* on the intestinal bacterial community, physiological, growth performance, and fillet colour characteristics in gilthead seabream.

The growth performance of gilthead seabream was not affected by the dietary supplementation of *G. gracilis* products/ by-products after 52 days of feeding. These results corroborate a previous study with gilthead seabream that described no significant differences in the growth performance when seabream was fed for 34 days at apparent satiation a diet supplemented with heat-treated *Gracilaria* at 5% (Magnoni et al. 2017). Similarly, in European seabass were observed no changes in growth performance by feeding fish with a supplemented diet with 5% AW for 80 days (Peixoto et al. 2019a).

To our best knowledge, the current study is the first attempt to evaluate, in gilthead seabream, the effect of dietary supplementation of *Gracilaria* ethanolic extracts and by-products (EEW and AW) in the intestinal bacterial community. A high-throughput sequencing approach targeting the 16S rRNA amplicon was successfully employed to characterize the intestinal bacterial communities in gilthead seabream. The results are in line with previous studies that reported Proteobacteria, Firmicutes, and Actinobacteria (in this order of abundance) as the most common phyla of intestinal bacteria in marine fish (Estruch et al. 2015; Butt et al. 2019; Rimoldi et al. 2020). Additionally, the diets utilized in the current study did not significantly change the intestinal bacterial structure, but the relative abundance of Firmicutes phylum increased in the 5% AW group. Firmicutes phylum includes several genera of lactic acid bacteria (LAB) like Streptococcus, Lactobacillus, and Leuconostoc. LAB are widely used as probiotics for fish and for other vertebrates due to their abilities to improve digestive function, immune response, disease resistance, and oxidative balance, among others (Ringø et al. 2018b; Vieco-Saiz et al. 2019; Mathur et al. 2020). Taking this into account, it is widely accepted that an increase in the abundance of LAB is desirable, as they may modulate the intestinal functions toward a healthier condition (Gatesoupe 2008). Previous studies using mannan-oligosaccharides additives reported an increase in LAB number in the intestinal bacterial community of Nile tilapia (*O. niloticus*) (Gonçalves et al. 2017), and rainbow trout (*O. mykiss*) (Levy-Pereira et al. 2018). However, the abundance of *Bacillus*, *Lactococcus*, and *Streptococcus* did not change in response to the dietary administration of EE, EEW,

and AW. Such result was not expected, especially for fish-fed diet EEW and AW, as these by-products may be potentially abundant in dietary fibre, a known substrate for LAB (Endo et al. 2011). In contrast, the *Clostridium* genera, also belonging to Firmicutes phylum, was higher in the intestine of fish fed 5% AW, especially in comparison to fish fed the 5% EEW. The *Clostridium* genus can produce butyrate that has been shown to have multiple beneficial effects in the animal intestine by improving intestinal function (Pryde et al. 2002). Additionally, butyrate possesses antimicrobial activity and has been used as feed additives to decrease the cumulative mortality in infected gilthead seabream (Piazzon et al. 2017). Since the butyrate is formed by intestinal fermentation of undigested dietary carbohydrates, such as resistant starch and dietary fibres (Luo et al. 2017; Bedford et al. 2018), it is plausible to infer that the increase of *Clostridium* bacteria, abundance in the seabream intestine measured in this study, could be linked to a higher dietary carbohydrate content in fish fed the AW supplemented diet. This observation is supported by previous studies relating the increase of *Clostridiales* members in the intestinal lumen of fish-fed plant-based supplements (Rimoldi et al. 2020).

Despite the differences found in the relative abundance of phylum Firmicutes, no major differences were detected in the richness and diversity of the intestinal bacterial communities in gilthead seabream fed the experimental diets, indicating that 5% *G. gracilis* by-products supplementation did not impact the intestinal bacterial composition. The results obtained in the current study corroborate a previous study that reported no changes on intestinal bacterial diversity of gilthead seabream fed 5% raw *Gracilaria cornea* supplemented diets, which is comparable to the level tested in the current study (Rico et al. 2016). However, the same authors showed that dietary supplementation with 15% raw *Gracilaria cornea* increased the intestinal bacterial community diversity, suggesting that higher levels of supplementation than those used in the present work (5%) are required to detect a dietary modulatory effect on the intestinal bacterial community diversity of gilthead seabream.

Post-prandial plasma metabolite levels are linked to the digestive process and the macronutrients and total energy content of the diets (Hamre et al. 2004; Du et al. 2005). Dietary supplementation of SW or their by-products is described by their rich content in mannan-oligosaccharides and other polysaccharides (Cheong et al. 2018; Saleh 2020). A previous study showed that in rainbow trout-fed diets supplemented with mannan-oligosaccharides (0.6%) up to 30 days the plasma triglycerides and glucose levels displayed variable responses in fish 24h after being fed (Gonçalves and Gallardo-

Escárate, 2017). In contrast, in this study, the dietary supplementation of SW by-products in gilthead seabream diets did not result in changes in plasma metabolites, such as glucose, triglycerides, and cholesterol 24h after being fed, indicating no variations in the digestibility of complex carbohydrates and lipids. These results were supported by the lack of differences detected in the intestinal amylase and lipase activities in seabream fed these different experimental diets, as these enzymes are important for starch and lipid digestion, respectively (Moreau et al. 2001; Kurtovic et al. 2009). Previously, Peixoto et al. (2016a) reported that the dietary supplementation of 5% of raw *Alaria* sp. is reflected in higher plasma lactate levels and an increased maximum metabolic rate in meagre (*Argyrosomus regius*). In contrast, the same study has shown that neither plasma glucose, cholesterol, triglycerides, and lactate levels nor the metabolic rate is changed in this species after being fed a diet supplemented with raw *Gracilaria sp.* at 5%. The study in meagre and the results presented here in seabream show that dietary supplementation with raw *Gracilaria sp.* or by-products do not alter the transport of nutrients nor the growth performance of both species.

In the current study, trypsin activity was significantly higher in fish fed 5% AW compared to those fed 0.5% EE diet. Intestinal trypsin activity has been positively associated with better growth performance in fish (Rungruangsak-Torrissen et al. 2006), but in the present study, no clear relation between the increase of trypsin activity and fish growth was observed. This may be linked as well to the lack of differences in the trypsin/chymotrypsin (T/C) ratio observed in the current study. The differences found in the trypsin activity between fish-fed 5% AW and 0.5% EE diets may suggest changes in the protein profile and/or the content of other components present in both diets. However, this study showed that both growth performance and final body composition remain similar in fish fed the different experimental diets after 52 days. Also, the lack of differences found in plasma metabolites after 24h of feeding suggests no differences in the levels of macronutrients transported in plasma. These results along with the lack of large differences in the bacterial community between the diets suggest that dietary supplementation with *G. gracilis* by-products does not have a large impact on the digestive process or fish performance under the conditions tested.

Colour measurement was undertaken in the skin of seabream as it is the first quality attribute of food evaluated by consumers, and is, therefore, an important component of food quality relevant to market acceptance (Hernández et al. 2009; Ünal Şengör et al. 2018). In the current study, the b* value in the skin of fish fed 2.5% AW diet was higher

than in fish fed the 0.5% EE diet. The difference detected could be important regarding the human perception as suggested earlier by Peixoto et al. (2019b). Particularly, an increase in the b* value represents a golden seabream skin which is preferable from the point of view of consumers (Gomes et al. 2002). This confirms previous results from Peixoto et al. (2019b) that observed, after 7 days at cold storage, an increase of skin b* value in European seabass fed a diet with a residue from *Gracilaria* methanolic extracts. This result may be related to a higher carotenoid deposition in the skin of fish (Gomes et al. 2002). As de novo synthesis of carotenoids does not take place in fish tissues (Hekimoglu et al. 2017), pigment deposition could be linked to dietary input. Conversely, fish fed 0.5% EE had the lowest b* value suggesting a lower pigment deposition. It is plausible to associate the lowest b* value to the higher LPO levels detected in the diet (0.5% EE). It is reasonable to infer pigments and other antioxidant compounds in 0.5% EE may have been oxidized during the period that the experimental diet was stored. The skin colour tended to decrease over time, regardless of the dietary treatment, and it is in agreement with Álvarez et al. (2012).

In the current study, TBARS levels did not vary in the fillet regardless of the dietary treatment, suggesting that EE, EEW, or AW had no significant impact on the lipid peroxidation process during cold storage. Similarly, Álvarez et al. (2012) showed that dietary rosemary (carvacrol) supplementation at 500 mg kg^{-1} diet had no impact on the LPO levels of gilthead seabream fillet stored at 4 ºC for 7 days. On the other hand, in the same study, LPO levels in the fillet decreased after 14 days of storage at 4 ºC in the fish-fed carvacrol supplemented diet (Álvarez et al. 2012). In our study, it seems that the storage duration was not long enough to detect an effect of dietary supplementation on fillet preservation, which may also relate to a lack of differences in LPO levels in fillets between fish-fed CTR and NO-AOX diets.

The current study showed that the LPO levels in fish fillet after 6 days storage at 4 ºC (~63 nmol g^{-1}) were within the typical range reported in chilled fish (69 and 110 nmol g^{-1}) (Secci et al. 2016). In addition, the increase of LPO levels during cold storage was expected since low temperatures decrease microbial growth but do not stop lipid oxidation (Payap 2011). Similar results have been reported for gilthead seabream (Tsironi et al. 2017), as well as for others species such as bream (*Megalobrama amblycephala*) (Song et al. 2011) and rainbow trout (*Oncorhynchus mykiss*) (Yildiz et al. 2006).

The increase of LPO levels detected during cold storage may be inversely related to both L* and a* values measured in the gilthead seabream fillet across the period that fish were

stored at 4 ºC. Earlier studies carried out in fillets of Nile Perch, *Lates niloticus*, (Demirbas et al. 2017) and anchovy marinade, *Engraulis encrasicolus*, (Turan et al. 2017) indicated the same relation between LPO and * and a* values during a storage 4 ºC. The decrease in a* value has been considered as one of the most important colour parameters to evaluate meat oxidation as indicates the transformation of oxymyoglobin (bright red colour) into brown metmyoglobin (Wetterskog et al. 2004; Chaijan et al. 2011).

An increase of b* value in the fillet of stored fish has been associated with increased levels of lipid oxidation products and free amino groups in proteins (Hamre et al. 2003; Sriket et al. 2018). However, in the current study, no changes were observed in the b* value of gilthead seabream fillet stored at 4 ºC for 6 days. This contrasts with previous works that reported an increase in b* value in fillet cold-stored for 8 days (Buchanan et al. 2008) and 18 days (Ünal Şengör et al. 2018), suggesting that the longer the storage period the higher the b* value in the fish fillet.

3.5. Conclusions

The results presented in the current study provide some evidence of the potential application of AW and EEW as a novel dietary supplement to be included in future aquaculture feed formulations. The 5% AW diet increase the relative abundance of *Clostridium* genera in the fish intestine, which may positively impact fish health in the long term. Despite the differences at the genera level, no major differences were demonstrated in the bacterial community diversity. The lack of negative effects on intestinal bacterial community diversity together with the absence of differences on fish growth performance, as shown in our study, can be considered desired features. In addition, fish fed the 2.5% AW and 5% EEW displayed the highest yellowness value (b*) for the skin colour of all fish, which could be relevant to market acceptance, although it was not significantly different from fish fed the CTR diet. On the other hand, AW and EEW had no impacts on lipid peroxidation progress during cold storage of fish fillet, at least during a short period of storage (6 days).

Further studies using EEW and AW at higher levels than those used in the present study may contribute to elucidate if by-products from *G. gracilis* may delay lipid peroxidation progression in chilled fish for a longer period of storage than that used in the present study.

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CHAPTER 4

Dietary supplementation with *Gracilaria gracilis* by-products modulates stress response, antioxidant and immune systems of gilthead seabream (*Sparus aurata*) exposed to crowding

Francisca Silva-Brito[1,2]; Francisco A. Guardiola[1,3]; Thais Cavalheri[1]; Rui Pereira[4]; Helena Abreu[4]; Anake Kijjoa[2]; Leonardo Magnoni[1]

[1]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208, Matosinhos, Portugal.
[2] Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Rua de Jorge Viterbo Ferreira, 225, 4050-313, Porto, Portugal.
[3] Department of Cellular Biology and Histology, Faculty of Biology, University of Murcia, 30100, Murcia, Spain. *Campus Regional de Excelencia Internacional* "*Campus Mare Nostrum*".
[4] ALGAplus - Produção e Comercialização de Algas e seus Derivados, Rua do Mar s/n, 3830-165, Ílhavo, Portugal.

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Dietary supplementation with *Gracilaria* sp. by-products modulates stress response, antioxidant and immune systems of gilthead seabream (*Sparus aurata*) exposed to crowding

Francisca Silva-Brito[1,2] · Francisco A. Guardiola[1,3] · Thaís Cavalheri[1] · Rui Pereira[4] · Helena Abreu[4] · Anake Kijjoa[2] · Leonardo Magnoni[1]

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ABSTRACT

By-products obtained from seaweed processing appear as a promissory source of bioactive compounds that could be included in aquafeed to improve the sustainability of aquaculture. Therefore, the present study aimed to evaluate the effects of dietary supplementation with by-products obtained from the seaweed *Gracilaria gracilis* on the stress response, antioxidant and immune systems of gilthead seabream (*Sparus aurata*) exposed to an acute crowding event. For this, fish were fed six experimental diets: *i)* a diet containing a commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% agar waste (2.5% AW) for 59 days. After this period, fish were exposed to 1 h of crowding (100 kg m^{-3}) and sampled immediately after 0 and at 24h post-crowding. Our results showed that the fish-fed AW and EEW diets showed lower plasma cortisol levels than those fed the CTR diet, suggesting an improved stress response to crowding on these groups. No differences were detected in hepatic lipid peroxidation levels between dietary groups after crowding. However, the fish-fed 0.5% EE diet showed lower hepatic glutathione reductase (GR) activity than fish fed the NO-AOX diet. Additionally, glutathione peroxidase (GPx) activity was decreased in the liver of fish fed 2.5% and 5% AW diets compared to the other dietary groups. Concerning immune parameters, the plasma peroxidase activity was higher in fish fed the 2.5% AW diet than fish fed NO-AOX or 5% EEW diets, suggesting a modulatory effect of dietary AW at 2.5% in the humoral immune response in crowded fish. Results suggest that dietary supplementation with AW, particularly when supplemented at 2.5%, could be used as a dietary tool to mitigate oxidative stress when improving immune response in gilthead seabream exposed to a stressor as crowding.

Keywords: *Gracilaria gracilis*; by-products; stress response; antioxidant compounds; immune system; gilthead seabream (*Sparus aurata*).

4.1. Introduction

Aquaculture management involves numerous procedures such as weighing, grading, and transporting fish, including maintaining animals at high densities for variable periods. The physiological consequences of these conditions in fish (e.g. crowding) have been described by previous studies, showing the occurrence of stress response in several cultured species (Barton 2002; Ashley 2007; Tort 2011; Zebral et al. 2015). As is known, stressors induce changes, affecting several functions, and the physiological responses are frequently divided into primary, secondary, and tertiary phases. The primary phase involves cortisol and catecholamine secretion, triggering several responses such as a boost on hepatic glycogen hydrolysis and systemic release of glucose, as well as increases in both heart rate and cardiac output (Barton 2002). The secondary phase involves metabolic changes in the blood (e.g. haematocrit and haemoglobin) and plasma parameters levels (e.g. glucose and lactate). In some cases, the physiological responses may be insufficient to overcome the stressor, becoming detrimental to the welfare of fish. Thus, if the adaptative response to the stressor is insufficient, fish enters into a tertiary phase, displaying impairments in growth, reproduction, or coping with an additional challenge (Barton 2002; Tort 2011). In consequence, the stressors may induce oxidative stress by enhancing free radical formation and by triggering the levels of pro-oxidant compounds in the animals, overcoming their antioxidant defence system, and decreasing the immune response capacity (Colitti et al. 2019).

Seaweeds (SW) contain several bioactive compounds with recognized antioxidant and immune-stimulant properties in fish (Jiménez-Escrig et al. 2012; Gomez-Zavaglia et al. 2019) that could be used as a nutritional tool to decrease the adverse effects of stressors associated with aquaculture procedures (Palstra et al. 2018). In this line, recent studies have shown the beneficial effects of dietary supplementation of SW and their extracts in the gilthead seabream (*Sparus aurata*) (Magnoni et al. 2017) as well as several other cultured fish species (Peixoto et al. 2019a), intending to improve the physiological responses of animals facing challenging conditions. For instance, gilthead seabream fed diets supplemented with 5% of heat-treated *Gracilaria* sp. exhibited enhanced tolerance to an acute hypoxia event (Magnoni et al. 2017). In the same way, European seabass (*Dicentrarchus labrax*) fed supplemented diets with aqueous extract from *Gracilaria* sp. showed an improvement in the immune and antioxidant systems response' against bacterial infection with *Photobacterium* sp. (Peixoto et al. 2019a). More concretely, these

authors observed increases in lysozyme activity as well as of some antioxidant enzymes like catalase in the fish fed supplemented diets with aqueous extract from *Gracilaria* sp. in contrast with those fed the control diet (Peixoto et al. 2019a).

Despite this use, the successful implementation of this nutritional tool depends on sustainable practices to avoid overlapping in the emergent use of SW by several industries and applications. The by-products generated by the production of phycocolloid from seaweeds appear as a promissory source of bioactive compounds for several applications including supplementation in aquafeeds. Agar extraction generates approximately 1600 tonnes per year of by-products, known as agar waste (AW), and uses only 40%, which suggests its sub-utilization (Ennouali et al. 2006; Pei et al. 2013; Meinita et al. 2017).

The red seaweed *Gracilaria* sp. is the main raw material from which agar is extracted by several industries (Ramalingam et al. 2002; Rocha et al. 2019). *Gracilaria gracilis* is cultivated in Integrated Multitrophic Aquaculture (IMTA) systems in Portugal, which involves increased sustainability and diversification in the production (Costa-Pierce 2010). In these systems, the metabolic products excreted by the fish are used to improve SW biomass production under controlled conditions. *G. gracilis* is particularly appropriate for use in IMTA systems due to the high efficiency in removing inorganic nutrients which is important for bioremediation (Abreu et al. 2009; Abreu et al. 2011; Torres et al. 2019). In addition to agar extraction, *Gracilaria* sp. is used to obtain ethanolic extracts (EE) for several applications in pharmaceutical and cosmetic industries (Torres et al. 2019), generating as well a by-product, the ethanolic extraction waste (EEW) (Do et al. 2014; Meinita et al. 2017). Therefore, this study investigated the potential effects of a *G. gracilis* ethanolic extract and the by-product included in a fish diet. Therefore, the present study aimed to investigate the modulatory effects of dietary supplementation of *G. gracilis* by-products on gilthead seabream exposed to an acute crowding event. More specifically, we evaluated the fish welfare through several biomarkers related to oxidative stress, antioxidant and immune system in gilthead seabream which is an opportunistic feeder that consumes SW as part of its natural diet (Arias 1980; Pita et al. 2002). Moreover, gilthead seabream has become an important commercial species in aquaculture in Europe, more specifically in Portugal, where intensive production conditions are implemented.

4.2. Materials and Methods

4.2.1. *Gracilaria gracilis* processing

The SW *Gracilaria gracilis* was produced by ALGAplus Lda. [Ílhavo, Portugal (40.604547 -8.667092)]. After two weeks of cultivation, *G. gracilis* was harvested, cleaned under tap water, and dried at 25 ºC for 24h. Dried *G. gracilis* was stored in sealed bags and transported to the Biomedical Sciences Institute of Abel Salazar (ICBAS) of the University of Porto (Portugal). *G. gracilis* was cleaned one more time under tap water, dried at 65 ºC for 48h. SW was ground using a hammer mill (Retsch), sieved first with 4 mm, and later with 0.5 mm meshes before extraction.

The agar extraction was carried out by adding 1L of distilled water at 95 ºC to the ground *G. gracilis*. The agar extracted was filtrated using a three-ply cheesecloth (Kumar et al. 2009) and the agar waste (AW) generated on this process was dried at 25 ºC for 48h to be included in the fish diets.

The ethanolic extraction was performed with ground *G. gracilis* and ethanol 96% in a 1:20 ratio. *G. gracilis* extraction took place at 25 ºC under continuous mixing (1500 rpm) for 3h in the dark, and the remaining *G. gracilis* was re-extracted and combined with the first fraction. The supernatant was filtered through Whatman No.1 filter paper and then evaporated in a rotary evaporator under reduced pressure at 50 ºC. The dry extract (EE) was stored in a sealed flask at 4 ºC in the dark. The waste obtained after the ethanolic extraction (EEW) was dried in the oven at 50 ºC for 48 h, ground, and sieved with a 0.5 mm mesh stored in a sealed flask at 4 ºC in the dark.

4.2.2. Experimental diets

Two different diets were manufactured by SPAROS Lda. (Olhão, Portugal), a diet containing a commercial antioxidant (0.2% Verdilox- Kemin) and a diet without antioxidant addition. Both diets were grounded below 500 µm in a hammer mill (Retsch GmbH, Haan, Germany) to prepare six experimental diets: *i*) a diet containing a commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% agar waste (2.5% AW).

Each experimental diet was mixed accordingly to the specified proportion in a paddle mixer (Alexanderwerk Inc.). For the EEW and AW diets, the mixture was humidified

with the addition of water (25%). For the preparation of the EE diet, the extract was dissolved into 96% ethanol and pulverized in the ground diet. All the experimental diets, including both CTR and NO-AOX, were dry-pelleted in a laboratory pellet mill through a 5.0 mm die (California Pellet Mill, CPM Crawfordsville, IN, USA). Compositional analyses of the experimental diets were made in duplicates. Dry matter content in the diets was quantified after heating at 105 °C for 24h in an oven, ash after combustion in a muffle furnace (550 °C for 12h), crude protein (Micro-Kjeldahl, Knigswinter, Germany N × 6.25) after acid digestion and lipid content by petroleum ether extraction (at Soxhlet 40-60 ºC). Ingredients and proximate composition of the experimental diets are presented in Table 14.

Table 14 Ingredients and proximate composition of six experimental diets.
i) a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% agar waste (2.5% AW).

Ingredients (%)	**CTR**	**NO-AOX**	**0.5% EE**	**5% EEW**	**2.5% AW**	**5% AW**
Fishmeal LT70	10.5	10.5	10.4	10.0	10.2	10.0
Fishmeal 60	15.0	15.0	14.9	14.3	14.6	14.3
Soy protein concentrate	12.0	12.0	11.9	11.4	11.7	11.4
Wheat gluten	6.0	6.0	6.0	5.7	5.9	5.7
Corn gluten	10.0	10.0	10.0	9.5	9.8	9.5
Soybean meal 48	12.0	12.0	11.9	11.4	11.7	11.4
Rapeseed meal	5.0	5.0	5.0	4.8	4.9	4.8
Wheat meal	12.7	12.9	12.8	12.3	12.6	12.3
Fish oil	4.4	4.4	4.4	4.2	4.3	4.2
Rapeseed oil	10.2	10.2	10.2	9.7	10.0	9.7
Vit. & Min. Premix	1.0	1.0	1.0	1.0	1.0	1.0
Antioxidant (Verdilox - Kemin)	0.2	0.0	0.0	0.0	0.0	0.0
MCP	0.6	0.6	0.6	0.6	0.6	0.6
DL-Methionine	0.2	0.2	0.2	0.2	0.2	0.2
L-Taurine	0.2	0.2	0.2	0.2	0.2	0.2
Gracilaria gracilis extract	-	-	0.5	-	-	-
Gracilaria gracilis ethanolic waste	-	-	-	5.0	-	-
Gracilaria gracilis agar waste	-	-	-	-	2.5	5.0
Proximate composition (%DM)						

Dry matter	94.29±0.02	96.56±0.01	97.12±2.28	95.49±0.05	93.46±0.02	95.49±0.05
Crude protein	46.91±0.05	47.12±0.11	46.99±0.01	46.24±0.19	47.90±0.25	45.93±0.01
Crude fat	18.46±0.06	17.32±0.18	15.25±0.05	16.63±0.02	15.07±0.10	17.23±0.20
Ash	8.81±0.01	8.61±0.00	8.92±0.19	8.43±0.02	8.93±0.07	8.47±0.05

Proximate composition values calculated as mean±SEM of two analyses. No significant differences were detected in proximate composition between diets (One-way ANOVA, $P < 0.05$).

4.2.3. Fish and rearing conditions

This study was conducted under the supervision of accredited experts 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 CIIMAR. Experiments were designed to comply with the ARRIVE (*Animal Research*: *Reporting* of *In Vivo Experiments*) guidelines for reporting animal research. The procedures implemented in the study were no likely to cause fish suffering, being equivalent to established procedures (weighing, grading, and transporting) applied in the culture of this species.

Juveniles gilthead seabream of approximately 9 g of body weight was provided by MARESA (Huelva, Spain), and transported to CIIMAR facilities. Fish were kept in quarantine for 15 days, fed twice a day with a commercial diet (SPAROS, Portugal) at 2% body weight day^{-1} before starting the trial. One week before the trial, 240 fish (initial body weight 10.0±0.2 g) were randomly distributed in 20 tanks (87 L, 1.4 g L^{-1}) for acclimation to experimental conditions. Photoperiod regime was 14h:10h (day: night cycle) and the temperature (17.40±0.13 ºC), salinity (35±0.04 ‰), pH (7.68±0.07), and dissolved oxygen (above 95%) were fixed and monitored every day. All the experimental tanks were connected to a common water recirculation system (RAS) at a flow rate of 257 L h^{-1}. Water ammonium and nitrite levels in the tanks were maintained below the limits for the species (0.05 mg L^{-1} and 0.5 mg L^{-1}, respectively).

Each experimental diet was tested in triplicates except the CTR diet that was tested in 5 replicas. Each dietary treatment was randomly assigned to the experimental tanks. Fish were hand-fed two meals per day (9:00h and 16:00h) for 59 days to apparent satiety. Fish was fasted for 24h before applying the acute crowding protocol.

4.2.4. Acute crowding experiment

The crowding stress protocol followed the procedure described by Yaghobi et al. (2015) and Naderi et al. (2018) with some modifications. Three fish per tank fed the CTR diet were sampled (n=9) to be used as a control for crowding (uncrowded). The crowding stress was later applied in all the fish of all the tanks, one tank at the time, every 15 minutes. For this procedure, the water circulation for the tank was disconnected and all the fish (6) were transferred to a smaller tank (1.5 L) to keep a 100 kg m^{-3} density for 1h. After this period, 3 fish per tank were immediately sampled and the remaining 3 fish were transferred to their original tank to be sampled 24h later. Sampled fish were anesthetized with MS-222 (0.1 g L^{-1}), buffered with $NaHCO_3$ (0.2 g L^{-1}). Blood was collected from caudal region using heparinised syringes. Plasma was obtained by centrifugation at 5 000 *x g* for 5 min and stored at -80 ºC. Fish were euthanized and the liver was sampled and immediately frozen in liquid nitrogen and stored at -80 ºC for analysis.

4.2.5. Fish performance

The body weight of each fish was measured before and at the end of the trial. Growth performance was determined by calculation of daily growth index (DGI), specific growth rate (SGR), feed conversion ratio (FCR), and daily feed intake (DFI), which were calculated for each experimental condition as follow: DGI = (final body weight$^{1/3}$ - initial body weight $^{1/3}$) number of days^{-1}) × 100; SGR (%) = [(Ln final body weight - Ln initial body weight) number of days^{-1}] × 100; FCR = (total feed intake) weight gain^{-1}; DFI = total feed intake (number of fish × number of days)$^{-1}$. The tank was used as the experimental unit.

4.2.6. Markers of stress measured in plasma

An ELISA kit based on the competitive link between cortisol and related monoclonal antibodies was used to quantify cortisol levels in plasma (RE 52061, IBL International, Hamburg, Germany). Lactate concentration in plasma was quantified using a commercial kit (UV method, AK00131, NZYTech, Lisbon, Portugal). Glucose levels in the plasma were determined using a commercial kit (UV method, 1001200, Spinreact, Girona,

Spain). All measurements were carried out in triplicates, following the recommendations provided by the manufacturers.

4.2.7. Markers of oxidative stress in liver

Liver samples were homogenized in 0.1 M phosphate buffer (pH 7.4) in a ratio of 1:10. Protein concentration was assayed in homogenates using bovine serum albumin as a standard (Bradford 1976). Lipid peroxidation (LPO) was quantified by using thiobarbituric acid as a substrate (Ohkawa et al. 1979). Catalase (CAT, EC 1.11.1.6.) activity was quantified using hydrogen peroxide (30%) as a substrate (Claiborne 1985). Glutathione reductase (GR, EC1.8.1.7) and glutathione peroxidase (GPx, EC 1.11.1.9.) activities were measured based on NADPH (Sigma, Portugal) oxidation at 340 nm (Mohandas et al. 1984; Cribb et al. 1989). Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate (Habig et al. 1974). Total and oxidized glutathione levels (TG and GSSG) were measured by the concomitant reaction of the reduced glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB) and read at 412 nm (Baker et al. 1990). In the GSSG assay, 2-Vinyl-pyridine was used to remove all the free thiols present in the sample leaving only GSSG (Griffith 1980). Finally, the GSH level was calculated as the result of subtracting the amount of GSSG to the TG. SOD was quantified using a commercial kit (19160-1KT, Merk, Germany), following the manufacture's instructions. Changes in absorption were measured at 25°C in a Power-Wave™ microplate spectrophotometer (BioTek Instruments, Inc., USA), and reactions were performed in triplicates.

4.2.8. Immune-related parameters measured in plasma

4.2.8.1. Peroxidase activity

The peroxidase activity in plasma was quantified according to Quade et al. (1997) with some modifications. Briefly, plasma was diluted with Hank's balanced salt solution (HBSS) without Ca^{+2} or Mg^{+2} in a proportion of 1:10. Then, TMB (20 mM) and H_2O_2 (5 mM) were added as a subtract. The reaction was stopped after 2 min by adding H_2SO_4 (2 M). The OD was read at 450 nm in a microplate reader (BioTek Instruments, Inc., USA).

Standard samples without plasma were used as blanks. One unit of activity was defined as a change of 1 unit of absorbance and expressed per mL^{-1} of plasma.

4.2.8.2. Protease activity

Protease activity in plasma was measured according to Guardiola et al. (2016) with some modifications. Samples were diluted three times with phosphate-buffered saline (PBS) and 125 µL of 2% azocasein (Sigma-Aldrich was added and incubated for 19 h at room temperature. The reaction was stopped by adding 10% trichloroacetic acid (TCA) and the mixture was incubated for 30 min at room temperature and centrifuged (10 000 *x g*, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µL $well^{-1}$ of 1 N NaOH. The OD was read at 450 nm using a microplate reader (BioTek Instruments, Inc., USA). The sample was replaced by trypsin (5 mg mL^{-1}, Sigma) in reference samples (100% of protease activity) or by buffer in blanks (0% activity).

4.2.8.3. Antiprotease activity

Total antiprotease activity was determined measured according to Guardiola et al. (2016) with some modifications. Briefly, aliquots of 30 µL of plasma were incubated for 10 min at 22 ºC with 10 µL of standard trypsin solution (5 mg mL^{-1}), and then 80 µL of PBS was added along with azocasein (Sigma-Aldrich) and incubated for 1h at room temperature. The reaction was stopped by adding 10% TCA. The mixture was incubated for 30 min at room temperature and centrifuged (10 000 *x g*, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µL $well^{-1}$ of 1 N NaOH. The OD was read at 450 nm using a microplate reader (BioTek Instruments, Inc., USA). The plasma was replaced by buffer in positive controls (100% protease), and buffer replaced both plasma and trypsin in negative controls (0% protease). The percentage of inhibition of trypsin activity for each sample was calculated.

4.2.8.4. Haemolytic complement activity

Natural haemolytic complement activity was evaluated as previously described by Sunyer et al. (1995) with some modifications. Three buffers were prepared: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatine; EDTA-GVB, as the previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{2+} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were washed four times in GVB and resuspended in the same buffer to a concentration of 1.93×10^8 cells mL^{-1}. Then, 10 µL of RaRBC suspension was added to 40 µL of serially diluted plasma in Mg-EGTA-GVB buffer in duplicates. The samples were then incubated for 120 min at room temperature with continuous shaking. After this period, 150 µL the cold EDTA-GVB was added to stop the reaction. Finally, samples were centrifuged for 2.5 min at 120 × *g* and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a microplate reader (BioTek Instruments, Inc., USA). The ACH_{50} units were defined as the concentration of plasma inducing 50% haemolysis of RaRBC.

4.2.9. Statistical analysis

All measurements were performed in three replicates, and the results are expressed as mean±standard error of the mean (SEM). Statistical analysis was performed using Sigma Stat 3.1. Data were checked for normality and homogeneity of variances (Shapiro-Wilk test and Brown-Forsyth test, respectively). A t-test analysis was performed to compare crowded and uncrowded fish fed the CTR diet at each time (0h or 24h) as well as to compare 0h and 24h within each group (crowded and uncrowded). The differences between experimental diets among crowding conditions were analysed by performing a one-way ANOVA analysis. The Tukey test was used to identify pairwise differences. A t-test analysis was used to test for differences over time for each experimental diet. Kruskal-Wallis factor ANOVA and Mann Whitney tests were used if the requirement for parametric statistics were not met. Significant differences were considered when $P<0.05$.

4.3. RESULTS

4.3.1. Growth performance

The one-way ANOVA results regarding the fish performance parameters of juvenile gilthead seabream fed the different diets for 59 days are presented in Table 15. All the dietary groups showed a similar final body weight after the feeding trial (P = 0.089). DFI index did not show variations between dietary groups (P=1.96).

Table 15 Zootechnical parameters in gilthead seabream.
Fish were fed for 59 days six experimental diets: *i)* a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% agar waste (2.5% AW).

Parameters	Experimental diets					
	CTR	NO-AOX	0.5% EE	5% EEW	5% AW	2.5% AW
IBW	10.12±0.08	10.11±0.07	10.14±0.20	9.70±0.17	10.10±0.12	10.26±0.13
FBW	25.67±0.10	24.56±1.37	25.06±1.94	21.56±1.82	24.17±0.42	27.83±0.39
DFI	0.31±0.01	0.33±0.02	0.29±0.02	0.32±0.03	0.31±0.02	0.32±0.02

IBW, Initial body weight; FBW, Final body weight; DFI, Daily feed intake. Values are the mean±SEM of parameters calculated in triplicate tanks, except for CTR (five tanks). No significant differences were detected between groups (One-way ANOVA, P <0.05).

4.3.2. Response to crowding

Figure 19 shows the cortisol (Fig. 19A), glucose (Fig. 19B) and lactate (Fig. 19C) levels in the plasma of fish fed the CTR diet and unexposed and exposed to crowding at 0 and 24h. The values of all markers of stress (cortisol, glucose, and lactate) were significantly increased in crowded fish compared to uncrowded fish at 0h. However, the values of these markers of stress decreased in plasma of crowded fish at 24h post-crowding, showing similar levels as in the uncrowded fish. On the other hand, all the markers analysed in the unexposed fish were unchanged across sampling times (0h and 24h). No mortality was registered during or after applying the crowding.

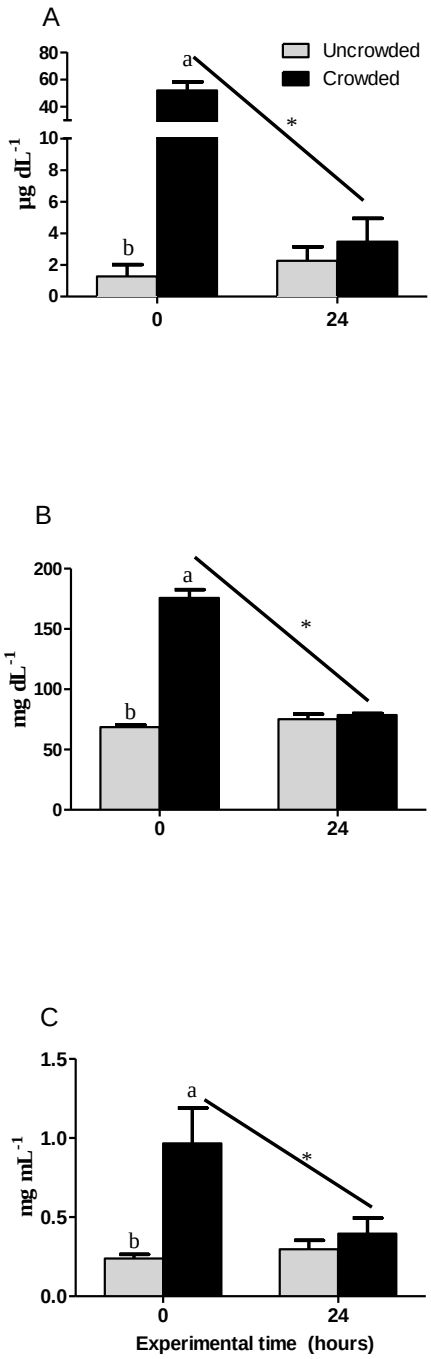

Figure 19 Metabolic and stress markers in plasma of gilthead seabream.

Fish fed a control diet at 0 and 24h after being exposed to crowding. A: cortisol (µg mL^{-1}); B: glucose (mg dL^{-1}); and C*:* lactate (mg mL^{-1}) levels. The bar represents the mean+SEM of each group ($n = 9$ fish). Different letters denote differences between uncrowded and crowded fish at each sampling time (T-test, $P<0.05$). The symbol * shows significant differences between sampling times for each dietary group (T-test, $P<0.05$).

4.3.3. Metabolic and stress-related markers in plasma

Fig. 20 presents the modulatory effect of experimental diets on cortisol (Fig. 20A), glucose (Fig. 20B), and lactate (Fig. 20C) levels in the plasma of seabream at 0h and 24h post-crowding. The effect of dietary treatments on the markers evaluated in the plasma of fish (cortisol, glucose, and lactate) increased at 0h after crowding. Results showed that the plasma cortisol levels in fish fed CTR diet were 1.7 times higher compared to fish fed EEW diet and 2 times higher compared to values found in the plasma of fish fed AW diet. In contrast, no variations were detected between 0.5% EE and other diets. Fish fed CTR diet also showed higher plasma glucose levels (~1.2 times) than other dietary groups, except for fish fed the 2.5% AW diet. On the other hand, no significant differences were detected in plasma lactate levels between dietary groups. Twenty-four hours after crowding, all the markers of stress returned to levels similar to those measured at 0h post-crowding. Additionally, at 24h post-crowding, all the markers of stress were no different from those detected in uncrowded fish that received the CTR diet.

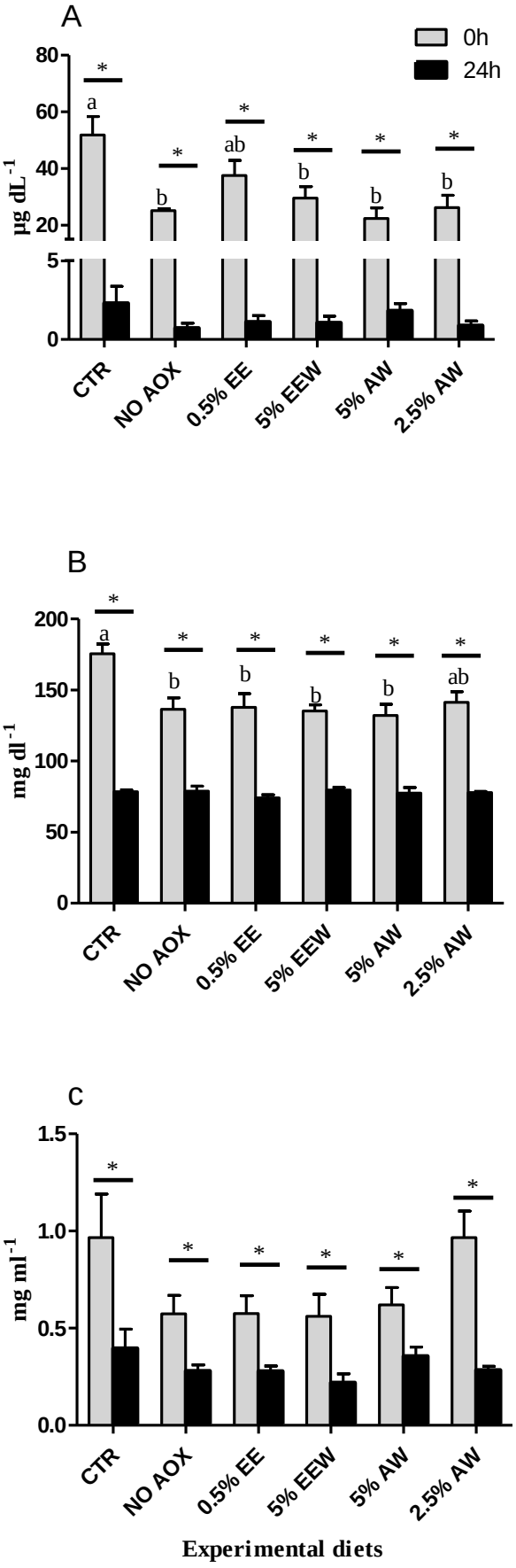
A
0h
24h
µg dL-1
80
60
40
20
5
0
a
b
ab
b
b
b
*
CTR
NO AOX
0.5% EE
5% EEW
5% AW
2.5% AW
B
mg dl-1
200
150
100
50
0
a
b
b
b
b
ab
C
mg ml-1
1.5
1.0
0.5
0.0
Experimental diets

Figure 20 Metabolic and stress markers in plasma of gilthead seabream.
Fish fed different diets, 0h, and 24h after being exposed to crowding. A: cortisol (µg mL^{-1}); B: glucose (mg dL^{-1}); and C: lactate (mg mL^{-1}) levels. Fish fed six experimental diets: *i)* a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% of agar waste (2.5% AW). Bars represent the mean+SEM of each group (n=9 fish). Different letters indicate differences between dietary groups for a given sampling time (Tukey test, $P<0.05$). The symbol * shows significant differences between sampling times for a given dietary group (T-test, $P<0.05$).

4.3.4. Oxidative stress markers in liver

The effect of experimental diets on lipid peroxidation (LPO) and several parameters of the antioxidant system in fish (SOD, CAT, GR, GPx, TG, GSSG, GSH, and GST) exposed to crowding are presented in Figs.21, 22, and 23. The antioxidant response was modulated by the experimental diets in response to acute crowding. In particular, the GPx activity was higher in fish fed 5% EEW diet than those fed 5% AW or 2.5% AW diets at 0h (Fig. 22A). Similarly, TG activity was higher in fish fed 2.5% AW diet than in the values observed in the liver of fish from the 5% EEW group (Fig. 23A). Also, the fish-fed NO-AOX diet presented a higher GR activity than in fish-fed the 0.5% EE diet (Fig. 22B). However, SOD, CAT, and GST activities (Fig. 21B, 21C, and 22C, respectively), as well as GSSG, GSH, and LPO levels in the liver of fish (Fig. 23B, 23C, and 21A, respectively) were similar among dietary groups at 0h.

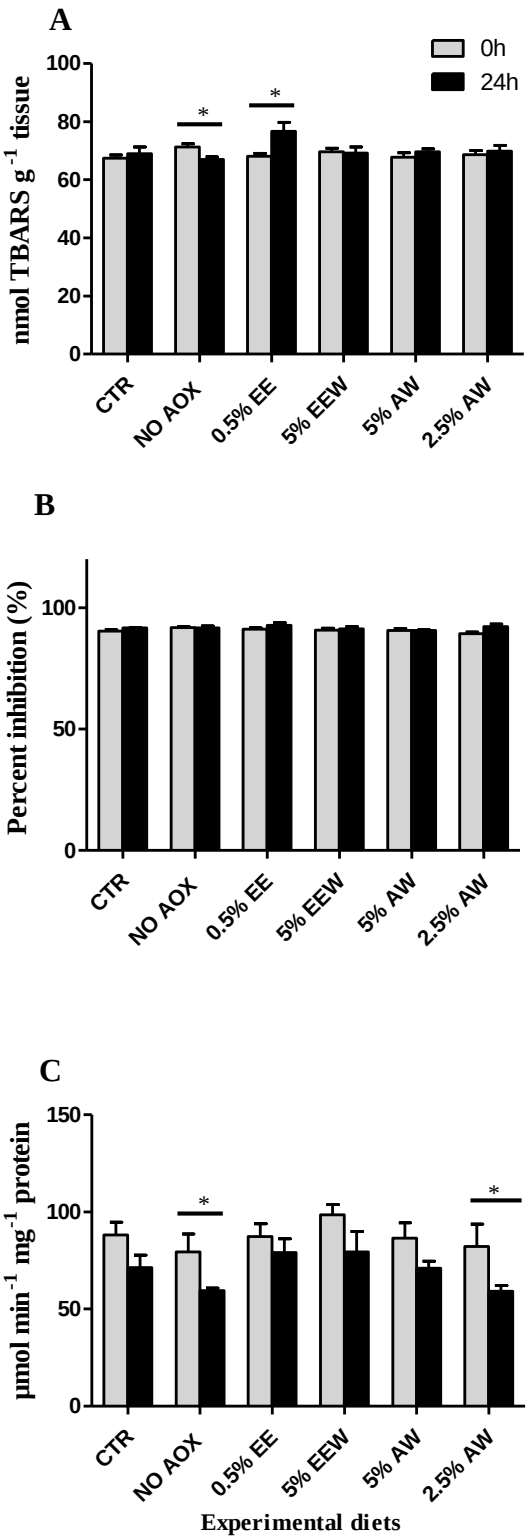

Figure 21 Lipid peroxidation level and some enzymatic antioxidant markers in the liver of gilthead seabream.

Fish fed different diets, 0h, and 24h after being exposed to crowding. A: Lipid peroxidation level (nmol TBARS g^{-1} tissue); B: superoxide dismutase (%); and C: catalase (µmol min^{-1} mg^{-1} protein). Fish fed six experimental diets: *i)* a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% of agar waste (2.5% AW). The bar represents the mean±SEM of each group (n = 9 fish). Different letters indicate differences between dietary groups for a given sampling time (Tukey test, P<0.05). The symbol * shows significant differences between sampling times for a given dietary group (T-test, P<0.05).

The current study suggests a modulatory effect of the diet on the antioxidant response 24h after crowding. In particular, the CAT activity in the liver of fish-fed NO-AOX or 2.5% AW diets was significantly lower at 24h compared to values registered at 0h in each dietary group (Fig. 21C). Similarly, hepatic GPx activity was lower in fish from CTR, NO-AOX, and 5% EEW groups at 24h with respect to values at 0h (Fig. 22A). At the end of the trial (24h), the fish fed a 0.5% EE diet showed an increase of hepatic GR activity in comparison to 0h post-crowding (Fig. 22B). On the opposite, fish fed 2.5% AW showed a decrease of hepatic GR activity at 24h post-crowding compared to 0h post-crowding (Fig. 22B). Additionally, the GSSG level in the liver of fish fed 0.5% EE diet was higher at 24h compared to fish sampled at 0h (Fig. 23B). Likewise, TG levels in the liver of fish fed 0.5% EE, 5% EEW, and 5% AW diets increased at 24h (Fig. 23A).

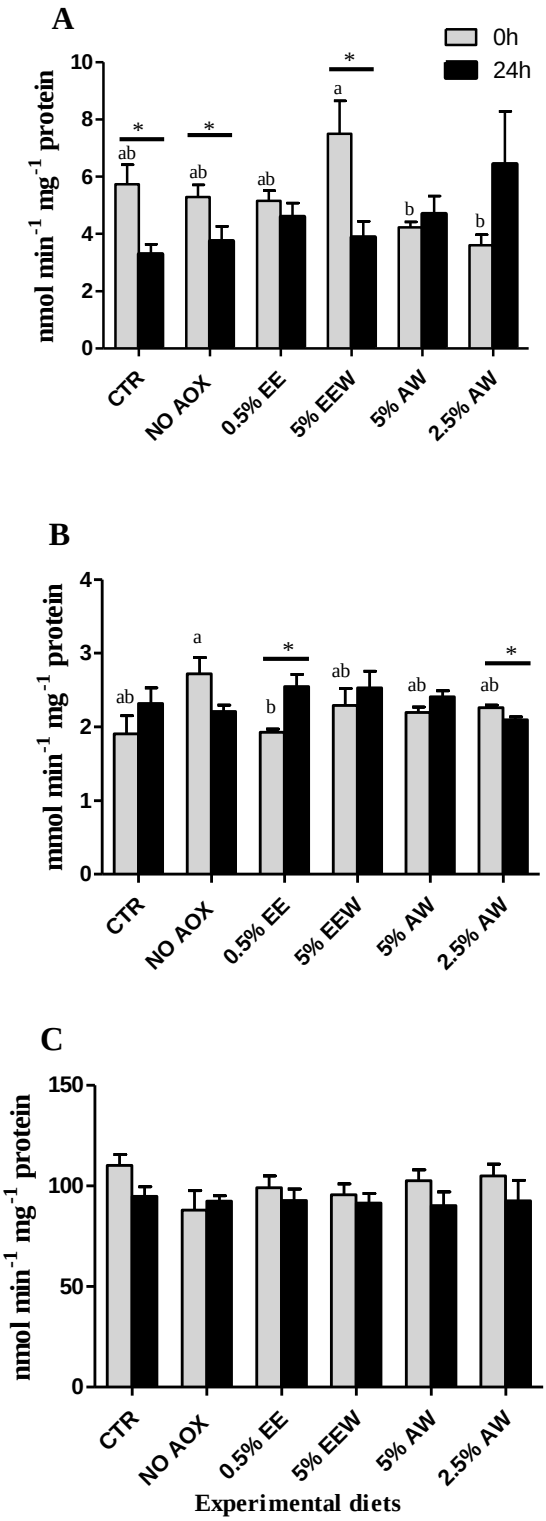

Figure 22 Glutathione system in the liver of gilthead seabream fed different diets, 0h, and 24h after being exposed to crowding.

A: glutathione peroxidase (nmol min^{-1} mg^{-1} protein); B: glutathione reductase (mmol min^{-1} mg^{-1} protein); and C: glutathione S-transferase (nmol min^{-1} mg^{-1} protein). Fish fed six experimental diets: *i)* a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% of agar waste (2.5% AW). The bar represents the mean+SEM of each group ($n = 9$ fish). Different letters indicate differences between dietary groups for a given sampling time (Tukey test, $P<0.05$). The symbol * shows significant differences between sampling times for a given dietary group (T-test, $P<0.05$).

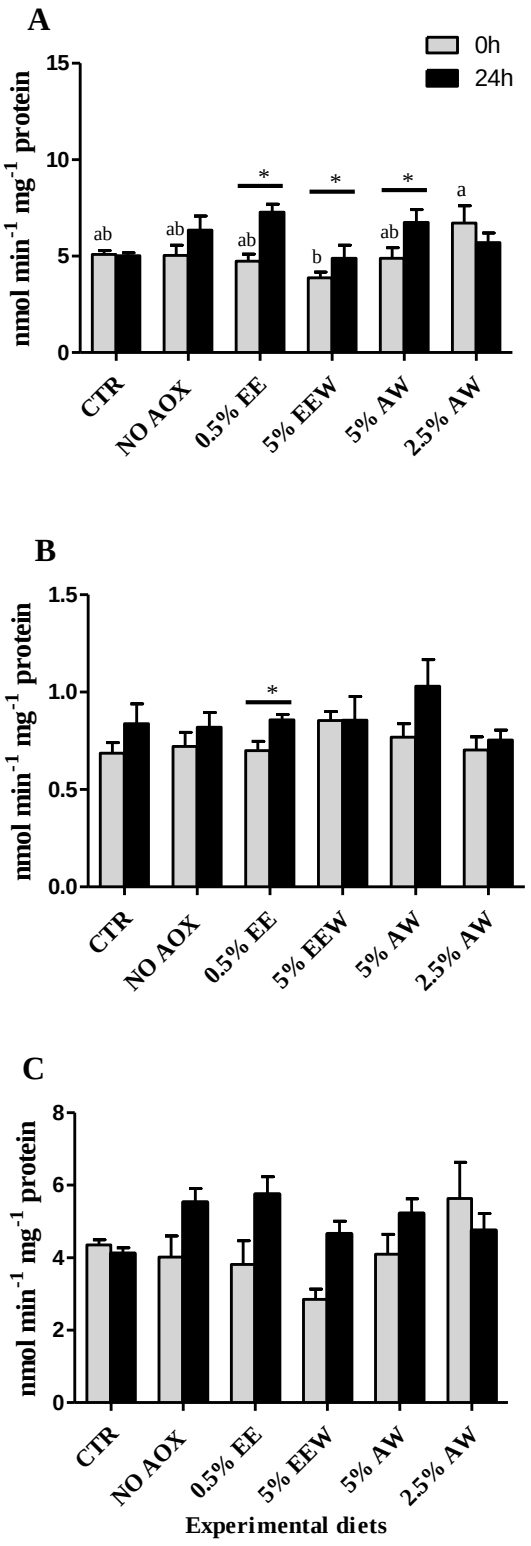

Figure 23 Non-enzymatic antioxidant markers in the liver of gilthead seabream.

Fish fed different diets, 0h, and 24h after being exposed to crowding. A: total glutathione (nmol min^{-1} mg^{-1} protein); B: oxidized glutathione (nmol min^{-1} mg^{-1} protein); and C: reduced glutathione

(nmol min^{-1} mg^{-1} protein). Fish fed six experimental diets: *i*) a diet with commercial antioxidant (CTR), *ii*) a diet without antioxidant addition (NO-AOX), *iii*) a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv*) a NO-AOX diet with 5% ethanolic waste (5% EEW), *v*) a NO-AOX diet with 5% agar waste (5% AW), *vi*) a NO-AOX diet with 2.5% of agar waste (2.5% AW). The bar represents the mean+SEM of each group (n = 9 fish). Different letters indicate differences between dietary groups for a given sampling time (Tukey test, $P<0.05$). The symbol * shows significant differences between sampling times for a given dietary group (T-test, $P<0.05$).

4.3.5. Immune-related parameters in plasma

The effects of experimental diets on several immune-related parameters of fish exposed to crowding are presented in Fig. 24. Immediately after applying the crowding (0h), the peroxidase activity of fish fed 2.5% AW diet increased compared to values observed in fish fed NO-AOX and 5% EEW diets (Fig. 24A). At the same experimental time, the antiprotease activity showed an increase in the plasma of fish fed 5% AW diet with respect to fish from the 2.5% AW group (Fig. 24B). However, no significant variations were observed in protease and haemolytic complement activities between dietary treatments (Fig. 24C and D).

After 24h of crowding, the results showed that the peroxidase activity significantly increased in the fish from the 5% AW group compared to the values measured at 0h (Fig. 24A). The haemolytic complement activity in plasma of fish fed NO-AOX, 5% EEW, and 2.5% AW diets were increased at 24h compared to 0h (Fig. 24D). Interestingly, the fish fed all dietary treatments exhibited lower antiprotease activity at 24h with respect to values observed at 0h whereas the fish fed NO-AOX diet exhibited lower antiprotease activity than those fed 5% AW diet at 24h (Fig. 24B).

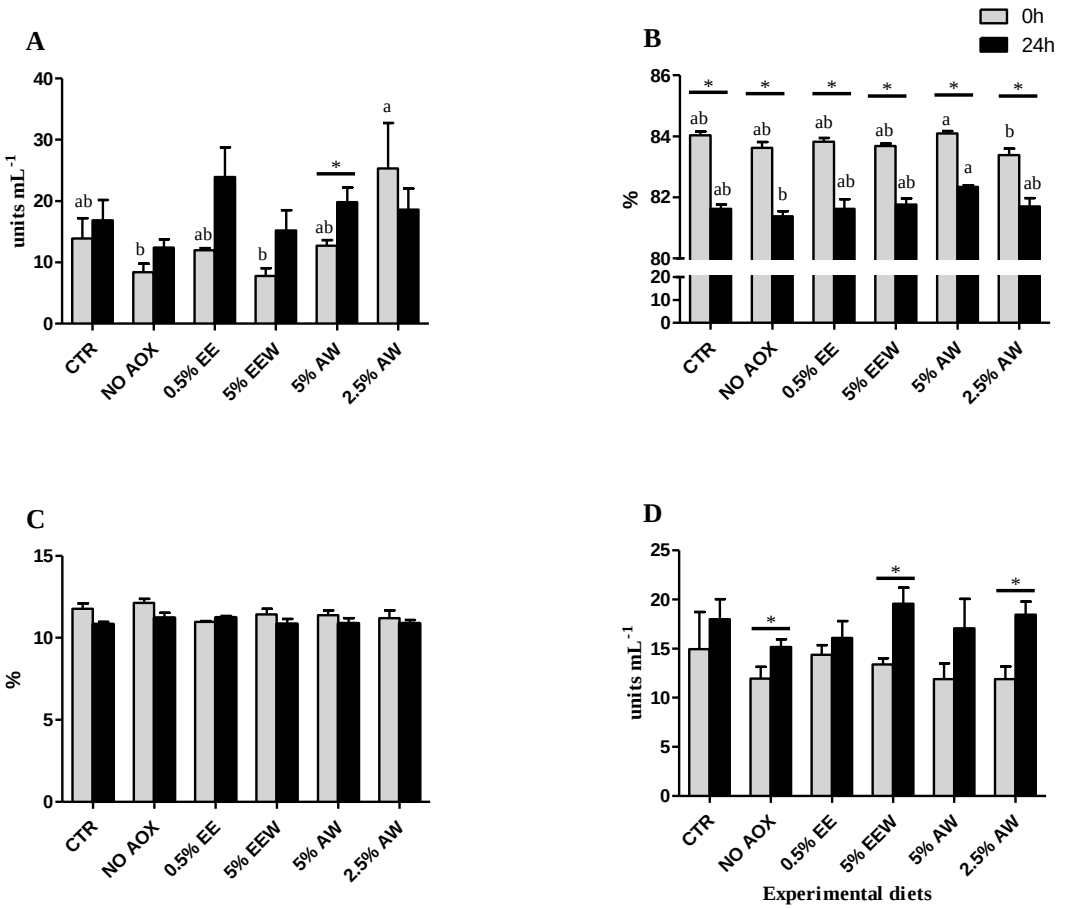

Figure 24 Immune-related markers in plasma of gilthead seabream.

Fish fed different diets, 0h, and 24h after being exposed to crowding. A: peroxidase (units mL^{-1}); B: antiprotease (%); C: protease (%); and D: alternative complement pathway activities (units mL^{-1}). Fish fed six experimental diets: *i)* a diet with commercial antioxidant (CTR), *ii)* a diet without antioxidant addition (NO-AOX), *iii)* a NO-AOX diet with 0.5% ethanolic extract (0.5% EE), *iv)* a NO-AOX diet with 5% ethanolic waste (5% EEW), *v)* a NO-AOX diet with 5% agar waste (5% AW), *vi)* a NO-AOX diet with 2.5% of agar waste (2.5% AW). The bar represents the mean+SEM of each group (n = 9 fish). Different letters indicate differences between dietary groups for a given sampling time (Tukey test, P<0.05). The symbol * shows significant differences between sampling times for a given dietary group (T-test, P<0.05)

4.4. Discussion

Crowding is present in aquaculture and may give rise to stress which negatively impacts fish welfare. The response is quite relevant for aquaculture as negative physiological outcomes are linked to the occurrence of stress that may lead to disease outbreaks and eventually death. In particular, acute crowding in the conditions implemented in this study has been shown to acts as a stressor in gilthead seabream. This is revealed by well-established hallmarks associated with a stress response, including increased cortisol, glucose, and lactate levels in plasma after implementing crowding for 1h. Previous studies have shown comparable effects in fish induced by crowding, triggering oxidative stress and resulting in immune suppression in seabream as well as in other cultured species (Uribe et al. 2011; Magnoni et al. 2019)

Results of the present study have shown that the plasma cortisol levels were increased in crowded versus uncrowded fish fed the control diet, suggesting the effectiveness of the acute stressor applied. In particular, fish showed a 40-fold increase in plasma cortisol levels immediately after acute crowding compared to the uncrowded group. Applying a similar protocol in gilthead seabream, Rotllant et al. (2001) reported a 50-fold increase in plasma cortisol levels after 1h of confinement. A protocol less severe was applied in the same fish species by Guardiola et al. (2016), where crowding was induced by reducing the water volume in the original tank, to attain a similar density as in our study. These authors found a 2-fold increase in cortisol levels after 2h of confinement. The stress response triggered by crowding involved cortisol release and several neuro-endocrine signals mediated by the hypothalamic-pituitary-interrenal (HPI) axis (Spiers et al. 2015). This, in turn, has been linked to increased glucose and lactate appearance in plasma of several fish species (Barton 2002; Fazio et al. 2015; Balasch et al. 2019; Herrera et al. 2019), a response that was observed in gilthead seabream after 1h of crowding in our study.

After 24h of crowding exposure, all the stress markers measured in plasma of crowded fish (cortisol, lactate, and glucose levels) were similar to the fish from the uncrowded group, suggesting that fish were able to recover after that time. Similar values of plasma cortisol to acute stressors have been reported in gilthead seabream exposed to confinement (70 kg m^{-3}) where this hormone peaked at 1h during confinement reaching pre-exposure levels at 2-24h (Arends et al. 1999). This stress response is comparable to that observed in several teleosts species exposed to different acute stressors (Barton

2002). For example, Costas et al. (2011) observed in the plasma of Senegalensis sole (*Solea senegalensis*) exposed to netting and air exposure for 3 min a peak of cortisol levels at 1h after stress, recovering between 4 and 24h after the stress. Similarly, Atlantic cod (*Gadus morhua*) was stressed by confinement and chasing for 15 min resulting in a peak of cortisol at 1h post-stress returning to basal levels at 12-24h post-stress (Olsen et al. 2008).

The dietary supplementation with *Gracilaria gracilis* by-products did not alter feed intake either fish growth performance of seabream during the trial. In addition, no mortality was registered during and after applying the crowing protocol in all the dietary treatments. Nevertheless, the dietary supplementation with *G. gracilis* by-products could reduce the stress levels by lowering plasma cortisol immediately after the crowding stress (0h). This is in line with the significantly lower levels of plasma cortisol in fish fed the diets supplemented with AW and EEW compared to the control diet. Dietary supplementation with *G. gracilis* and other SW have been considered to attenuate the negative effects of stressors in several cultured fish species due to antioxidant effect (Magnoni et al. 2017) and immunostimulant properties (Peixoto et al. 2016a; Peixoto et al. 2016b). Some beneficial effects of dietary SW supplementation could be attributed to the presence of bioactive compounds such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Awad et al. 2017), which may act by decreasing the negative outcomes of stressors. Most of the bioactivity of SW and their by-products appear to be associated with the antioxidant compounds, which may effectively act against ROS burst and free radicals production induced by stressors (Spiers et al. 2015).

Concerning stress response, LPO is recognized as a reliable marker of oxidative stress (Pérez-Sánchez et al. 2013). Antioxidant mechanisms consist of multiple enzymatic and non-enzymatic systems protecting all organisms against oxidative damage (Kabel 2014), including LPO. In this study, hepatic LPO levels were similar between fish fed the experimental diets. This could suggest that the antioxidant mechanisms were efficient to control ROS and free radical formation to minimize lipid damage even after acute crowding stress. The antioxidant system is composed of the glutathione system (GPx, GST, and GR) and other enzymes such as SOD and CAT that are important antioxidant mechanisms in fish (Kabel 2014). In the current study, both SOD and CAT enzyme activities in the liver of fish remained similar between the dietary groups after crowding. However, GPx activity was lower in the liver of fish-fed AW supplemented diets compared to other experimental diets. Also, fish-fed diets supplemented with EEW and

AW showed lower GR activity than fish fed the NO-AOX diet. Reduced activity of those enzymes measured on these groups suggests that SW by-products may improve the antioxidant capacity, more likely by the presence of bioactive compounds. Some of these SW compounds with antioxidant capacity has been associated to pigments, polysaccharides, and polyphenols, which may act as electron scavengers during the free radical formation (Gomez-Zavaglia et al. 2019). This, in turn, may lower down the requirement of GPx activity to decrease hydrogen peroxide and organic hydroperoxide formation (Kabel 2014) and the requirements for GR activity, enzymes that reduce GSSG to GSH, generating reducing power in tissues (Couto et al. 2016). Therefore, lower GR activity could suggest that the compounds with antioxidant capacity in diets supplemented with SW by-products may assist the endogenous antioxidant mechanisms implemented to maintain the oxidative stress homeostasis.

A previous study in which gilthead seabream fed diets supplemented with 5% AW obtained from *G. gracilis* and subsequently exposed to 24h hypoxia and 24h normoxia (recovery), have shown a protective effect of this SW by-product (Magnoni et al. 2017). In that study, lower LPO levels were measured in the liver of fish-fed 5% AW diet at recovery indicating decreased lipid peroxidation compared to fish-fed control diets. Besides, these authors have shown that gilthead seabream fed 5% AW from *Gracilaria* sp. displayed a decrease of hepatic GPx and CAT activities during the hypoxia experiment. Our results confirm that dietary supplementation with AW has a protective effect in fish feeding these diets, although the protective effect at the level of oxidative damage (LPO) appears to depend on the severity of the stressor. The hypoxia-normoxia protocol applied by Magnoni et al. (2017) showed to be associated with high mortality during the trial, particularly in fish fed the control diet (without SW supplementation). In contrast to the hypoxia-normoxia protocol applied by Magnoni et al. (2017), the crowding protocol applied in the present study appears to be a less severe stressor. No mortality was registered in our study, although fish displayed all the hallmarks associated with a response to a stressor, such as high plasma cortisol, glucose, and lactate levels. Additionally, the absence of mortality after feeding the different diets, as well as the lack of differences in the GST activity in the liver, could suggest that the dietary supplementation with SW by-products has not negative consequences on gilthead seabream welfare.

The current study also analysed the impact of dietary SW by-product supplementation on the immune response of gilthead seabream exposed to crowding. In the case of haemolytic

complement and protease activities, the values remained similar after crowding in fish fed the experimental diets. In contrast, our results showed that the antiprotease activity was higher in fish fed the 5% AW diet compared to the group fed the 2.5% AW diet, suggesting that higher levels of AW produce a better response for neutralizing proteolytic enzymes, such these secreted by a bacterial pathogen (Rebl et al. 2018). Similarly, peroxidase activity was increased in fish fed the 2.5% AW diet compared to fish fed NO-AOX and 5% EEW diets. This enzyme is important to remove the excess of H_2O_2 formed as a defence mechanism in fish (Veen et al. 2009; Guardiola et al. 2014). Therefore, the results could indicate that the AW diet supplemented at 2.5%, acted as a preventive treatment that kept the peroxidase at high levels in the seabream plasma. The immunostimulant properties of SW have been associated with the presence of bioactive compounds (Pal et al. 2014). For example, polysaccharides, highly abundant in SW, their extracts, and by-products, have been linked to enhancements of the fish immune system (Peixoto et al. 2016b).

The effect of dietary SW on the immune response and disease resistance has been investigated earlier in fish (Peixoto et al. 2016b). In particular, methanolic extracts obtained from *G. gracilis* improved some immune-related parameters such as the alternative complement pathway in the European seabass (*Dicentrarchus labrax*) (Peixoto et al. 2019b). These authors also reported an improvement in resistance after a bacterial challenge in European seabass-fed diets supplemented with 5% *Gracilaria sp.* aqueous extract (Peixoto et al. (2019a), which is analogous to the AW implemented in the diet of the current study. However, variations in the immune-related parameters were observed in fish-fed SW or their by-products (Peixoto et al. 2019a; Peixoto et al. 2019b). For instance, Peixoto et al. (2019b) reported an increase in the haemolytic complement activity in the plasma of European seabass fed supplemented diets with *Gracilaria sp.* methanolic extracts where not variations were observed in the values of peroxidase activity. On the other hand, Peixoto et al. (2019a) described a decrease of peroxidase activity in the plasma of fish fed 5% *Gracilaria sp*. aqueous extract after 10 days of intraperitoneal injection with a bacterial pathogen (*Photobacterium damselae* subsp. piscicida). This may suggest that the stressor types (e.g. crowding vs. bacterial challenge), sampling time, and type/quantity of extract/by-product supplemented in the diet may be reflected in different modulation of the immune-related parameters in fish.

Conclusions

In summary, the present study confirmed the potential of AW to improve the stress response of gilthead seabream when exposed to acute crowding. Fish-fed diets supplemented with SW by-products (5% EEW, 5% AW, and 2.5% AW) exhibited a decrease of stress markers after crowding, which could be advantageous for the potential application in aquaculture. Our results showed that dietary AW supplementation may provide a reinforced antioxidant response to cope with the oxidative stress as suggested by lower GPx and GR activities measured in the liver. No differences for antiprotease, protease, and haemolytic complement activities were detected in the plasma of fish fed the different experimental diets. In contrast, peroxidase activity was increased in plasma of fish fed 2.5% AW diet, which suggests an immune response. The findings might not be enough to have a complete appraisal of the modulatory effects of SW by-products on the immune response of juvenile seabream. However, the results suggest that dietary supplementation with natural by-products, particularly with AW and EEW, could improve the response of cultured fish to stressors as well as be a sustainable alternative to the use of antioxidant supplements included currently added into commercial diets. Further studies should characterize the specific compound(s) present in AW, as well as investigating the mechanisms mediating the observed modulatory role on the antioxidant and immune responses in gilthead seabream.

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CHAPTER 5

Improving agar properties of farmed *Gracilaria gracilis* by using filtered sunlight

Francisca Silva-Brito[1,2]; Sara G. Pereira[3]; Cristina M.R. Rocha[3]; Elisabete da Costa[4,5]; M. Rosário Domingues[4,5]; Andreina Azevedo[6], Anake Kijjoa[2]; Helena Abreu[6]; Leonardo Magnoni[1]

[1]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208, Matosinhos, Portugal.

[2] ICBAS - Institute of Biomedical Sciences of Abel Salazar, University of Porto, Rua de Jorge Viterbo Ferreira, 225, 4050-313, Porto, Portugal.

[3] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[4] Centro de Espetrometria de Massa, Departamento de Química & QOPNA, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal;

[5] Departamento de Química & CESAM & ECOMARE, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[6] ALGAplus - Produção e Comercialização de Algas e seus Derivados, Rua do Mar s/n, 3830-165, Ílhavo, Portugal.

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Improving agar properties of farmed *Gracilaria gracilis* by using filtered sunlight

Francisca Silva-Brito[1,2] · Sara G. Pereira[3] · Cristina M. R. Rocha[3] · Elisabete da Costa[4,5] · M. Rosário Domingues[4,5] · Andreina Azevedo[6] · Anake Kijjoa[2] · Helena Abreu[6] · Leonardo Magnoni[1]

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ABSTRACT

The effects of different light spectra on agar yield, composition and gel properties, pigment content, and antioxidant activity of *Gracilaria gracilis* cultivated in a land-based IMTA-system were investigated. *G. gracilis* was cultivated for 4 weeks applying neutral (NT), blue (BL) and red (RD) spectral filters in outdoor tanks in duplicates. BL light (13% agar extraction yield) increased 1.3 and 2 times the agar yield compared to both NT and RD conditions, respectively. BL light produced an agar gel strength (1037.9 g cm^{-2}) similar to the NT condition (960.0 g cm^{-2}). Light spectra had no significant effect on melting (~93.5 ºC) nor gelling temperatures (~44.1 ºC) of agar, also comparable to the agar obtained from *G. gracilis* before the growth trial (WD). However, the productivity of *G. gracilis* farmed under the BL condition (0.70% day^{-1}) decreased when compared to the NT condition (1.14% day^{-1}), while RD condition (0.93% day^{-1}) presented a middling growth. BL condition increases the total carbohydrate content of *G. gracilis* compared to NT, RD, and WD conditions (78.72, 68.48, 27.41, and 67.68 mg Glc Eq. g^{-1} SW, respectively). The content of phycoerythrin, phycocyanin, and chlorophyll-*a* in *G. gracilis* was similar among different light spectra. Total phenol (13.24 - 15.93 mg PE g^{-1} SW), as well as the antioxidant activity, remained similar in *G. gracilis* growing under the different light spectra. Results show that the agar yield and gel strength from farmed *G. gracilis* is improved when applying light filters despite a decrease in growth. Also, this study shows that light filters did not affect antioxidant activity in farmed *G. gracilis*.

Key-words: Agar gel strength, agar yield; antioxidant activity, *Gracilaria gracilis*; IMTA-system.

5.1. INTRODUCTION

The seaweed farming sector represents one of the most important industries among aquatic production activities. The world seaweed production increased from 22 million tonnes to 31 million tonnes from 2011 to 2015, with 40% of that production channelled toward the manufacture of hydrocolloids (Ferdouse et al. 2018). The *Gracilaria* sp. is the most important raw material for the extraction of agar, supplying approximately 53% of the market demands. Although *Gracilaria* sp. is a valuable source used for agar extraction, this seaweed appears to generate gels with low strengths (Murano 1995), which reduces the commercial value of *Gracilaria* sp. for high-end applications (Sousa et al. 2010).

In Portugal, *G. gracilis* is produced in an IMTA-system using the effluent from a fish farm, rich in inorganic nutrients (Abreu et al. 2009; Abreu et al. 2011; Da Costa et al. 2017). Therefore, IMTA systems are a sustainable form to produce seaweed which represents to be highly valuable in terms of biomass production. Despite these benefits, farmed seaweeds appear to have a low proportion of carbohydrates to protein content (Ashkenazi et al. 2019). Consequently, the agar obtained from *G. gracilis* farmed in IMTA-systems tends to have higher quality (high gel strength) but lower agar yields (Sousa et al. 2010; Lee et al. 2017). From the point of view of the agar industry, it is important to find a trade-off between the agar yield and quality to optimize its production. The business of farming *Gracilaria* in Europe for agar extraction it is not viable at this point, and the biomass produced is currently sold for direct consumption in the retail and distribution channels. However, if production strategies of increasing agar yield and quality are found, it can open the door for upscaling *G. gracilis* biomass production towards polysaccharide extraction under a biorefinery approach (ALGAplus information).

Several studies have investigated the effect that environmental conditions may have on the economic value of seaweed and agar yield and quality, including temperature and salinity (Bird 1988; Daugherty et al. 1988), light intensity (Bunsom et al. 2012) and nutrient supply (Rocha et al. 2019). It was established that a combination of moderate nutrient levels and high light intensity appears to be the main factors influencing the agar quality of *G. gracilis* farmed in IMTA-systems (Araño et al. 2000; Mensi et al. 2020). The nature and intensity of the light have a major impact on the photosynthetic activity of the seaweed and ultimately on its growth. For example, green and blue lights were both

shown to improve growth in several seaweed species, including *Halymenia floresii*, *Gracilaria furcata and Porphyra haitanensis* (Godínez-Ortega et al. 2008; Wu 2016; Zhang et al. 2020). Also, blue light appears to trigger the synthesis of the pigments such as phycobiliproteins in *Halymenia floresii* and *Porphyra umbilicalis* (Figueroa et al. 1995; Godínez-Ortega et al. 2008), while red light improved the antioxidant response in *Porphyra haitanensis* (Wu 2016). In this regard, modulating the light conditions of farmed *G. gracilis* may be a convenient tool to improve agar yield and gel strength, which may represent superior commercial characteristics.

The agar industry, mostly based on *G. gracilis* biomass, generate a considerable quantity of by-product from the extraction process (AW), which is under-valued. Thirty percent of the total seaweed biomass producing agar account for the equivalent quantity of AW generated (Meinita et al. 2017). Today, only 40% of the AW generated has an application (Ennouali et al. 2006; Pei et al. 2013; Meinita et al. 2017). Reusing the AW could be a way to recovery bioactive compounds, including natural antioxidants (Sindhi et al. 2013; Pal et al. 2014). As far as we know, it has not yet been evaluated the presence of antioxidant compounds and/or antioxidant activity in the AW obtained from *G. gracilis* farmed in an IMTA-system. Although *G. gracilis* biomass is usually used for agar extraction, previous studies confirmed the antioxidant activity of aqueous and ethanolic extracts obtained directly from *Gracilaria birdiae*, *Porphyra leucosticta*, *Gracilaria conferta* and *Hydropuntia cornea* (Korbee et al. 2005; Figueroa et al. 2010; Souza et al. 2011; Figueroa et al. 2012).

This study aimed to study the effect of different light spectra on agar yield and quality, pigments and antioxidant activity of *G. gracilis* farmed in the IMTA-system.

5.2. MATERIALS AND METHODS

5.2.1. *Gracilaria gracilis* origin

The initial cultivars of *G. gracilis* (Gracilariales, Rhodophyta) were hand-harvested at Ria de Aveiro, always from the same location, to reduce potential variations. Then *G. gracilis* was transported into plastic bags to the ALGAplus Lda company. This company, localized in northern Portugal, is specialized in the production of seaweed in an Integrated Multi-Trophic Aquaculture (IMTA) system. The cultivation system and protocols were the ones established by the company.

5.2.2. Tanks setting and environmental parameters

The experiment was conducted over four weeks during winter (January - February 2020). *Gracilaria* (~12 kg, FW) was distributed into six polyethylene tanks (500 L, footprint of 1 m^2 each, light transparency≈0 %). Water inflow to each tank was adjusted to 126 L h^{-1}, and the seaweeds were kept in constant movement with air diffusers to assure a homogeneous exposure of the seaweed to incident irradiance. Each tank was set up to receive one of the three conditions: neutral (NT), blue (BL), or red (RD). For that purpose, the filters (NT, BL or RD, LEE Filters USA & Latin America) were positioned between two crystal acrylic screens, 3 mm width each (Laços globais LDA, Portugal). Light spectra of each filter were measured using an OceanOptics spectrometer (Flame model, wavelength range 190–1100 nm) and the values obtained are shown in Fig. 25.

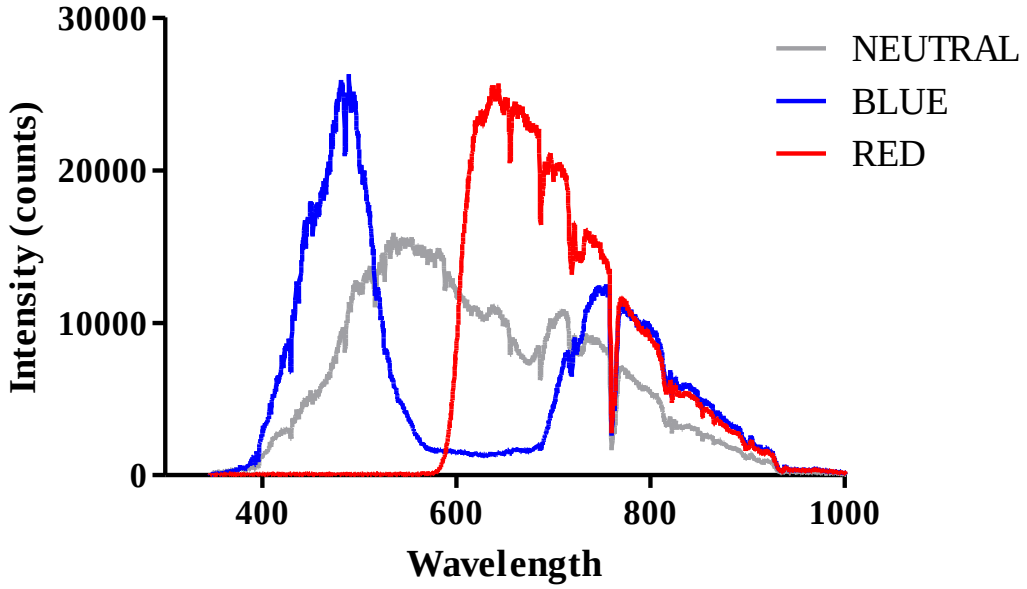

Figure 25 Transmission spectra of each filter.

The screens with the filters were inserted 20 cm above each tank to minimize possible greenhouse effects. Furthermore, the screens with the filters were positioned in a way that the sunlight was filtered and only specific wavelengths could reach the tanks where the *G. gracilis* was cultivated. The tanks were distributed in different positions to receive similar amounts of irradiance across the experiment. Each experimental condition was tested in duplicate.

All parameters were measured in each tank, including oxygen levels, pH and temperature, which were monitored five days a week, twice a day (in the morning at 9.00 and in the afternoon at 15.00) with a multi-parametric sensor (multi 340i/set, WTW, Germany). Salinity was measured with a multi-parametric sensor (multi 340i/set, WTW, Germany) and the values were occasionally registered, especially on rainy days. The incident sunlight was measured five days a week with a light sensor (Quantum meter NQ-100 Series, Apogee Instruments, Inc, USA), with measurements performed at midday below the water surface of each tank. Photosynthetic Photon Flux (PPF) was measured in micromoles per square meters per second (µmols m^{-2} s^{-1}) as an estimation of the number of photons between 400 nm and 600 nm (radiant power) to evaluate the potential for photosynthesis.

Water samples were taken from the inflow and the outflow of each tank five days a week, twice a day during the morning and in the afternoon. Water samples were analysed for ammonium (AP 152), nitrite (AP 109) and nitrate (AP 163) content by spectrophotometric methods by using commercial kits (Palintest water analysis technologies) according to manufacture recommendations. Results were expressed as mg L^{-1}. At the end of the trial, the biomass from each tank was dried following the procedure of the ALGAplus company (forced air-tunnel set at 30 ºC). A sample of *G. gracilis*, collected at the beginning of the experimental trial (WD) was dried following the procedure of ALGAplus company to be analysed in parallel to the samples obtained from the experimental trial.

5.2.3. *Gracilaria gracilis* growth and productivity

G. gracilis biomass from each tank was harvested weekly, drained to constant weight (FW, fresh weight) and re-stocked to the initial density. Relative growth rate (RGR) and productivity (P) of *G. gracilis* were calculated at 7, 14, 21 and 30 days according to the equations:

$$RGR\ (\%\ day^{-1}) = \ln(B_t/B_0)/D \times 100;$$

$$P\ (g\ FW\ m^2\ w^{-1}) = [(B_t - B_o)/W]/A;$$

where B_t is the final fresh biomass (kg), B_o is the initial inoculated fresh biomass (kg), W is the time (weeks), A is the surface area of the tank (m^2) and D is the time (days).

5.2.4. Agar yield and quality

5.2.4.1. Agar extraction

Agar extractions were performed at least in triplicate as described by Villanueva et al. (2010). The dried *G. gracilis* biomass was pre-treated with 6% NaOH (w/w) at 85 °C for 3.5h with a sample: solvent ratio of 1: 25 and a final volume of 800 mL. *G. gracilis* was washed several times with tap water and neutralized with an equivalent volume of 0.5% acetic acid (w/w) at room temperature for 1h. The agar extraction was performed in 800 mL of distilled water for 2.5h at 85 °C. The hot mixture obtained was filtered using a cotton filter cloth and then frozen at -20 °C. The agar was recovered from this fraction through freeze-thawing, dehydrated with ethanol (96%) and dried overnight at 60 °C. Agar yield was calculated based on the formula described by Kumar et al. (2009):

$$\text{Agar Yield \%} = \text{Dry weight of agar (g)} / \text{Dry weight of seaweed (g)} \times 100.$$

5.2.4.2. Agar gel strength

Gel strength was determined as described by Villanueva et al. (2010) using a texture analyser (TA.HD plus, Stable Micro Systems, Surrey, England) equipped with a cylindrical probe with 10 mm of diameter. Agar-enriched fraction (15 g) was milled with a coffee grinder (150 W) and dissolved in boiling distilled water (1.5% w/w) until complete solubilization. The hot solution was transferred into a cylindrical container with 30 mm of diameter and kept at room temperature for 20h. Gel strength was considered to be the stress required to break the gel surface. The rate of penetration used was 0.2 mm s^{-1}. The analysis was carried out in triplicate and the results were expressed in g cm^{-2}.

5.2.4.3. Agar gelling and melting temperatures

Gelling and melting temperatures were measured according to Rocha et al. (2019) in a controlled stress rheometer HR-1 (TA Instruments, New Castle, USA) fitted with a parallel plate geometry (40 mm diameter, gap 1000 µm). The agar was solubilized with distilled water (1.5% w/w) at boiling temperature until complete dissolution as described above. The solution was de-gassed and poured onto the pre-heated plate of the rheometer at 80 °C. Liquid paraffin oil was used to prevent water loss. A temperature ramp from 80 °C to 20 °C at the rate of 2 °C min^{-1} was applied. The sample was equilibrated for 30 min at 20 °C and heated from 20 °C to 95 °C with the same rate of 2 °C min^{-1}. Storage and

viscous moduli were recorded at the end of the equilibration time at 20 °C. The analysis was carried out in triplicate and in all the tests a frequency of 6.28 rad s^{-1} and a 1.0% strain were applied.

5.2.4.4. Agar sugar composition

Aliquots with solubilized agar (1%) were subjected to quantitative post-hydrolysis for oligosaccharide determination (20 min at 121 ºC and 4% H_2SO_4). The liquid resulting from this post-hydrolysis was filtered through 0.22 µm membranes and analysed by high-performance liquid chromatography (HPLC) to quantify the solubilized compounds, including glucose, galactose and arabinose. The conditions used in the HPLC analysis were as follows: refractive index; Aminex HPX-87H column at 60 ºC; mobile phase of 0.05 M H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Analyses were carried out in triplicates and glucan, galactan and arabinan content in the samples were calculated from the concentrations of glucose, galactose and arabinose.

5.2.4.5. Agar structure

Functional groups and bonding arrangement of constituents present in the agar samples were determined by Fourier Transform Infrared Spectroscopy (FTIR) using an ALPHA II- Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell. The FTIR spectra were recorded in the range of 4000-400 cm^{-1}, by acquiring 60 scans cycles per sample with 4 cm^{-1} resolution. Analyses were carried out in triplicate.

5.2.5. *Gracilaria gracilis* moisture, ash, and protein content

G. gracilis biomass (250 mg) was weighed into ceramic crucibles and dried overnight (16 h at 105 °C). Crucibles were cooled to room temperature and weighed for moisture determination. Ash determination was performed in the same *G. gracilis* biomass samples. Seaweed biomass was pre-incinerated by maintaining the crucibles on a heated plate for 20 min and transferred to a muffle furnace at 575 °C for 6 h. After cooling to room temperature, the crucibles were weighted and the ash content was determined.

The nitrogen (N) elemental composition of the seaweed biomass was evaluated using a Leco Truspec-Micro CHNS 630-200-200 elemental analyser in a combustion furnace at

1075 °C and afterburner at 850 °C. Seaweed biomass (2 mg) was burned in an oxygen/carrier gas mixture to full combustion and the conversion of by-products to N for gas analysis was detected using thermal conductivity. The elemental analysis allowed the estimation of the protein content by the use of a nitrogen-to-protein conversion factor. A nitrogen-to-protein conversion factor of 5 was applied, as this value has been proposed to be more adequate for seaweed (Angell et al. 2016). Analyses were carried out in triplicate and the results were expressed as the percentage of dry weight (DW).

5.2.6. Total carbohydrate content

The total carbohydrate content of agar was determined by the method of phenol-sulfuric acid according to Wang et al. (2017) with minor modifications. For 96-well plates, 50 µL of the sample (0.0625%) or standard was added, followed by 150 µL of sulfuric acid (96%) and 30 µL of phenol (5% in H_2O). The plates were incubated in the dark at 60 ºC with stirring at 120 rpm for 1h and the absorbance was read at 490 nm. Glucose was used as a standard (0.025-1.00 mg mL^{-1}). The analyses were carried out in triplicate and results were expressed in mg of glucose equivalent per gram of dry weight of seaweed (mg Glc Eq. g^{-1} SW).

5.2.7. *Gracilaria gracilis* phycobiliproteins and chlorophyll-*a*

Phycobiliproteins and chlorophyll-*a* were quantified following the procedure described by Pereira et al., 2006 and Kumar et al. (2010), respectively, with slight modifications. For phycobiliproteins, dried *G. gracilis* biomass was grounded in phosphate buffer (1:5 w/v) and pH 6.8 in a mortar with sand. The homogenates were incubated overnight at 4 ºC and then centrifuged at 10 000 x *g* for 20 min at 4 ºC. Phycocyanin and phycoerythrin contents were estimated using the equations optimized for aqueous crude extracts of red seaweeds (Beer et al. 1985). For chlorophyll-*a* quantification, a dried *G. gracilis* biomass was grounded in 80% acetone (1:5 w/v) in a mortar with sand. The homogenates were incubated overnight at 4 ºC and then centrifuged at 3 000 x *g* for 10 min at 4 ºC. The supernatant was transferred to a microplate and the absorbance was recorded at 665 nm by using a Synergy™ HT Multi-Mode Microplate Reader (BioTek® Instruments, Inc., Vermont, USA). The extinction coefficient (11.9) was used for calculating chlorophyll-*a* content. All the procedures described above were performed in dark and cold conditions

(4 ºC). Pigments determinations were carried out in 5 replicas per tank (n=10 per treatment).

5.2.8. Preparation of samples extracts for determination of antioxidant activity in *Gracilaria gracilis*

G. gracilis was extracted using the procedure described by Chew et al. (2008) with some modifications. One gram of dried seaweed was grounded in ethanol (1:2 w/v) in a mortar with sand. Then, the homogenate was shaken continuously on an orbital shaker for 1h. The extract was then filtered with filter paper and was stored at -20 ºC for further analysis. Extractions were performed in 3 replicas per tank (n=6).

5.2.8.1. Gracilaria gracilis total phenolic content

Total phenolic compounds (TPC) were measured using Folin Ciocalteu's method as described by Chan et al. (2015). TPC content of each extract was quantified using phloroglucinol as a standard (0.01-0.5 mg mL^{-1}). The results were expressed as mg phloroglucinol equivalent g^{-1} seaweed (mg PE g^{-1} SW, n= 10).

5.2.8.2. Gracilaria gracilis total flavonoid content

The total flavonoid content (TFC) was determined using the $AlCl_3$ colourimetric method as described by Chan et al. (2015). TFC was quantified in triplicates using rutin (Santa Cruz Biotechnology) as a standard (2-70 µg mL^{-l}). The results were expressed as mg rutin equivalent g^{-1} seaweed (mg RE g^{-1} SW). Analyses were carried in five replicas per tank (n=10 per treatment).

5.2.8.3. Gracilaria gracilis free radical scavenging activity

The DPPH assay was performed according to Chan et al. (2015) with slight modifications. Serial dilutions of the sample were incubated with DPPH (α, α-diphenyl-β-picrylhydrazyl) at a final concentration of 0.1 mM. A sample blank was performed in the same manner but without DPPH. An equal amount of ethanol and DPPH was used as a control. The DPPH curve standard was performed to determine DPPH concentration in each well. The scavenging activity was calculated using the following equation:

DPPH scavenging activity (%) = $100 - (100 \times [A_{517sample} - A_{517blank}/(A_{517DPPH}-A_{517solvent})])$

The half-maximal inhibitory concentration (IC_{50}) was determined by linear regression analysis of the dose-response curve plotted between scavenging activity (%) against sample concentration. The IC_{50} is defined as the concentration of sample scavenging 50 % of the initial DPPH radical (Chan et al. 2015). Analyses were carried in 9-10 replicas per treatment.

5.2.8.4. Gracilaria gracilis inhibitory capacity for lipid peroxidation

Thiobarbituric acid-reactive substances formation inhibition assay (TBARS) was performed based on the method described by Zeb et al. (2016) with slight modifications. The lipid substrate used in the TBARS assay was a homogenate of fish liver (*Dicentrarchus labrax*). Five microliters of the sample were added to the liver homogenate (150 µl, diluted 1:10) and incubated overnight (16 h) at 50 ºC. The antioxidant activity was expressed as equivalent mM of malonaldehyde (MDA) kg^{-1} of the sample. TBARS concentration was calculated using a standard curve based on MDA (200 µM – 6.25 µM). The results were expressed as mM of TBARS by g of the liver. Analyses were carried in 9-10 replicas per treatment.

5.2.9. Statistical analysis

Statistical analysis was performed using Sigma Stat 3.1 applying an $\alpha = 0.05$. Data were checked for normality and homogeneity of variances (Shapiro-Wilk test and Brown-Forsyth test, respectively). Differences between conditions regarding agar yield, agar gel strength, gelling and melting temperatures of agar, sugars composition, total carbohydrates, protein quantification, pigments content and antioxidant activity were tested by applying a one-way ANOVA (Analysis of variance) analysis. Differences for irradiance and water salinity between experimental tanks were tested by applying a one-way ANOVA analysis. A Tukey post-hoc test was performed to assess the pairwise multiple comparisons among the experimental conditions and tanks.

A one-way repeated measures ANOVA (RM-ANOVA) were performed to check the significant effect of the light condition on the growth and productivity of *G. gracilis*. Multiple comparisons among the experimental conditions were tested by applying a Tukey test. A two-way ANOVA was performed to analyse the effect of light condition

and the daytime (morning or afternoon) on pH, O_2 levels and temperature of the water in the experimental tanks. When the effects of the factors were significant, the Tukey test was applied.

5.3. RESULTS

5.3.1. *Gracilaria gracilis* growth rate and productivity

Fig. 26 presents the effect of different light spectra on relative growth rate (RGR) (Fig. 26A) and productivity (Fig. 26B) of *G. gracilis* farmed under an IMTA-system. The BL condition significantly decreased RGR and productivity of *G. gracilis* compared to the NT condition (P<0.05). On the other hand, RD condition exhibited similar RGR and productivity to those *G. gracilis* produced under NT and BL conditions (P>0.05).

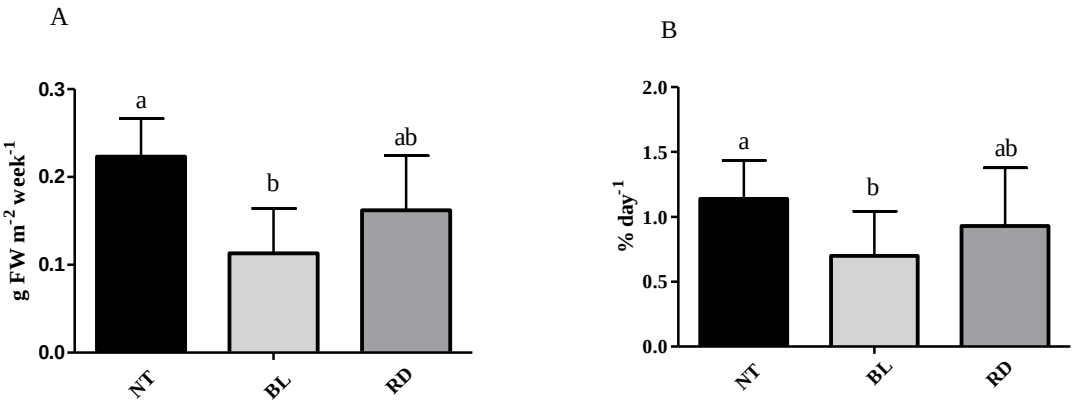

Figure 26 Productivity (A) and growth (B) of *Gracilaria gracilis* farmed during 4 weeks in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

Columns represent mean+SEM (n=2). Different letters indicate significant differences after RM-ANOVA analysis (P>0.05).

Table 16 presents the environmental parameters during the growth trial. Light conditions did not significantly affect temperature, pH and oxygen levels in the tanks (P>0.05). During the experiment, the temperatures of the water oscillated between 11 °C and 17 °C for all light conditions (average temperature ~ 15 ºC), with lower temperature in the morning and higher in the afternoon (P<0.001). The pH of the water during the trial was between 8.1 and 8.2, reaching a minimum of 7.92 and a maximum of 8.69. Maximum pH

values were registered in the afternoon coinciding with a peak in dissolved O_2 levels in the tanks (P<0.001). The water salinity during the trial ranged from 20.61 to 26.0 ‰.

The daily mean irradiance measured was significantly higher in the tanks assigned to the NT condition (370.6 µmol m^{-2} s^{-1}) compared to both BL (206.4 µmol m^{-2} s^{-1}, P<0.001) and RD (150.2 µmol m^{-2} s^{-1}, P<0.001) conditions. The irradiance incident was 1.4 times higher on the tanks assigned to the BL condition than those under RD condition (P<0.05).

Table 17 presents the mean nitrogen content (including NH_3/NH_4^+, NO_3^- and NO_2^-) of water input and output in all the tanks during the trial (NT, BL and RD conditions). No significative differences were detected in the content of nitrogen compounds in the input and the output water of the tanks during the trial (P>0.05). The nitrite levels measured in the morning were significantly higher than those measured in the afternoon regardless of the light conditions (P=0.007).

Table 16 Environmental parameters in the experimental tanks during 4 weeks of growth trial in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

			Light condition				
			NT	**BL**	**RD**	**Two-way ANOVA**	
						Factors	**P-value**
Temperature (ºC)	Time	Morning	13.59±0.19*	13.72±0.05*	13.68±0.03*	**Light (L)**	0.224
		Afternoon	15.60±0.19#	15.85±0.01#	15.90±0.09#	**Time (T)**	**<0.001**
						L*T	0.681
		Max	16.65±0.05	17.05±0.05	16.95±0.15		
		Min	11.05±0.65	11.90±0.10	11.85±0.05		
		Mean	14.6±0.26	14.7±0.02	14.7±0.00		
pH	Time	Morning	8.07±0.01*	8.07±0.01*	8.07±0.01*	**Light (L)**	0.134
		Afternoon	8.30±0.03#	8.23±0.00#	8.27±0.00#	**Time (T)**	**<0.001**
						L*T	0.152
		Max	8.69±0.13	8.47±0.02	8.54±0.06		
		Min	7.92±0.01	7.94±0.01	7.92±0.01		
		Mean	8.2±0.02	8.1±0.00	8.2±0.00		
Dissolved O_2 (mg L^{-1})	Time	Morning	11.39±0.09*	11.27±0.03*	11.28±0.02*	**Light (L)**	0.123
		Afternoon	10.95±0.03#	10.86±0.00#	10.91±0.02#	**Time (T)**	**<0.001**
						L*T	0.753
		Max	12.02±0.27	11.85±0.08	11.85±0.06		
		Min	10.38±0.01	10.35±0.04	10.35±0.04		

	Mean	11.2±0.08	11.1±0.02	11.1±0.02
Salinity (‰)	Max	29.75±0.07	29.70±0.00	29.75±0.05
	Min	20.62±0.01	20.61±0.00	20.61±0.00
	Mean	26.0±0.23	25.9±0.02	25.9±0.01
Irradiance ($\mu mol\ m^{-2}s^{-1}$)	Max	550.0±0.00	360.5±69.50	180.0±0.00
	Min	161.0±69.30	83.0±0.00	90.0±0.00
	Mean	370.6±0.18[a]	206.4±20.94[b]	150.2±4.19[c]

Values represent mean±SEM of two tanks (n=2) for each experimental condition. Different superscript letters indicated significant differences among light spectra ($P<0.05$). Different superscript symbols represent significant differences between different periods of the day (morning and afternoon) regardless of the light condition ($P<0.05$).

Table 17 Nitrogen (N) compounds content (mg L^{-1}) in the water of tanks of the *Gracilaria gracilis* growth trial in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

	Inflow water		Outflow water (per light condition)						Two-way ANOVA (P-value)		
			NT		BL		RD		Factors		
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Light (L)	Time (T)	L*T
NH_3	0.24±0.34	0.13±0.02	0.48±0.43	0.11±0.00	0.14±0.04	0.15±0.02	0.09±0.01	0.14±0.02	0.724	0.673	0.609
NO_3-	0.07±0.03	0.05±0.02	0.06±0.00*	0.04±0.00$^{\#}$	0.09±0.00*	0.05±0.00$^{\#}$	0.05±0.01*	0.04±0.02$^{\#}$	0.065	**0.007**	0.475
NO_2-	1.87±0.31	1.79±0.21	1.71±0.12	1.65±0.03	2.04±0.25	1.88±0.09	1.99±0.09	1.91±0.04	0.121	0.603	0.878

Values are mean±SEM of two tanks (n=2) for each light condition averaged during the experimental trial (4 weeks). The absence of letters indicated no significant differences among light spectra (P>0.05). Different superscript symbols represent significant differences between different periods of the day (morning and afternoon) regardless of the light condition (P<0.05).

5.3.2. Agar yield and gel strength

Fig. 27 shows the agar yield and agar gel strength obtained from *G. gracilis* before the growth trial (WD) and after 4 weeks of culture in the IMTA-system under different light conditions. The WD condition (14.03%) showed a similar agar yield to that measured in the BL condition (13.15%, $P>0.05$). The highest agar yield was obtained in the BL condition compared to NT (10.63%, $P=0.018$) and RD (6.73%, $P=0.003$) conditions. The gels strength of the BL condition (1037.9 g cm^{-2}) was higher than that obtained from the WD condition (720.4 g cm^{-2}, $P=0.038$). Also, the BL condition produced a higher agar gel strength than that obtained from the RD condition (585.5 g cm^{-2}). No differences were detected on gel strength between BL and NT conditions (960.0 g cm^{-2}, $P>0.05$). RD condition produced the lowest gel strength among all the conditions ($P<0.05$).

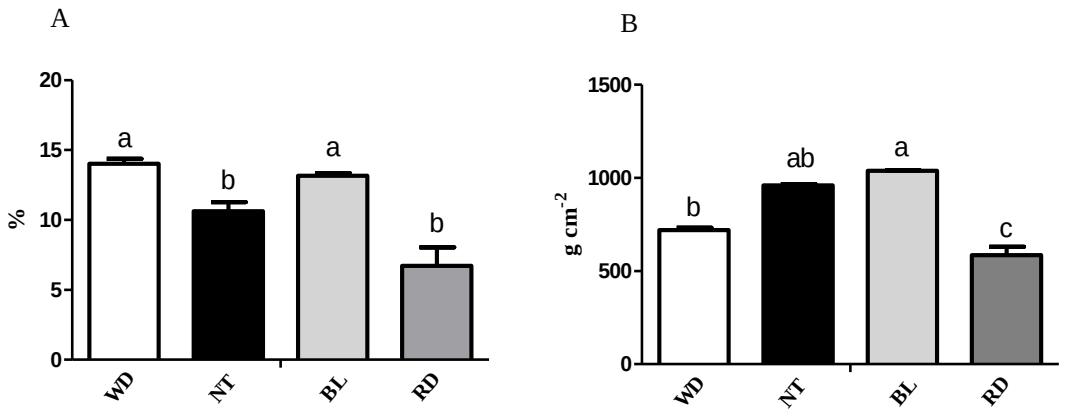

Figure 27 Agar yield (A) and gel strength (B) of *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

Columns represent mean+SEM, (n=3). Different letters indicate significant differences between conditions after one-way ANOVA analysis ($P<0.05$).

5.3.3. Agar melting and gelling temperatures

The melting and gelling temperatures of agar extracted from *G. gracilis* before the growth trial (WD) or after applying different light conditions (BL, NT and RD) are presented in Table 18. Results showed no significant effects of light conditions on melting temperatures of agar ($P>0.05$), with values ranging from 92.85 ºC to 94.1 ºC. Similarly, the gelling temperature was similar between all light conditions ($P>0.05$) and varied

between 40.7 ºC and 47.45 ºC. Both melting and gelling temperatures measured in agar extracted from *G. gracilis* in the WD condition were similar to those verified after 4 weeks of culture in an IMTA-system (P>0.05).

Table 18 Melting (T_m) and gelling (T_g) temperatures of agar extracted from *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

	Condition			
Temperatures (ºC)	**WD**	**NT**	**BL**	**RD**
T_m	93.15±0.55	94.1±0.20	93.75±0.25	92.85±0.35
T_g	42.15±3.05	47.45±2.85	46±3.60	40.7±2.70

Values represent mean±SEM of three replicas (n=3) for each experimental condition. The absence of letters indicated no significant differences among the experimental condition (P>0.05).

5.3.4. Agar sugar composition

The sugar composition of agar obtained from *G. gracilis* under the four conditions is shown in Table 19. The BL condition (107.0 mg g^{-1}) significantly increased the glucan content of agar compared to NT (37.7 mg g^{-1}, P>0.001) and RD (34.2 mg g^{-1}, P>0.001) conditions. Also, SW farmed under the BL condition showed a significant increase in glucan content compared to the WD condition (34.6 mg g^{-1}, P>0.001). In contrast, no significant differences between NT, RD and WD conditions were recorded regarding glucan content (P>0.05).

BL light (394.0 mg g^{-1} SW) displayed a significant increase of galactan content compared to all the experimental conditions (P<0.05). Both BL (394.0 mg g^{-1}, P<0.001) and NT (291.0 mg g^{-1}, P=0.008) conditions represented a significant increase of galactan content when compared with the WD condition (244.0 mg g^{-1}). In contrast, no significant differences in galactan content between WD and RD (235.0 mg g^{-1}) conditions were detected (P>0.05).

The highest arabinan content was detected in the agar extracted from *G. gracilis* in the BL condition (182.0 mg g^{-1}, P<0.05), followed by the NT condition (110.0 mg g^{-1}, P<0.05). Both BL and NT conditions displayed a significantly higher arabinan content than in the WD condition (25.5 mg g^{-1}, P<0.05). In contrast, the RD condition (7.4 mg g^{-1}) produced the agar with the lowest arabinan content among all conditions (P<0.05).

The BL condition significantly increases the total content of sugar in agar (683 mg g^{-1}) in comparison to those detected in both NT and RD conditions (439 mg g^{-1} and 276 mg g^{-1}, respectively, $P<0.05$). Both BL and NT conditions significantly increased the total sugar content in the agar compared to the WD condition (304 mg g^{-1}), contrasting to the RD condition, which displayed similar content to that detected in the WD condition ($P<0.05$).

Table 19 Sugar composition of the agar extracted from *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light conditions, using neutral (NT), blue (BL) and red (RD) light filters.

	Condition			
Sugar composition (mg g^{-1})	**WD**	**NT**	**BL**	**RD**
Glucan	34.6±0.76^{b}	37.7±2.62^{b}	107.0±1.45^{a}	34.2.0±0.24^{b}
Galactan	244.0±1.73^{c}	291.0±4.69^{b}	394.0±13.5^{a}	235.0±2.36^{c}
Arabian	25.5±0.90^{c}	110.0±2.67^{b}	182.0±2.98^{a}	7.4±0.20^{d}
Total sugar	304.0±3.07^{c}	439.0±9.93^{b}	683.0±16.6^{a}	276.0±2.77^{c}

Values represent mean±SEM of three replicas (n=3) for each experimental condition. Different superscript letters indicate significant differences among experimental conditions ($P<0.05$).

5.3.5. Agar structure

FTIR spectra of agar obtained from *G. gracilis* under the four different conditions are presented in Fig. 28. All experimental conditions exhibited similar peaks at 715, 740, 770, 890 and 930 cm^{-1} (Baghel, Reddy, & Jha, 2014), 1250 and 1370 cm^{-1}, which are all characteristic peaks of agar. Similarly, the peaks at 715 cm^{-1}, related to C-S stretch, were noted in all the treatments (Coates, 2006). The other two peaks at 740 and 770 cm^{-1} could be associated with skeletal bending of the galactose ring (Rhein-Knudsen et al. 2017). Absorption at 890 cm^{-1} was referred to as the C-H bending, while the peaks at 930 cm^{-1} were related to the C-O vibration of LA units.

The absorption at 1250 and 1370 cm^{-1}, related to total sulphate (S=O stretching vibration and ester sulphates, respectively) (Sousa et al., 2012), was identified in all polysaccharides. No peaks at 805, 830 cm^{-1} and 820 cm^{-1} were detected. The broad absorption region between 1000 and 1100 cm^{-1} as well as the peak at 1150 cm^{-1} was common to all polysaccharides and could be assigned C-O-H bending and to C-O and C-

C stretching (Warren et al. 2013). A peak at 1640 cm^{-1} was assigned to C=O stretching (Selvalakshmi et al. 2017).

The group frequency between 2500 and 2600 cm^{-1} was assigned to the S-H stretching (Coates 2006), however, no peaks were detected in the ATR spectra between 2400 and 2800 cm^{-1} of the agars. The peak at 2920 cm^{-1} corresponded with the C-H, being a good measure of total sugar content (Rochas et al., 1986) and the peak at 2890 cm^{-1} could also be linked to C-H stretching in the methylene groups. The small shoulder at 2850 cm^{-1} was typical of highly methylated agars (Rocha et al., 2019) and was present in all agars, though it seemed to be more pronounced for WD and RD agars.

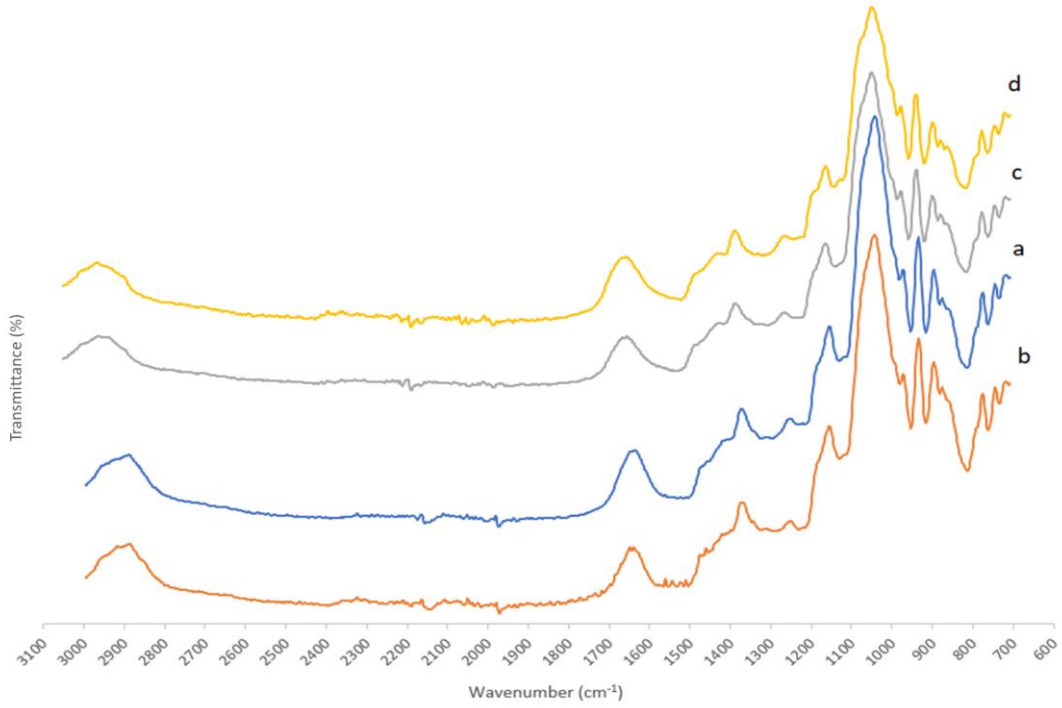

Figure 28 FTIR spectra of agar from *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

5.3.6. *Gracilaria gracilis* protein and carbohydrate contents

Fig. 29 shows the protein (A) and the carbohydrate (B) content in *G. gracilis* under four different conditions. Results showed that light spectra had no significant effect on the protein content of *G. gracilis* (28.97 - 29.46%, $P>0.05$). Regardless of light conditions,

the protein content tended to increase in seaweed farmed in the IMTA system in comparison to the WD condition (25.59%) (P>0.05, Fig. 29A).

Culturing *G. gracilis* in the IMTA system under different light spectra produced a significant effect on the carbohydrate content. The BL condition (78.72 mg Glc Eq. g^{-1} SW) significantly increased the total carbohydrates in comparison to the NT (68.48 mg Glc Eq. g^{-1} SW, P=0.002) and RD (27.41 mg Glc Eq. g^{-1} SW, P<0.001) conditions. Seaweed farmed under RD condition present the lowest total carbohydrates content among all conditions (P<0.05). Concerning the beginning of the trial (67.68 mg Glc Eq. g^{-1} SW), the BL condition increased the total carbohydrate content (78.72 mg Glc Eq. g^{-1} SW), while the RD condition significantly decreased that content (P<0.05). Interestingly, the NT condition did not change the total carbohydrate content in relation to the beginning of the trial (P>0.05, Fig. 29B).

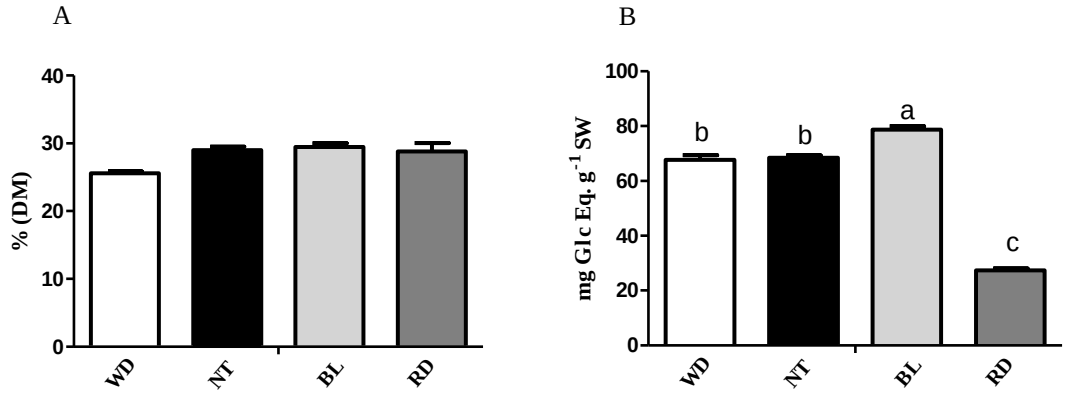

Figure 29 Protein (A) and carbohydrates contents (B) of *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.

Columns represent mean+SEM (n=3-6). Different letters indicate significant differences between conditions after one-way ANOVA analysis (P>0.05).

5.3.7. *Gracilaria gracilis* pigments content

Fig. 30 shows the phycocyanin, phycoerythrin (A) and chlorophyll-*a* (B) contents in *G. gracilis* under the four conditions. The phycocyanin content in *G. gracilis* significantly decreased in farmed *G. gracilis* for all light conditions, representing a 50% decrease in the content of this pigment compared to that measured at the beginning of the growth trial (WD) (P<0.001). No differences were found between light conditions, although *G.*

gracilis farmed under the RD condition appears to show lower phycocyanin content (P>0.05). Phycoerythrin content did not display changes between the four conditions. Chlorophyll-*a* content in *G. gracilis* was not different between the four conditions (P>0.005), although the content of this pigment tended to decrease over the experimental period regardless of light condition.

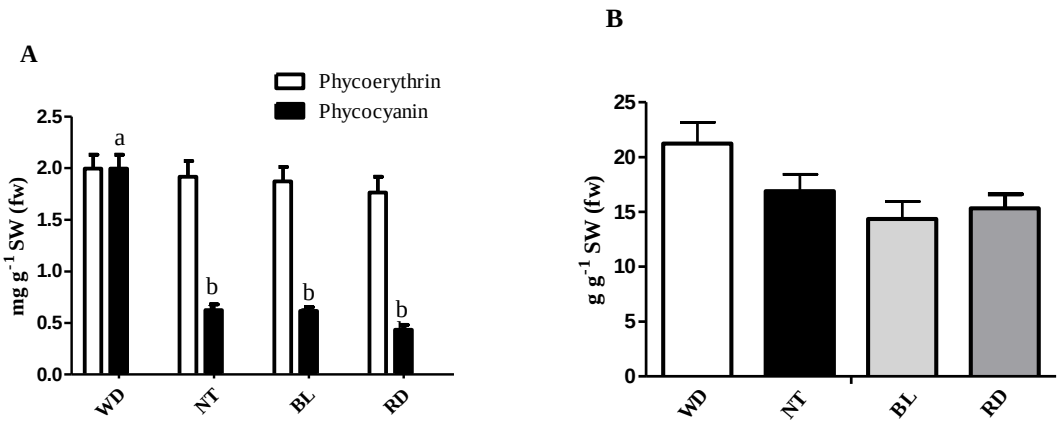

Figure 30 Phycocyanin, phycoerythrin (A) and chlorophyll-*a* (B) contents of *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.
Columns represent mean+SEM (n=10). Different letters indicate significant differences between conditions after one-way ANOVA analysis (P>0.05).

5.3.8. *Gracilaria gracilis* antioxidant activity

Fig. 31 shows the total phenol content (TPC), flavonoid content, antiradical activity (DPPH assay) and lipid peroxidation inhibitory capacity (TBARS assays) of *G. gracilis* obtained from the four conditions. The total phenol content and the flavonoid content measured in seaweed were similar between the four conditions (P>0.05). Also, both antiradical activity and lipid peroxidation inhibitory capacity of *G. gracilis* were not different between the four conditions (P>0.05).

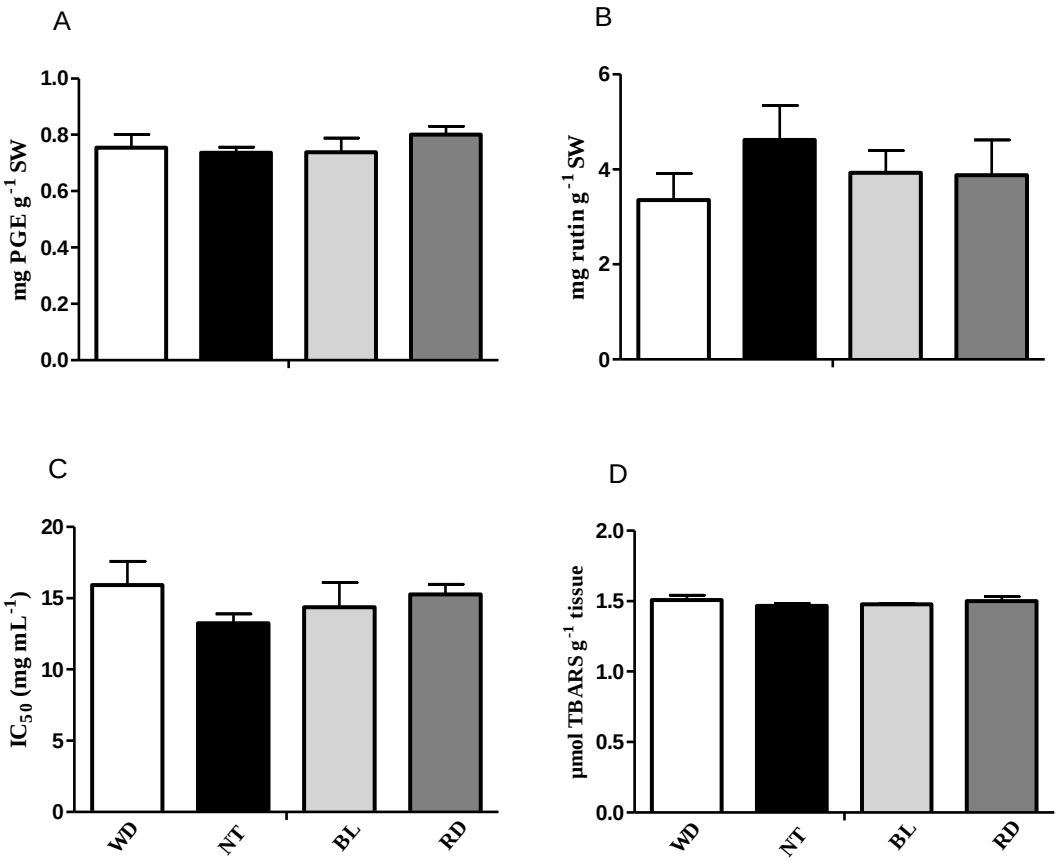

Figure 31 Total phenol content (A), flavonoid content (B), DPPH (C) and TBARS (D) assays of *Gracilaria gracilis* before (WD) and after 4 weeks of growth in an IMTA-system under three different light spectra, using neutral (NT), blue (BL) and red (RD) light filters.
Columns represent mean+SEM, n=10. The absence of letters indicates no significant differences after one-way ANOVA analysis (P>0.05).

5.4. Discussion

The present study revealed a significant modulatory effect of light spectrum on both agar yield and gel strength. The most remarkable result to emerge from the data is that the BL condition increased the agar yield compared to both NT and RD conditions. The results reinforce the previous studies supporting the regulatory effect that blue light has on carbohydrate metabolism (Matthijs et al. 1996; Marchetti et al. 2013; Habibi et al. 2019). In this study, this is shown by an increase in *G. gracilis* carbohydrates content, particularly when the seaweed is farmed under the BL condition. As carbohydrates are the major intermediates in the photosynthetic carbon reduction cycle and the photorespiratory carbon oxidation cycle, the increase observed in the carbohydrate

content may be due to a decrease in the photosynthetic activity (Raven et al. 2004). This outcome is supported by previous studies reporting lower photosynthetic rates in *Gracilaria tenuistipitata* (Mercado et al. 2002), *Porphyra leucosticta* (Korbee et al. 2005) and *Palmaria palmata* (Parjikolaei et al. 2013) farmed under blue light.

The increase of agar gel strength detected in the BL condition (1038 g cm^{-2}) compared to both NT (960 g cm^{-2}) and RD (585 g cm^{-2}) conditions suggest a modulatory effect of light spectrum on agar gelling properties. The increase of agar gel strength observed in *G. gracilis* farmed under the BL condition compared to the WD condition (720 g cm^{-2}, WD) also reinforces such possibility. In this study, the contrast found between BL and RD conditions regarding agar yield and agar gel strength suggests that the light spectrum was the main factor affecting agar properties of seaweed, despite a reduction on the irradiance level in both conditions when compared to the NT condition. The higher gel strength value obtained for *G. gracilis* farmed under the BL condition in this study is a promising result, as are greater than other commercial agars (> 800 g cm^{-2}) (Arvizu-Higuera et al., 2008), suggesting an improvement in the agar quality produced. Interestingly, the agar obtained from the BL condition showed the highest sugar (carbohydrates) content among all the conditions, including also the highest galactose content (main monosaccharide present in agar), thus corroborating the results for the agar strength. A similar relation between carbohydrate content and gel strength was previously described by Ellis et al. (2019), showing that carbohydrates (sucrose) can bind to agarose through hydrogen bonds, decreasing the size and increasing the number of cross-links during gelation.

The agar structure, analysed by the Fourier Transform Infrared Spectroscopy (FTIR), revealed a great similarity between agars extracted from seaweed in the four conditions, suggesting that both IMTA-production and light spectrum did not produce major alterations in the agar structure. This was reinforced by similar values for the T_g and T_m among all experimental conditions. The absence of peaks at 805 and 830 cm^{-1}, the small shoulder at 850 cm^{-1} and the absence of a peak at 820 cm^{-1} indicated very low sulphate content of the agar extracted from *G. gracilis* under the four conditions (Rochas et al. 1986; Romero et al. 2008), which may be linked to the alkali pretreatment.

Agars from all the conditions presented gelling temperatures above 40 °C, which in the commercial agars are typically below 40 °C (Skriptsova et al. 2009). Agars with melting temperatures of ~90 °C (Tm >85 °C) are suitable as agar standards (United States Pharmacopeia, USP) and the agars from all conditions showed temperatures between 93 ºC and 94 °C (Arvizu-Higuera et al., 2008). The agar properties are similar to previous

studies carried out in *Gracilaria tikvahiae* and *Gracilaria vermiculophylla* (Arvizu-Higuera et al. 2008; Villanueva et al. 2010; Rocha et al. 2019).

Changes in phycobiliproteins and chlorophyll-*a* contents of seaweed farmed under different light conditions have been associated with an adaptative response to altered irradiances. These changes involve the accumulation of pigments in red seaweed as a photo-adaptive mechanism (Figueroa et al. 1995; Wu 2016; Chen et al. 2019; Borlongan et al. 2020) which could improve their photosynthetic efficiencies and growth (Wu 2016). However, in the present study, pigment content (phycobiliproteins and chlorophyll-*a*) in farmed *G. gracilis* was unchanged by differences in the light spectrum despite BL and RD inducing a reduction in the irradiance levels in the tanks. Thus, the lack of changes in photosynthetic apparatus (pigment content) may sustain the decrease found in the growth performance of seaweed growing under lower irradiance (BL and RD) when compared to the NL condition. Similarly, others studies have shown that LEDs providing blue light (at 350- 500 nm wavelength) result in decreased growth in *Palmaria palmata* (Barufi et al. 2015), *Halymenia foresii* (Godínez-Ortega et al. 2008), *Porphyra leucosticta* (Korbee et al. 2005) and *Gracilaria tikvahiae* (Kim et al. 2015) under laboratory conditions. Lower growth of seaweed under the blue light spectrum may be due to low photosynthetic efficiency, possibly at the water-splitting complex of the PSII and/or a lower carbon utilization (Hanelt et al. 2003; Korbee et al. 2005). The phycocyanin content in *G. gracilis* was significantly higher in the WD condition than in the NT, BL, or RD conditions. This result suggests that *G. gracilis* under the WD condition, before being introduced into the IMTA system, may have displayed changes in their photosynthetic apparatus to optimize light absorption (e.g., increasing phycocyanin content), perhaps to minimize the risks associated with high solar radiation and air exposure, as seaweed in our study was collected during low tide. A similar scenario has been suggested for others seaweed in natural environments (Figueroa et al. 1995; Figueroa et al. 2010).

The TPC level of *G. gracilis* measured in this study (0.74 - 0.80 mg PGE g^{-1} SW) was within the range of values reported for this seaweed (0.49 - 1.46 mg gallic acid equivalent g^{-1} SW, mg GAE g^{-1} SW) in independents growth trials but using the same IMTA-system (Neto et al. 2018; Silva et al. 2019). Therefore, the TPC level in farmed *G. gracilis* can vary for a given IMTA-system, implying that further studies should assess the potential factors associated with changes on this and other compounds present in *G. gracilis* and that could be linked to antioxidant activity.

The antioxidant activity reported in the present study for *G. gracilis* under the four different conditions was ~15 mg mL^{-1}, which was between the values reported to *G. gracilis* (76.27 mg mL^{-1}) (Reboleira et al. 2020) and *Gracilaria corticata* (60-70 µg mL^{-1}) (Sundaramurthy et al. 2017), reinforcing that *G. gracilis* has a considerable antioxidant activity aiming for future applications. As the TFC reported in the current study was lower than that detected in *Gracilaria corticata* (26.18±0.34 µg quercetin equivalent (EQ)) (Sundaramurthy et al. 2017) it may be plausible that other compounds than flavonoids may be involved in the antioxidant activity measured in *G. gracilis*.

In this study, growing *G. gracilis* under different light conditions in an IMTA-system did not result in changes in both total polyphenols and flavonoid levels, neither was reflected in differences in the antioxidant activity. Other studies growing seaweed under N supplemented systems and exposing them to UV light resulted in a significant increase of phenolic compounds and antioxidant activity (DPPH scavenging activity) in several red seaweeds (Duval et al. 1999; Barufi et al. 2011; Bonomi Barufi et al. 2012; Zubia et al. 2014; Álvarez-Gómez et al. 2019). Such differences were expected, as UV light induces both DNA and protein damage with deleterious effects on seaweed if antioxidant mechanisms are not induced to preserve the structure and functions of these biomolecules (Bischof et al. 2003). This may suggest that a different light condition than that applied in the current study (e.g., UV) may be required to induce significant changes in the antioxidant activity of farmed *G. gracilis*.

Conclusions

This study showed that culturing *G. gracilis* in an IMTA-system after applying the BL condition for 4 weeks resulted in improved agar yield and gel strength, both key attributes in agar production. Light conditions did not induce changes in seaweed pigment content nor in the antioxidant activity, although growth was decreased in the BL condition when compared to the NT condition. Despite this limitation, BL condition may prove useful if applied at later stages in the seaweed production cycle in IMTA-systems, as a means of modulating agar properties when seaweed biomass production reaches target levels. Finally, the residue resulting from agar extraction (AW) contains a suitable antioxidant activity. Futures studies should focus on AW supplementation in animal feeds as a means of improving the economic and ecological sustainability of the aquaculture and hydrocolloid industries.

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CHAPTER 6

Dietary supplementation with *Gracilaria gracilis* by-products modulates the immune status and oxidative stress response of gilthead seabream (*Sparus aurata*) stimulated with *Photobacterium damselae* subsp. *piscicida*

Francisca Silva-Brito[1,2*]; Alexandra Cardoso[3], Marina Machado[1], Lourenço Ramos-Pinto [1], Mariana Hinzmann [1]; Helena Abreu[4]; Benjamín Costas[1,2], Leonardo Magnoni[1]

[1]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208, Matosinhos, Portugal.

[2]ICBAS - Institute of Biomedical Sciences of Abel Salazar, University of Porto, Rua de Jorge Viterbo Ferreira, 225, 4050-313, Porto, Portugal.

[3] FCUP - Faculty of Sciences, University of Porto, Porto, Portugal, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal.

[4]ALGAplus - Produção e Comercialização de Algas e seus Derivados, Rua do Mar s/n, 3830-165, Ílhavo, Portugal.

*Corresponding author: fisbrito@gmail.com

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ABSTRACT

This study evaluated the effects of agar waste (AW) dietary supplementation, obtained from the seaweed *Gracilaria gracilis* cultivated under two different spectral lights, neutral (NT) and blue (BL), on haematological parameters, inflammatory response, and antioxidant biomarkers of gilthead seabream (*Sparus aurata*). Three diets were prepared: *i*) a basal diet (CTR), *ii*) a diet supplemented with 2.5% NT, and *iii*) a diet supplemented with 2.5% BL. After 15 days of feeding, fish were injected with PBS (placebo) or inactivated *Photobacterium damselae* subsp. *piscicida* (stimulated) and sampled at 4h and 24h post-stimulus.

Results indicated that fish fed NT and BL supplemented diets had lower Ht value and mean corpuscular volume (MCV) than fish fed the CTR diet, regardless of the stimulus and the sampling time. No differences in mean corpuscular haemoglobin (MCH) were found between fish fed the different diets, while the mean corpuscular haemoglobin concentration (MCHC) increased in fish fed AW supplemented diets compared to fish fed the CTR diet, regardless of the stimulus and the sampling time. In response to inflammation, fish fed the NT diet displayed higher neutrophils count in blood when compared to the CTR group, regardless of the stimulus and the sampling time. Thrombocyte count was higher in fish fed NT and BL diets than in the CTR group, especially in the stimulated fish (Diet*injection (D*I), P=0.004). An increase in plasma protease activity was detected in fish fed NT or BL diets in both placebo and stimulated fish regardless of the sampling time. Hepatic catalase activity was higher in fish fed the NT and BL than in the CTR group, particularly in the stimulated fish (D*I, P<0.001). Also, both stimulated and placebo fish that received the BL diet showed an increase in hepatic GR activity compared to the CTR group, regardless of the sampling time.

Dietary supplementation with AW by-products obtained from *G. gracilis* cultured under NT and BL conditions showed to improve the inflammatory and antioxidant mechanisms in gilthead seabream in response to a UV-killed bacterial stimulus, having valuable applications for the sustainable use of seaweed toward improving the health and welfare of cultured fish.

Key-words: Agar waste, immunomodulation, antioxidant system; *Gracilaria gracilis*; sustainability.

6.1. INTRODUCTION

Antibiotics have been the first line of defence in aquaculture fish to control disease outbreaks. However, the sustained use of antibiotics in fish has led in various cases to bacterial resistance (Vignesh et al. 2011; Chen et al. 2020), raising as well concerns on food safety and environmental risks (WHO 2000). With this in mind, an increasing number of studies have focused on ways to apply preventive measures to avoid the occurrence of diseases in cultured fish rather than treating them (Subasinghe et al. 2004). Boosting the fish's natural defence through the prophylactic administration of immunostimulants and antioxidant supplements can result in beneficial and practical preventive actions available to the producers (Labh et al. 2014), essential to avoid economic losses when increasing animal welfare as well as the public acceptance of cultured fish.

Several seaweeds species have been shown to have immunostimulant and antioxidant properties, being in the spotlight to improve robustness of farmed fish without compromising growth when supplemented into the diets (Pavaraj et al. 2011; Peixoto et al. 2016; Lobo et al. 2018; Palstra et al. 2018; Peixoto et al. 2019a). However, increased demand in the use of seaweeds for multiple applications had driven efforts to find sustainable ways to produce them by culturing without over-exploiting the wild stocks. In Portugal, *Gracilaria gracilis* have been cultured in a sustainable form using an Integrated Multitrophic system (IMTA), involving the recycling of the nutrients included in the water-waste generated by fish farming (uneaten food and excretion products) to enhance seaweed production. This kind of production has beneficial impacts on the economic, environmental, and social sustainability of this activity (Troell et al. 2009; Sasikumar et al. 2015).

Gracilaria sp. biomass is mainly used for agar production, generating globally 9600 tons of agar per year (Bixler et al. 2011; Porse et al. 2017). The residue obtained from the agar extraction process (AW) represents 30% of the initial seaweed processed biomass (Meinita et al. 2017). However, only 40% of the generated AW is used for applications including paper, bioethanol, and fertilizers production (Ennouali et al. 2006; Meinita et al. 2017), with a considerable quantity being available for other applications, such as the production of aquafeeds. A recent study reported that feeding gilthead seabream (*Sparus aurata*) diets supplemented with 2.5% AW for 59 days resulted in an improved humoral immunity response and displaying reduced plasma cortisol levels when fish are subjected

to crowding stress. These results suggest that bioactive compounds contained in seaweed are present in AW and these have beneficial effects on the immune status, by improving fish robustness and welfare (Silva-Brito et al. 2020). However, the potential effects of dietary AW supplementation in gilthead seabream when exposed to a bacterial stimulus remains to be investigated. The bacteria selected for this study, *Photobacterium damselae* subsp. *piscicida* (*Phdp*), is the etiological agent of photobacteriosis, a threatening disease for marine fish species such as gilthead seabream (*Sparus aurata*) (Andreoni et al. 2014), one of the most important species in Mediterranean aquaculture (Manchado et al. 2016). Despite its economic value, the average mortality rate in the gilthead seabream farms of the Mediterranean is approximately 10–15%, although in some fish farms mortality reached 30% (Doménech et al. 1997). The fact that this species is an opportunistic feeder that consumes seaweeds as part of its natural diet (Arias 1980; Pita et al. 2002), including *Gracilaria* species (Pita et al. 2002), makes it ideal to study the immunomodulatory effect of SW.

SW growth, composition, and the synthesis of bioactive compounds can be influenced by light condition and other environmental factors (Barufi et al. 2015; Bonomi Barufi et al. 2015; Álvarez-Gómez et al. 2017; Álvarez-Gómez et al. 2019). Modulating and controlling the conditions in which seaweed are cultured may allow tailoring its production towards different applications. A recent study showed that growing *Gracilaria gracilis* in an IMTA system using filtered sunlight resulted in improved agar yield and agar gel strength without modifying the antioxidant content or properties in the AW generated (Silva-Brito et al. 2021) However, to the best of our knowledge, the immunostimulants properties of the AW generated from this cultured SW when supplemented in aquafeeds remain to be investigated, having in mind the modulation of the antioxidant response and immune status in cultured fish exposed to a bacterial stimulus.

The present study evaluated the effects that short-term dietary supplementation with 2.5% AW obtained from *Gracilaria gracilis* cultured in two different sunlight conditions, neutral (NT) or blue (BL), may have on gilthead seabream stimulated with inactivated *Phdp*. Specifically, it was assessed how feeding gilthead seabream an AW supplemented diet can modulate the immune status and antioxidant response by evaluating several parameters in plasma and tissues of fish at 4h and 24h post-stimulus.

6.2. MATERIALS AND METHODS

This study was conducted under the supervision of accredited experts 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 CIIMAR aquatic care facilities (BOGA). Experiments were designed to comply with the ARRIVE guidelines (*Animal Research*: *Reporting* of In Vivo Experiments) for reporting animal research. The procedures implemented in the study were no likely to cause fish suffering, being equivalent to established procedures (weighing, grading, and transporting) applied in the culture of this species. No mortality was detected during the experimental trial.

6.2.1. Seaweed origin and processing

A preliminary experiment was carried out at the ALGAplus Lda company (Ilhavo, Portugal) to produce *Gracilaria gracilis* in an Integrated Multi-Trophic Aquaculture (IMTA) system growing the seaweed under two different spectral light conditions by using a neutral filter (NT) or a blue filter (BL). The details of culture conditions were described by Silva-Brito et al. (2021) After 4 weeks of cultivation, the SW produced under both light conditions were dried, following the procedure established by the company, and transported to the Laboratory of Clean Technologies, University of Minho, Portugal, to perform the agar extractions. The procedure of agar extractions was previously described by Silva-Brito et al. (2021). The by-product resulting from the agar extraction processes were stored in plastic bags for the preparation of the experimental diets.

6.2.2. Experimental diets

A commercial diet was formulated and produced by SPAROS Lda. (Olhão, Portugal) and it was used as a basal diet to prepare the experimental diets. At the Sciences Faculty of University of Porto facilities, the basal diet was grounded below 500 µm in a hammer mill (Retsch GmbH, Haan, Germany) to prepare three experimental diets: *i*) a control diet (CTR, commercial diet), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5%

NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL). Each experimental diet was mixed accordingly to the specified proportion in a paddle mixer (*Alexanderwerk* Inc.). All the experimental diets were dry-pelleted in a laboratory pellet mill through a 5.0 mm die (California Pellet Mill, CPM Crawfordsville, IN, USA). Ingredients of the experimental diets are presented in Table 20.

Table 20 Ingredients of the experimental diets.
i) A control diet (CTR), *ii)* a diet with 2.5% agar waste from NT condition (2.5% NT) and *iii)* a diet with 2.5% agar waste from BL condition (2.5% BL).

Ingredients (%)	**CTR**	**2.5% NT**	**2.5% BL**
Fishmeal LT70	10.5	10.2	10.2
Fishmeal 60	15.0	14.6	14.6
Soy protein concentrate	12.0	11.7	11.7
Wheat gluten	6.0	5.9	5.9
Corn gluten	10.0	9.8	9.8
Soybean meal 48	12.0	11.7	11.7
Rapeseed meal	5.0	4.9	4.9
Wheat meal	12.7	12.6	12.6
Fish oil	4.4	4.3	4.3
Rapeseed oil	10.2	10.0	10.0
Vit & Min Premix	1.0	1.0	1.0
Antioxidant (Verdilox - Kemin)	0.2	0.0	0.0
MCP	0.6	0.6	0.6
DL-Methionine	0.2	0.2	0.2
L-Taurine	0.2	0.2	0.2
Agar waste from NT condition	-	2.5	-
Agar waste from BL condition			2.5

6.2.3. Bacteria inactivation

Photobacterium damselae subsp. *piscicida* (*Phdp*), strain PP3 was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and

isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). To prepare the inoculum for intraperitoneal (i.p.) injection, stocked bacteria were cultured for 48 h at 22 °C on tryptic soy agar supplemented with NaCl to a final concentration of 1% (w/v, TSA-1) and then inoculated into tryptic soy broth supplemented with NaCl to a final concentration of 1% (w/v, TSB-1) and cultured overnight at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation (3 500 × *g* for 30 min), resuspended in sterile HBSS, and adjusted to 5×10^5 colony forming units (cfu) ml^{-1} according to Machado et al. (2015). Bacteria were then killed by UV exposure for 2h. Loss of bacterial viability following UV was confirmed by plating resulting cultures on TSA-1 plates and failing to see any bacterial growth.

In this study, the use of the inactivated bacteria represented an efficient strategy to study the inflammatory response mechanisms with minimal negative impacts on fish welfare, as inactivated bacteria did not induce the disease (Lee et al. 2012).

6.2.4. Feeding Trial

Gilthead seabream were provided by Pedro Pousão (Fish Farming Pilot Station, Olhão, Portugal), and maintained for 15 days at the aquatic care facilities (BOGA) of CIIMAR under controlled conditions (see below) before starting the trial. After the quarantine period, a group of 96 fish (~70 g) was randomly distributed into 12 tanks (87 L, 6 kg m^{-3}). Photoperiod regime was 14h:10h (L:D) and water physical-chemical parameters were fixed and monitored every day, including (mean±SEM) temperature (18.05±0.04 ºC), salinity (35.12±0.07 ‰), pH (7.37±0.05), and dissolved oxygen (>95%). All the experimental tanks were connected to a common recirculation aquaculture system (RAS) with a water renewal rate of 257 L h^{-1}. Water ammonium and nitrite levels in the tanks were maintained below the limits for the species (<0.05 mg L^{-1} and <0.5 mg L^{-1}, respectively).

Each diet was tested in quadruplicate, being randomly assigned to each tank. Fish were hand-fed twice per day (9:00 h and 16:00 h) a daily amount calculated as 2.5% of their body weight. The feeding trial was performed for 15 days to be the required time to induce a dietary modulatory effect on the innate immune response during an inflammatory response (Falco et al. 2012; Pionnier et al. 2013).

6.2.5. Bacterial stimulus trial

At the end of the 15 days feeding trial all the fish in each tank (8 individuals) were fasted for 24h and anaesthetized with MS-222 (0.05 g L^{-1}) buffered with $NaHCO_3$ (0.1 g L^{-1}). Four fish from each tank were intraperitoneally injected with 100 µl of PBS (placebo group) or UV-inactivated *Phdp* (UV-inactivated *Phdp*, 5×10^4 cfu mL^{-1}, stimulated group).

Fish in these two groups (placebo and stimulated) were re-allocated in different tanks connected to a RAS system with similar conditions as the RAS system used for the feeding trial (20±0.5°C, 35 ‰, pH 7.4, 14h: 10h L: D). Each tank was housing 8 fish in total, by combining 4 fish from 2 different tanks, which were previously fed the same diet. This redistribution of fish during the bacterial stimulus trial resulted in duplicate tanks per experimental condition, which consisted of 3 dietary groups including fish in the placebo (6 tanks) or stimulated groups (6 tanks).

6.2.6. Samples collection

Four fish per tank (8 fish per experimental condition) were sampled at 4h or 24h post-injection and euthanized by an overdose of anaesthetic (MS-222). The blood was sampled from the caudal region with heparinized syringes. One part of the blood was used for haematological analysis and the remaining blood was centrifuged at 10 000 × *g* for 5 min and plasma stored at –80 °C until analyses. Peritoneal exudates were collected according to the procedure described by Machado et al. (2015) with some modifications. Two millilitres of cold HBSS supplemented with 30 U heparin was injected intraperitoneally in the ventral midline, midway between the pelvic and pectoral fins. Then, the abdominal area was massaged for about 30s to disperse the peritoneal cells in the injected PBS. The intraperitoneal PBS containing suspended cells was collected with a syringe fitted with a 19 gauge needle and placed in ice until processed. Haemorrhagic peritoneal washings were discarded.

The liver and the anterior (AI) and posterior (PI) sections of the intestine were collected and immediately frozen in liquid nitrogen. AI was taken as a segment that starts immediately after pyloric caeca and finishes in the middle of the intestine while the PI was taken as a segment from the middle of the intestine until the rectum (Calduch-Giner et al. 2016).

6.2.7. Haematological parameters

Immediately after blood collection, the haematological profile was evaluated and consisted in this study were total white (WBC) and red blood cell (RBC) counts, haematocrit (Ht), and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were also calculated as follows:

$$MCV\ (\mu m^3) = (Ht/RBC) \times 10$$

$$MCH\ (pg\ cell^{-1}) = (Hb/RBC) \times 10$$

$$MCHC\ (g\ 100\ mL^{-1}) = (Hb/Ht) \times 100$$

6.2.8. Differential blood cells count

After blood collection, blood smears were prepared by air drying, fixing with formol-ethanol (10% of 37% formaldehyde in absolute ethanol). Afterward, detection of peroxidase activity was carried out as described by Afonso et al. (1998) to facilitate the detection of neutrophils. Blood smears were then stained with Wright's stain (Haemacolor; Merck) and examined (1000×), and at least 200 leukocytes were counted per slide and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. The relative percentage and absolute value ($\times 10^4\ \mu l^{-1}$) of each cell type were calculated.

6.2.9. Humoral Innate Immune-related parameters in plasma

6.2.9.1. Peroxidase activity

The peroxidase activity in plasma was quantified according to Quade et al. (1997) with some modifications. Briefly, plasma (1:10 diluted with HBSS without Ca^{+2} or Mg^{+2}) was transferred to a flat-bottomed 96-well plate. Then 50µl of 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, 20 mM) and H_2O_2 (5 mM) were added as substrates. The reaction was stopped after 2 min by adding H_2SO_4 (2 M). The OD was read at 450 nm in a microplate reader (BioTek Instruments, Inc., USA). HBSS instead of plasma was used as a blank. One unit of activity was defined as a change of 1 unit of absorbance and expressed per mL^{-1} of plasma.

6.2.9.2. Protease activity

Protease activity in plasma was measured according to Guardiola et al. (2016) with some modifications. Samples were diluted with phosphate-buffered saline (PBS) and 125 µL of 2% azocasein (Sigma-Aldrich) was added and incubated for 24h at room temperature. The reaction was stopped by adding 250 µL of 4.6% trichloroacetic acid (TCA, 4 ºC) and the mixture was incubated for 30 min at room temperature and centrifuged (10 000 *x g*, 10 min). An aliquot of 100 µL of the supernatant was transferred to a 96-well plate containing 100 µL of 1 N NaOH per well. The OD was read at 450 nm using a microplate reader (BioTek Instruments, Inc., USA) and measured in triplicates. Samples were replaced by PBS and 10 µL of trypsin (5 mg mL^{-1}, Sigma) or just PBS to evaluate 100% and 0% of protease activity, respectively.

6.2.9.3. Antiprotease activity

Total antiprotease activity was determined measured according to Machado et al. (2015) with some modifications. Briefly, aliquots of 110 µL of plasma were incubated for 10 min at 22 ºC with 10 µL of standard trypsin solution (5 mg mL^{-1}), and then 80 µL of PBS was added along with 125 µL of azocasein (Sigma-Aldrich) and incubated for 1h at room temperature. The reaction was stopped by adding 250 µL of 4.6% TCA (4 ºC). The mixture was incubated for 30 min at room temperature and centrifuged (10 000 *x g*, 10 min). An aliquot of 100 uL of the supernatant was transferred to a 96-well plate in triplicate containing 100 µL of 1 N NaOH per well. The OD was read at 450 nm using a microplate reader (BioTek Instruments, Inc., USA). The sample was replaced by PBS in positive controls (100% protease), and both sample and trypsin were replaced by PBS in negative controls (0% protease). The percentage of inhibition of trypsin activity for each sample was calculated.

6.2.9.4. Haemolytic alternative complement pathway

Haemolytic alternative complement (ACH) activity was evaluated as previously described by Sunyer et al. (1995) with some modifications. Three buffers were prepared: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatine; EDTA-GVB,

as the previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{2+} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were washed four times in GVB and resuspended in the same buffer to a concentration of 1.93×10^8 cells mL^{-1}. Then, 10 μL of RaRBC suspension was added to 40 μL of serially diluted plasma in Mg-EGTA-GVB buffer in duplicates. The samples were then incubated for 120 min at room temperature with continuous shaking. After this period, 150 μL of EDTA-GVB at 4º C was added to stop the reaction. Finally, samples were centrifuged for 2.5 min at 120 × *g* and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a microplate reader (BioTek Instruments, Inc., USA). The ACH units were defined as the concentration of plasma inducing 50% haemolysis of RaRBC (ACH_{50}).

6.2.10. Markers of oxidative stress in the liver, anterior and posterior intestine

The liver and the anterior and posterior sections of the intestine were homogenized in 0.1 M phosphate buffer (pH 7.4) in a ratio of 1:10. Protein concentration was assayed in homogenates using bovine serum albumin as a standard (Bradford 1976). Lipid peroxidation (LPO) was quantified by using thiobarbituric acid as a substrate (Ohkawa et al. 1979). Catalase (CAT, EC 1.11.1.6.) activity was quantified using hydrogen peroxide (30%) as a substrate (Claiborne 1985). Glutathione reductase (GR, EC1.8.1.7) and glutathione peroxidase (GPx, EC 1.11.1.9.) activities were measured based on NADPH (Sigma, Portugal) oxidation at 340 nm (Mohandas et al. 1984; Cribb et al. 1989). Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate (Habig et al. 1974). Total and oxidized glutathione levels (TG and GSSG) were measured by the concomitant reaction of the reduced glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB) and read at 412 nm (Baker et al. 1990). In the GSSG assay, 2-Vinyl-pyridine was used to remove all the free thiols present in the sample leaving only GSSG (Griffith 1980). The GSH level was calculated as the result of subtracting the amount of GSSG to the TG. Changes in absorption were measured at 25°C in a Power-Wave™ microplate spectrophotometer (BioTek Instruments, Inc., USA), and reactions were performed in duplicates. Finally, the GSH/GSSG ratio was calculated.

6.2.11. Statistical Analysis

Statistical analysis was performed using Sigma Stat 3.1. Data were checked for normality and homogeneity of variances (Shapiro-Wilk test and Brown-Forsyth test, respectively). A three-way ANOVA was performed to analyse the effect of experimental diet (D), injection (I), and sampling time (T) on haematological parameters, humoral innate immune, and antioxidant responses. When the effects of the factors were significant, the Holm-Sidak method was applied. Significant differences were considered when $P<0.05$. The results are expressed as the Least square means (LSM)±standard error of the LSM (SE).

6.3. RESULTS

6.3.1. Haematological parameters

The effects of the diets on the haematological parameters of gilthead seabream at 4h and 24h post-stimulus are presented in Fig. 32. The outcome of the three-way ANOVA analysis is presented in Table 21. Ht value was significantly lower in fish fed the BL diet compared to fish fed the CTR ($P<0.001$) or NT diets ($P<0.001$) (Fig. 32A). Also, fish fed the NT diet presented a lower Ht value than those fed the CTR diet ($P=0.004$). Stimulated fish showed lower Ht than the placebo group for all the dietary groups. No differences between 4h and 24h were detected in both placebo and stimulated fish, regardless of the diets ($P>0.005$).

The RBC count was similar between the dietary groups ($P>0.05$) (Fig. 32B). On the other hand, the RBC count significantly decreased in the blood of stimulated fish compared to the placebo group ($P=0.006$). This haematological parameter was higher at 24h than at 4h ($P<0.001$), regardless of the diet.

The MCV was higher in fish fed the CTR diet compared to fish fed the NT ($P=0.002$) or BL diets ($P=0.001$) (Fig. 32C). However, the MCV value was similar between fish fed the NT and BL diets ($P>0.05$). Additionally, stimulated fish showed lower MCV than in the placebo group ($P<0.001$), regardless of the diet and the sampling time.

The MCH was not significantly modulated by the diets, neither nor by the sampling time ($P<0.05$) (Fig. 32D). On the other hand, the MCH was significantly decreased in the stimulated fish compared to the placebo group ($P=0.024$).

The MCHC increased in fish fed the BL diet compared to the CTR group, regardless of the bacterial stimulus ($P<0.001$) (Fig. 32E). Fish fed the NT diet displayed similar MCHC values to those fed the CTR and BL diets ($P>0.05$). For all dietary treatments, the MCHC was higher at 24h than at 4h ($P=0.008$). Also, bacterial stimulus increased the MCHC in fish regardless of the diets ($P=0.013$).

Hb concentration was not significantly modulated by the diets ($P>0.05$), remaining unchanged by the bacterial stimulus as well ($P>0.05$) (Fig. 32F). On the other hand, the Hb concentration increased at 24h in all the treatments ($P<0.001$).

Table 21 Three-way ANOVA analysis results for haematocrit, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) and haemoglobin in the blood of gilthead seabream.
Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (Placebo) or inactivated *Phdp* (stimulated).

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
Haematocrit	0.222	**<0.001**	**0.014**	0.172	0.254	0.274	0.943
RBC	**<0.001**	0.245	**0.006**	0.832	0.222	0.284	0.602
MCV	0.419	**<0.001**	**<0.001**	0.749	0.951	0.139	0.777
MCH	0.413	0.307	**0.020**	0.653	0.392	0.071	0.148
MCHC	**0.008**	**0.001**	**0.013**	0.428	0.921	0.648	0.143
Haemoglobin	**<0.001**	0.900	0.526	0.123	**0.041**	0.591	0.408

Values represent the *P*-value.

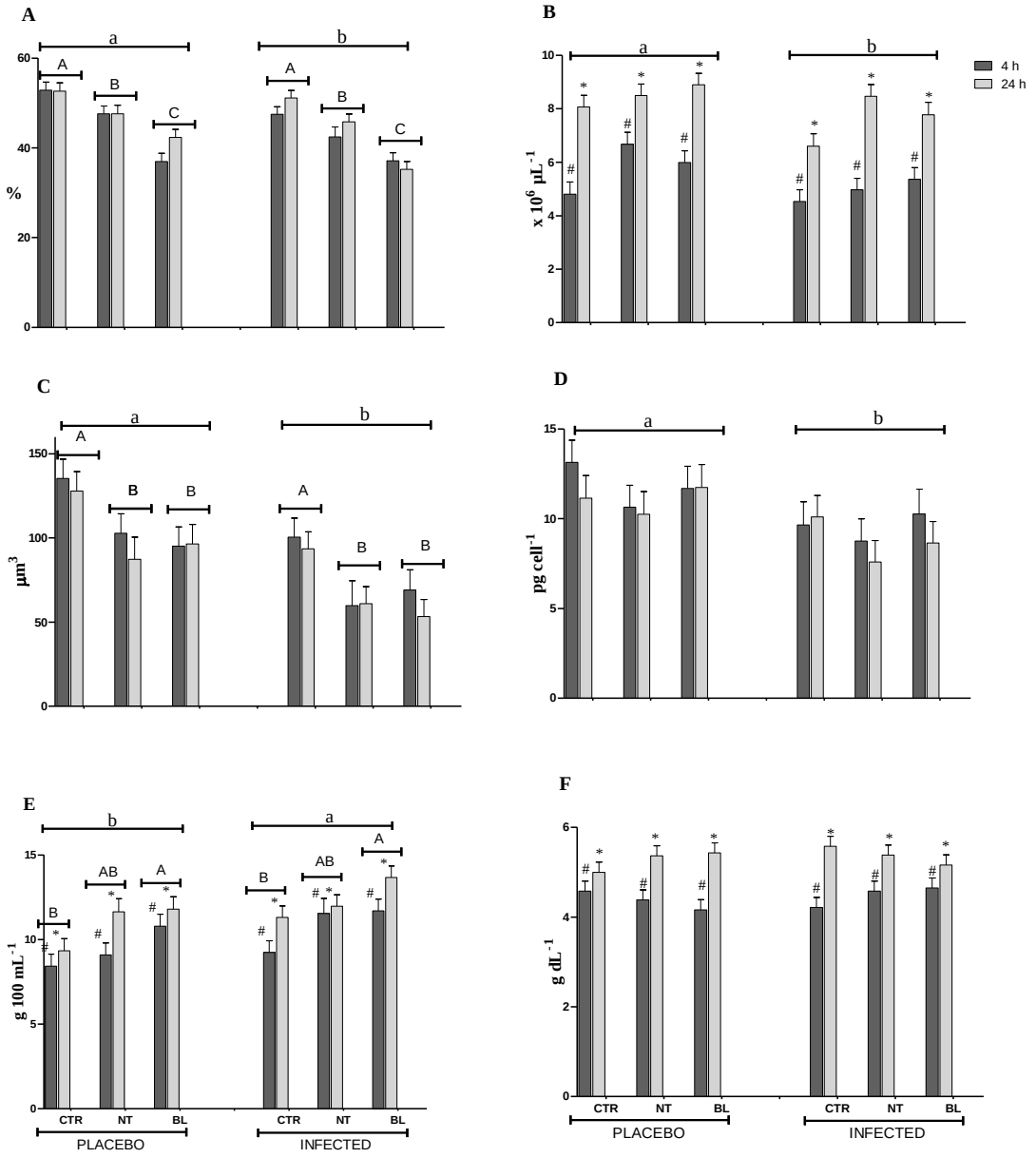

Figure 32 A) Haematocrit, B) red blood cells (RBC), C) mean corpuscular volume (MCV), D) mean corpuscular haemoglobin (MCH), E) mean corpuscular haemoglobin concentration (MCHC), and F) haemoglobin of gilthead seabream at 4h and 24h post-injection with PBS (placebo) or *Phdp* (stimulated).

Fish were fed with three diets: *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL). Values are presented as LSM+SE (n = 6-8). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-

stimulus, regardless of the diet and the stimulus. Significant differences were considered when $P<0.05$.

6.3.2. Total peritoneal and differentiated peripheral leukocytes counts

The effects of the diets on the white blood cells (WBC), differentiated peripheral leukocytes as well as the total peritoneal leukocytes counts of gilthead seabream at 4h and 24h post-stimulus are presented in Fig. 33. The outcome of the three-way ANOVA analysis is presented in Table 22.

The experimental diets did not impact the WBC count in gilthead seabream ($P>0.05$), although it was lower in stimulated fish ($P<0.05$) (Fig. 33A). On the other hand, the WBC count significantly increases at 24h in placebo and stimulated fish ($P<0.001$).

The various peripheral leukocytes were differently modulated by the diet and bacterial stimulus in the fish blood. The neutrophil count was positively modulated in fish fed the NT diet compared to the CTR group ($P=0.015$) for both sampling times, but no significant differences were detected between the CTR and BL groups (Fig. 33B). In addition, stimulated fish displayed an increase in the number of neutrophils compared to the placebo group, regardless of the diet and the sampling time ($P=0.022$). Regarding monocyte count, these cells were not modulated by the experimental diets either nor sampling time ($P=0.05$) despite the bacterial stimulus increased its number ($P=0.003$) (Fig. 33C). The BL diet displayed a higher lymphocytes count than NT and CTR, only in the placebo group at 4h post-stimulus (D*T*I, $P=0.029$; Fig. 33D). In addition, the number of lymphocytes at 4h was higher than at 24h post-stimulus ($P<0.001$), regardless of the diet and the stimulus. The infection reduced the lymphocyte count, especially at 4h post-stimulus, regardless of the experimental diets (T*I, $P=0.030$). The thrombocyte count increased in the blood of fish in the BL ($P=0.019$) and NT groups ($P=0.007$) in relation to the CTR diet, but only in the placebo group. In addition, the number of thrombocytes was higher at 4h than at 24h, regardless of the diet and the bacterial stimulus ($P<0.001$). Also stimulated group presented higher thrombocytes than placebo fish ($P=0012$) (Fig. 33E).

The total peritoneal leukocyte count was reduced by the BL diet compared to the NT diet ($P=0.017$) (Fig. 33F), regardless of the challenge and the sampling time. At 24h post-stimulus, the peritoneal leukocytes count of fish fed BL diet was significantly lower than those fed the CTR ($P=0.008$) and NT diets ($P=0.000$), regardless of the challenge (T*D,

P=0.025). Stimulated fish presented a higher leukocyte count than the placebo group, regardless of the diet and the sampling time (*P*<0.001).

Table 22: Three-way ANOVA analysis results for white blood cells (WBC), neutrophils, monocytes, lymphocytes and thrombocytes counts in the blood, and the total peritoneal leukocyte count of gilthead seabream.

Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (Placebo) or inactivated *Phdp* (stimulated).

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
WBC	**<0.001**	0.196	0.079	0.711	0.160	**0.036**	0.554
Neutrophils	0.301	0.178	**0.022**	0.523	**0.028**	0.327	0.698
Monocytes	0.621	0.540	**0.003**	0.929	0.380	0.133	0.919
Lymphocytes	**<0.001**	0.906	0.403	0.828	**0.030**	0.255	**0.029**
Thrombocytes	**<0.001**	**0.014**	**0.012**	0.189	0.509	**0.021**	0.780
Peritoneal leukocytes	0.645	**0.027**	**<0.001**	**0.025**	0.743	0.213	0.920

Values represent the *P*-value.

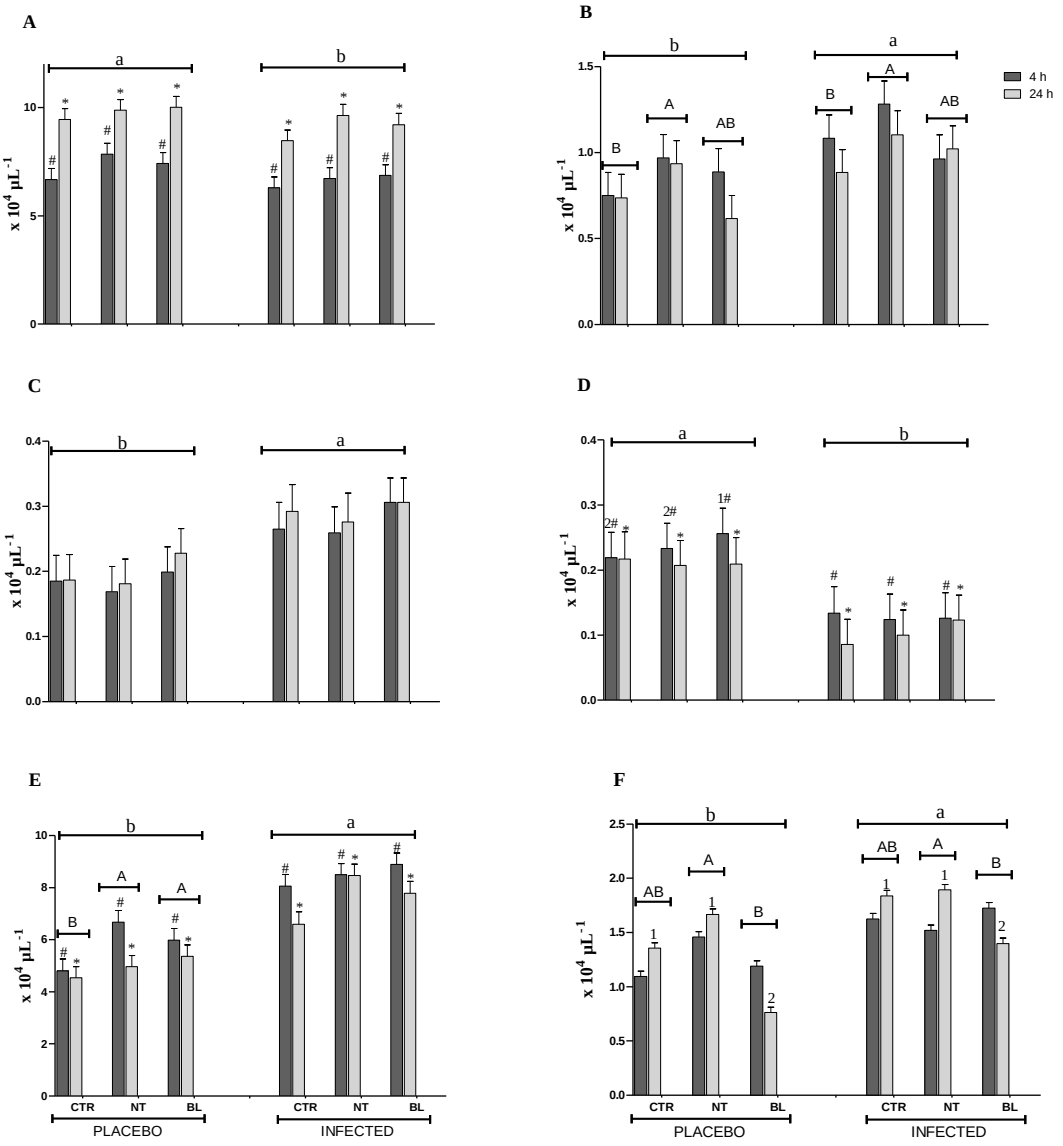

Figure 33 A) White blood cells (WBC), B) neutrophils, C) monocytes, D) lymphocytes, E) thrombocytes counts in the blood, and F) absolute values of the total peritoneal leukocytes of gilthead seabream at 4h and 24h post-injection with PBS (placebo) or Phdp (stimulated).

Fish were fed with three diets: *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) Values are presented as LSM+SE (n = 6). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-stimulus, regardless of the diet and the stimulus. The numbers 1 and 2 represent significant differences between experimental diets within a specific sampling time. Significant differences were considered when $P<0.05$.

6.3.3. Humoral innate immune response

The effects of the diets on the humoral innate immune response of gilthead seabream at 4h and 24h post-stimulus are presented in Fig. 34. The outcome of the three-way ANOVA analysis is presented in Table 23.

Peroxidase and ACH_{50} activities in plasma were not altered by the diets, regardless of the sampling time and the bacterial stimulus (P>0.05) (Fig. 34A and B, respectively). On the other hand, the protease activity was significantly higher in fish fed the NT (P=0.041) or BL diets (P=0.049) compared to groups fed the CTR diet (Fig. 34C), regardless of the bacterial stimulus and the sampling time. Fish sampled at 24h displayed higher ACH_{50} and protease activities than those sampled at 4h for all the dietary groups (P<0.05), regardless of the bacterial stimulus. An opposite pattern was shown by the peroxidase activity (P=0.032).

The antiprotease activity of fish fed the BL diet was significantly lower than that measured in fish fed the CTR (P=0.008) or NT (P=0.017) diets (Fig. 34D), only at 24h post-stimulus. Also, in fish fed the BL diet the antiprotease activity was lower at 24h than that measured at 4h (T*D, P=0.005).

Table 23: Three-way ANOVA analysis results for peroxidase, haemolytic alternative complement, protease, and antiprotease activities in plasma of gilthead seabream.

Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (Placebo) or inactivated *Phdp* (stimulated).

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
Peroxidase	**0.032**	0.217	0.258	0.667	0.132	0.683	0.067
ACH_{50}	**0.006**	0.458	0.427	0.886	0.841	0.496	0.189
Protease	**<0.001**	**0.027**	0.743	0.178	0.350	0.296	0.530
Antiprotease	0.785	0.275	0.266	**0.005**	0.639	0.485	0.589

Values represent the *P*-value.

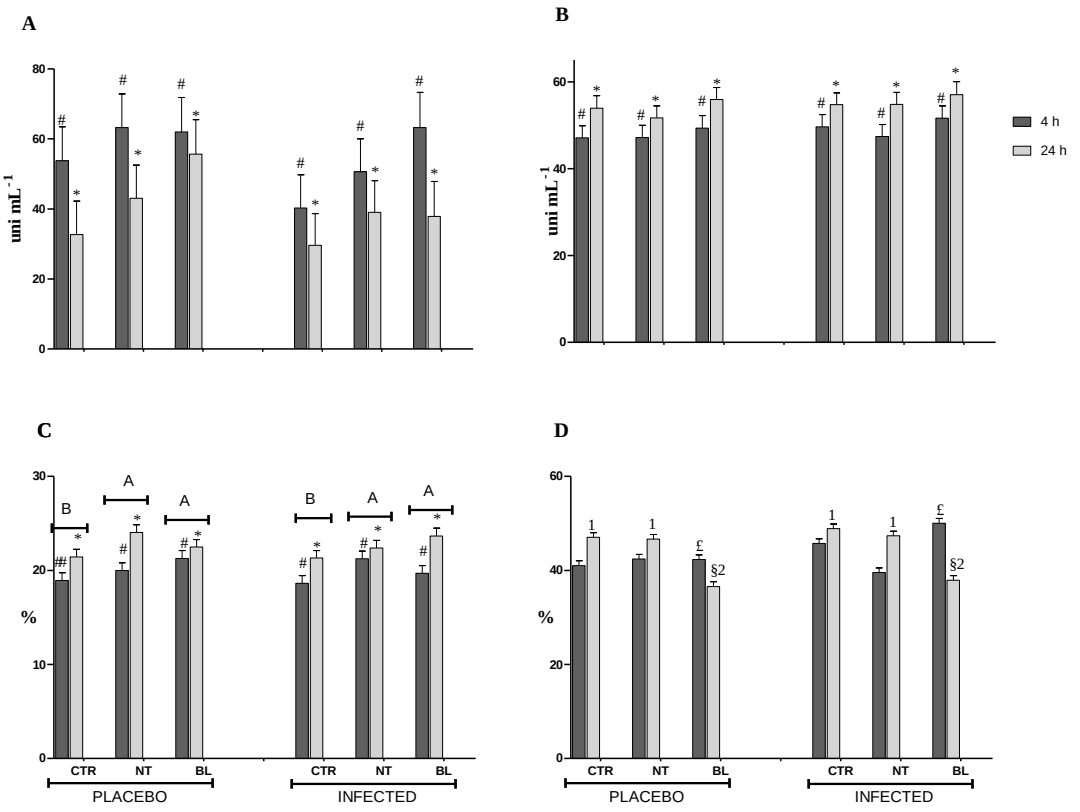

Figure 34 A) Peroxidase, B) haemolytic alternative complement, C) protease and D) antiprotease activities in the plasma of gilthead seabream at 4h and 24h post-injection with PBS (placebo) or *Phdp* (stimulated).

Fish were fed with three diets: *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL). Values are presented as LSM+SE (n = 8). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-stimulus, regardless of the diet and the stimulus. The numbers 1 and 2 represent significant differences between experimental diets within a specific sampling time. The symbols £ and § represent significant differences between sampling time within a specific experimental diet, regardless of the stimulus. Significant differences were considered when $P<0.05$.

6.3.4. Markers of oxidative stress in liver

The effects of the diets on the oxidative stress markers in the liver of gilthead seabream at 4h and 24h post-stimulus and the outcome of the three-way ANOVA analysis are presented in Table 24.

The CAT activity significantly increased in fish fed AW supplemented diets, being higher in both NT (P=0.018) and BL groups (P<0.001) when compared to fish fed CTR diet, regardless of the bacterial stimulus and the sampling time. After challenge with inactivated *Phdp*, CAT activity remained higher in fish fed supplemented diets than in CTR diet (D*I, P<0.001). CAT activity was similar between stimulated and placebo groups at 4h or 24h, regardless of the diet (P>0.05).

The GR activity was significantly higher in fish fed the BL diet compared to the CTR group (P=0.003), regardless of the bacterial stimulus and the sampling time. No differences were found in the activity for this enzyme between fish fed the BL or NT diets (P>0.05). In addition, the activity of GR was similar between stimulated and placebo groups at 4h and 24h post-stimulus (P>0.05).

The GPx and GST activities were not altered by the diet either nor by the bacterial stimulus (P>0.05). On the other hand, the GPx and GST activities significantly increased at 24h, regardless of the diet and the bacterial stimulus (P<0.05). Despite the lack of differences between dietary groups, a significant interaction between T*D*I was detected, showing that GST tended to increase at 24h post-stimulus, especially in stimulated fish fed the BL diet.

The GSSG level was not changed by the diet (P>0.05). On the other hand, the GSSG levels decreased in the stimulated fish compared to placebo, regardless of the diet and the sampling time. At 24h post-stimulus, the levels of GSSG increased, regardless of the diet and the bacterial stimulus (P= 0.007).

The GSH level was lower in fish fed the NT diet than in the CTR (P=0.019) or BL groups (P=0.047) in both stimulated and placebo fish, regardless of the sampling time. The GSH level was lower in stimulated than placebo groups (P=0.027), remaining unchanged with sampling time (P>0.05).

The GSH/GSSG ratio and LPO level were similar in fish fed the different diets (P>0.05). LPO level increased at 24h (P=0.029), while GSH/GSSG ratio showed the opposite pattern (P=0.001). The bacterial stimulus had no significant effect on LPO level and GSH/GSSG ratio (P>0.05).

Table 24: Catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities, oxidized glutathione (GSSG), reduced glutathione (GSH) levels, GSH/GSSG ratio and lipid peroxidation (LPO) level in the liver of gilthead seabream.
Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (Placebo) or inactivated *Phdp* (stimulated).

Parameters	**Injection**	**Dietary treatment**					
		CTR		NT		BL	
		4h	24h	4h	24h	4h	24h
CAT	Placebo	114.06±5.65 ^{B}	132.57±5.78 ^{B}	133.01±5.65 ^{A}	138.62±5.67 ^{A}	143.00±5.91 ^{A}	141.01±5.78 ^{A}
(µmol min^{-1} mg^{-1} protein)	*Phdp*	120.72±5.69 ^{B}	116.97±5.58 ^{B}	126.77±5.58 ^{A}	135.92±5.58 ^{A}	129.16±5.69 ^{A}	145.90±5.84 ^{A}
GR	Placebo	5.34±0.28 ^{B}	6.10±0.29 ^{B}	6.05±0.28 ^{AB}	6.24±0.28 ^{AB}	6.44±0.29 ^{A}	6.62±0.29 ^{A}
(mmol min^{-1} mg^{-1} protein)	*Phdp*	5.60±0.28 ^{B}	5.58±0.28 ^{B}	5.74±0.28 ^{AB}	6.30±0.28 ^{AB}	6.12±0.28 ^{A}	6.69±0.29 ^{A}
GPx	Placebo	23.64±1.03*****	29.84±1.06$^{\#}$	25.03±1.03*****	28.59±1.04$^{\#}$	25.45±1.08*****	27.71±1.06$^{\#}$
(mmol min^{-1} mg^{-1} protein)	*Phdp*	24.51±1.041*****	26.32±1.02$^{\#}$	23.25±1.02*****	27.71±1.02$^{\#}$	22.39±1.08*****	28.13±1.07$^{\#}$
GST	Placebo	112.20±5.85*****	124.23±5.98$^{\#}$	116.97±5.85*****	124.30±5.86$^{\#}$	126.17±6.11*****	134.72±5.98$^{\#}$
(nmol min^{-1} mg^{-1} protein)	*Phdp*	115.66±5.87*****	122.24±5.77$^{\#}$	115.73±5.77*****	127.01±5.77$^{\#}$	126.16±5.89*****	136.21±6.04$^{\#}$

GSSG	Placebo	0.38±0.03^{a*}	0.49±0.03$^{a\#}$	0.44±0.03^{a*}	0.48±0.03$^{a\#}$	0.42±0.03^{a*}	0.47±0.03$^{a\#}$
(nmol min^{-1} mg^{-1} protein)	*Phdp*	0.37±0.03^{b*}	0.39±0.03$^{b\#}$	0.35±0.03^{b*}	0.45±0.03$^{b\#}$	0.35±0.03^{b*}	0.43±0.03$^{b\#}$
GSH	Placebo	4.61±0.29^{Aa}	4.89±0.29^{Aa}	4.34±0.29^{Ba}	3.88±0.29^{Ba}	4.70±0.29^{Aa}	4.60±0.29^{Aa}
(nmol min^{-1} mg^{-1} protein)	*Phdp*	4.49±0.30^{Ab}	4.02±0.29^{Ab}	3.48±0.29^{Bb}	3.75±0.29^{Bb}	4.20±0.29^{Ab}	4.11±0.29^{Ab}
GSH/GSSG ratio	Placebo	24.91±1.83*	18.94±1.87$^{\#}$	20.53±1.87*	15.62±1.87$^{\#}$	23.22±1.87*	19.94±1.91$^{\#}$
	Phdp	24.35±1.88*	20.88±1.81$^{\#}$	21.03±1.97*	16.50±1.91$^{\#}$	25.35±1.84*	19.19±1.894$^{\#}$
LPO	Placebo	68.29±1.85*	68.15±1.83$^{\#}$	68.27±1.88*	72.54±1.87$^{\#}$	66.12±1.88*	71.40±1.91$^{\#}$
(nmol TBARS g^{-1} tissue)	*Phdp*	65.22±1.80*	71.63±1.87$^{\#}$	69.61±1.90*	71.61±1.83$^{\#}$	68.47±1.88*	69.47±1.83$^{\#}$

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
CAT	0.091	**0.003**	0.302	0.156	0.563	**<0.001**	0.054
GR	0.083	**0.011**	0.556	0.444	0.454	0.090	0.892

GPx	**<0.001**	0.973	0.098	0.129	0.640	0.282	**0.048**
GST	**0.040**	0.071	0.869	0.903	0.597	0.260	**0.012**
GSSG	**0.007**	0.785	**0.023**	0.443	0.073	0.175	0.273
GSH	0.657	**0.040**	**0.027**	0.388	0.949	0.505	0.097
GSH/GSSG ratio	**0.001**	0.059	0.630	0.731	0.291	0.088	0.501
LPO	**0.029**	0.421	0.884	0.248	0.314	0.051	0.569

Values are presented as LSM±SE (n = 8). *P*-values from three-way ANOVA (*P*<0.05). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the bacterial stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-stimulus, regardless of the diet and the bacterial stimulus.

6.3.5. Markers of oxidative stress in the anterior intestine

The effects of the diets on the oxidative stress markers in the anterior intestine of gilthead seabream at 4h and 24h post-stimulus and the outcome of the three-way ANOVA analysis are presented in Table 25.

The CAT activity was similar between all the dietary treatments and no differences between sampling times were detected ($P>0.05$). The bacterial stimulus decreased its activity compared to the placebo group ($P<0.001$).

The GR activity was increased in fish fed the BL diet compared to the CTR group ($P=0.013$), regardless of the bacterial stimulus and sampling time. No significant difference was detected in the GR activity of fish fed the BL or NT diets. Stimulated fish showed a decrease in the GR activity regardless of the experimental diet ($P=0.034$). Also, GR activity remained unchanged with sampling time ($P>0.05$).

GST activity was significantly higher in fish fed the BL diet compared to fish fed CTR ($P=0.003$) or NT ($P=0.014$) diets, especially in stimulated fish (D*I, $P=0.024$). However, the GST activity was similar in the stimulated and placebo groups ($P>0.05$). The GST activity decreased at 24h post-stimulus($P=0.046$).

The GPx activity was similar in all dietary groups at 4h or 24h ($P>0.05$). However, the GPx activity in stimulated fish was lower than in the placebo group ($P<0.001$), regardless of the diet and the sampling time.

The GSSG level was similar in all dietary groups and remained unchanged by the bacterial stimulus ($P>0.05$). However, the GSSG level increased at 24h ($P=0.013$), regardless of the diet and the bacterial stimulus.

Fish fed the BL diet showed higher GSH levels than the CTR ($P=0.003$) and NT ($P=0.014$) groups, regardless of the bacterial stimulus and the sampling time. The GSH level was higher at 4h, regardless of the diets ($P=0.011$). The GSH level remained unchanged after the challenge ($P>0.05$).

The GSH/GSSG ratio and LPO level were similar across all the experimental conditions ($P>0.05$).

Table 25: Catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities, oxidized glutathione (GSSG), reduced glutathione (GSH) levels, GSH/GSSG ratio and lipid peroxidation (LPO) level in the anterior intestine of gilthead seabream.
Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (Placebo) or inactivated *Phdp* (stimulated).

Parameters	**Injection**	**Dietary treatment**					
		CTR		NT		BL	
		4h	24h	4h	24h	4h	24h
CAT	Placebo	46.07±4.03^{a}	46.07±4.11^{a}	49.41±4.11^{a}	46.49±4.11^{a}	51.11±4.11^{a}	43.65±4.11^{a}
(µmol min^{-1} mg^{-1} protein)	*Phdp*	38.94±4.19^{b}	32.02±4.03^{b}	39.36±4.03^{b}	35.36±4.11^{b}	36.52±4.03^{b}	37.06±4.11^{b}
GR	Placebo	15.74±1.02^{Ba}	13.60±1.04^{Ba}	15.67±1.04^{ABa}	15.78±1.04^{ABa}	17.56±1.04^{Aa}	16.63±1.04^{Aa}
(mmol min^{-1} mg^{-1} protein)	*Phdp*	12.89±1.06^{Bb}	13.07±1.02^{Bb}	15.08±1.02^{ABb}	13.00±1.04^{ABb}	15.93±1.02^{Ab}	14.87±1.04^{Ab}
GPx	Placebo	0.65±0.03^{a}	0.63±0.03^{a}	0.60±0.03^{a}	0.62±0.03^{a}	0.61±0.03^{a}	0.65±0.03^{a}
(mmol min^{-1} mg^{-1} protein)	*Phdp*	0.52±0.03^{b}	0.57±0.03^{b}	0.51±0.03^{b}	0.52±0.03^{b}	0.55±0.03^{b}	0.53±0.03^{b}
GST	Placebo	112.72±4.72^{B*}	95.04±4.97$^{B\#}$	109.45±4.93^{B*}	103.40±4.88$^{B\#}$	117.23±5.09^{A*}	118.50±4.88$^{A\#}$
(nmol min^{-1} mg^{-}1 protein)	*Phdp*	97.19±4.93^{B*}	99.90±4.66$^{B\#}$	105.55±4.66^{B*}	96.63±5.04$^{B\#}$	120.66±4.66^{A*}	104.41±5.03$^{A\#}$

GSSG	Placebo	2.12±0.20*	2.67±0.21#	2.12±0.21*	2.48±0.21#	2.26±0.21*	2.54±0.21#
(nmol min^{-1} mg^{-1} protein)	*Phdp*	2.07±0.21*	2.32±0.20#	1.89±0.20*	2.32±0.21#	1.94±0.20*	2.46±0.21#
GSH	Placebo	112.72±4.72 B*	95.04±4.97 B#	109.451±4.93 B*	103.40±4.88 B#	117.30±5.09 A*	118.50±4.88 A#
(nmol min^{-1} mg^{-1} protein)	*Phdp*	97.19±4.93 B*	99.90±4.66 B#	105.55±4.66 B*	96.63±5.04 B#	120.66±4.66 A*	104.41±5.03 A#
GSH/GSSG ratio	Placebo	2.48±0.47	2.18±0.47	3.22±0.48	2.21±0.47	2.20±0.47	2.44±0.47
	Phdp	2.93±0.45	2.52±0.45	2.96±0.45	3.25±0.48	3.18±0.45	2.23±0.45
LPO	Placebo	75.31±3.39	79.00±3.39	83.33±3.39	76.94±3.39	84.97±3.46	81.55±3.46
(nmol TBARS g^{-1} tissue)	*Phdp*	78.30±3.37	70.53±3.37	76.24±3.37	78.55±3.37	80.85±3.44	80.19±3.44

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
CAT	0.267	0.879	**<0.001**	0.613	0.575	0.405	0.578

GR	0.213	**0.045**	**0.034**	0.504	0.229	0.281	0.768
GPx	0.626	0.592	**<0.001**	0.516	0.071	0.261	0.774
GST	**0.046**	**0.006**	0.152	0.111	0.083	**0.024**	0.943
GSSG	**0.013**	0.849	0.207	0.766	0.850	0.552	0.376
GSH	**0.046**	**0.006**	0.152	0.111	**0.083**	**0.024**	0.943
GSH/GSSG ratio	0.313	0.585	0.269	0.359	**0.068**	0.890	0.794
LPO	0.431	0.163	0.290	0.257	0.117	0.621	0.246

Values are presented as LSM±SE (n = 8). *P*-values from three-way ANOVA (*P*<0.05). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the bacterial stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-stimulus, regardless of the diet and the bacterial stimulus.

6.3.6. Markers of oxidative stress in the posterior intestine

The effects of the diets on the oxidative stress markers in the posterior intestine of gilthead seabream at 4h and 24h post-stimulus and the outcome of the three-way ANOVA analysis are presented in Table 26.

Diet had no significant effect on CAT, GR, GST, and GPx activities ($P>0.05$). Similarly, CAT, GR, and GST activities were not altered by the bacterial stimulus ($P>0.05$) while GPx activity was negatively modulated by the bacterial stimulus ($P=0.021$), regardless of the diet and sampling time. In addition, the activities of all these enzymes were lower at 24h ($P<0.05$), regardless of the diet and the bacterial stimulus.

The GSSG levels were higher in fish fed the BL diet compared to the CTR ($P=0.018$) or NT ($P=0.027$) groups, regardless of the sampling time and the bacterial stimulus. However, the GSSG level was similar between CTR and NT dietary groups ($P>0.05$). The GSSG level was significantly increased at 24h, regardless of the diets or the stimulus. The diet and the bacterial stimulus had no significant effects on the GSH level neither nor on the GSH/GSSG ratio ($P>0.05$). On the other hand, both the GSH level and the GSH/GSSG ratio decreased at 24h ($P<0.001$), regardless of the diet and the bacterial stimulus.

The LPO level was higher in fish fed the BL diet than in the CTR ($P=0.031$) or NT ($P=0.007$) groups, regardless of the bacterial stimulus and the sampling time. The LPO level decreased at 24h in fish fed the CTR and NT diets, whereas fish fed the BL diet displayed the opposite pattern (T*D*I, $P=0.003$).

Table 26: Catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities, oxidized glutathione (GSSG), reduced glutathione (GSH) levels, GSH/GSSG ratio and lipid peroxidation (LPO) level in the posterior intestine of gilthead seabream.
Fish were fed with *i*) a control diet (CTR), *ii*) a basal diet with 2.5% agar waste from NT condition (2.5% NT) and *iii*) a basal diet with 2.5% agar waste from BL condition (2.5% BL) and sampled at 4h and 24h post-injection with PBS (placebo) or *Phdp* (stimulated).

Parameters	**Injection**	**Dietary treatment**					
		CTR		NT		BL	
		4h	24h	4h	24h	4h	24h
CAT	Placebo	47.23±3.10*****	41.21±3.15$^{\#}$	49.32±3.16*****	38.94±3.10$^{\#}$	51.47±3.16*****	44.74±3.23$^{\#}$
(µmol min^{-1} mg^{-1} protein)	*Phdp*	44.98±3.08*****	35.58±3.13$^{\#}$	42.70±3.13*****	37.66±3.08$^{\#}$	48.51±3.27*****	39.82±3.08$^{\#}$
GR	Placebo	12.57±0.93*****	10.66±0.95$^{\#}$	13.47±0.93*****	12.27±0.91$^{\#}$	13.87±0.94*****	11.99±0.98$^{\#}$
(mmol min^{-1} mg^{-1} protein)	*Phdp*	12.51±0.93*****	11.09±0.89$^{\#}$	14.13±0.92*****	11.98±0.89$^{\#}$	13.84±1.03*****	12.40±0.91$^{\#}$
GPx	Placebo	0.89±0.06^{a}*****	0.74±0.06$^{a\,\#}$	0.85±0.06^{a} *****	0.67±0.03$^{a\,\#}$	0.73±0.06^{a} *****	0.75±0.07$^{a\,\#}$
(mmol min^{-1} mg^{-1} protein)	*Phdp*	0.72±0.06^{b}*****	0.68±0.06$^{b\,\#}$	0.66±0.06^{b}*****	0.63±0.06$^{b\,\#}$	0.74±0.06^{b}*****	0.51±0.06$^{b\,\#}$
GST	Placebo	874.81±66.45*****	736.45±67.32$^{\#}$	921.79±66.45*****	729.11±66.01$^{\#}$	920.85±67.75*****	926.44±66.01$^{\#}$
(nmol min^{-1} mg^{-}1 protein)	*Phdp*	824.89±66.89*****	746.28±65.57$^{\#}$	817.55±65.57*****	793.26±68.18$^{\#}$	1014.88±65.57*****	792.32±66.89$^{\#}$

GSSG	Placebo	2.55±0.20 ^{B*}	2.91±0.20 $^{B\#}$	2.61±0.20 ^{B*}	2.92±0.20 $^{B\#}$	2.94±0.20 ^{A*}	3.42±0.20 $^{A\#}$
(nmol min^{-1} mg^{-1} protein)	*Phdp*	2.52±0.20 ^{B*}	2.92±0.20 $^{B\#}$	2.52±0.20 ^{B*}	2.98±0.20 $^{B\#}$	3.02±0.20 ^{A*}	3.32±0.20 $^{A\#}$
GSH	Placebo	2.18±0.31*	1.43±0.30$^{\#}$	1.95±0.31*	0.69±0.30$^{\#}$	1.72±0.34*	0.52±0.31$^{\#}$
(nmol min^{-1} mg^{-1} protein)	*Phdp*	2.54±0.29*	1.15±0.31$^{\#}$	1.80±0.29*	0.92±0.28$^{\#}$	1.64±0.30*	0.69±0.42$^{\#}$
GSH/GSSG ratio	Placebo	1.41±0.21*	0.75±0.20$^{\#}$	1.47±0.21*	0.54±0.19$^{\#}$	1.13±0.23*	0.15±0.19$^{\#}$
	Phdp	1.67±0.20*	0.62±0.21$^{\#}$	1.45±0.18*	0.68±0.18$^{\#}$	1.07±0.19*	0.34±0.30$^{\#}$
LPO	Placebo	117.50±6.34 ^{B}	98.56±6.22 ^{B}	109.60±6.34 ^{B}	99.96±6.11 ^{B}	111.66±6.34 ^{A}	130.22±6.58 ^{A}
(nmol TBARS g^{-1} tissue)	*Phdp*	94.66±6.30 ^{B}	106.92±6.07 ^{B}	96.06±5.95 ^{B}	99.02±6.30 ^{B}	126.32±6.43 ^{A}	101.08±6.30 ^{A}

Parameters	**Three-Way ANOVA (*P*-value)**						
	Time (T)	**Diet (D)**	**Injection (I)**	**T x D**	**T x I**	**D x I**	**T x D x I**
CAT	**0.002**	0.309	0.101	0.719	0.878	0.518	0.648

GR	**0.021**	0.227	0.793	0.889	0.284	0.243	0.351
GPx	**0.036**	0.419	**0.021**	0.199	0.243	0.677	0.571
GST	**0.034**	0.129	0.691	0.257	0.360	0.225	0.186
GSSG	**0.013**	**0.030**	0.951	0.904	**0.013**	0.771	0.684
GSH	**<0.001**	0.061	0.867	0.606	0.969	0.953	0.525
GSH/GSSG ratio	**<0.001**	0.092	0.691	0.650	0.667	0.690	0.622
LPO	0.483	**0.019**	0.131	**0.006**	0.857	**0.016**	**0.003**

Values are presented as LSM±SE (n = 8). *P*-values from three-way ANOVA (*P*<0.05). A different lower case letter indicates a significant difference for placebo and stimulated groups, regardless of the diet and the sampling time. A different capital letter indicates a significant difference among diets, regardless of the bacterial stimulus and the sampling time. The symbols * and # represent significant differences at 4h and 24h post-stimulus, regardless of the diet and the bacterial stimulus.

6.4. Discussion

This study showed a significant modulatory effect of short-term dietary supplementation with different *Gracilaria gracilis* by-products in gilthead seabream, in particular when the fish was stimulated with an inactivated bacterial pathogen (*Phdp*). Differential response of fish to inflammation when fed the three diets was revealed by changes across time of several haematological parameters, humoral innate immune, and antioxidant responses.

Results showed that gilthead seabream fed AW supplemented (NT and BL) diets displayed lower Ht values (35-48%) than in the CTR group (47-53%), which may be linked to lower levels in MCV measured on these two dietary groups. Despite the decreased MCV values, all the dietary groups showed similar MCH values, indicating that the haemoglobin content expressed per RBC did not decrease in fish fed AW supplemented diets. Additional support for this observation is provided by MCHC, reflecting the haemoglobin per unit volume of RBC (Clark et al. 1990), that was increased in fish fed supplemented diets. Attending to the fact that erythrocyte volume, but not haemoglobin content, decreased in fish fed supplemented diets, it is plausible to infer that the capacity to transport oxygen was boosted in the bloodstream of gilthead seabream, based on the principle that larger surface area of volume ratios and shorter diffusion distances allows more rapid oxygenation, and deoxygenation of haemoglobin (Dal'Bó et al. 2015). This enhancement of oxygenation capacity is important to improve the response of fish to several biotic and abiotic challenges, such as exposure to bacteria or parasites that typically induce hypochromic microcytic anaemia (Harikrishnan et al. 2012; Alsaid et al. 2015; Ahmed et al. 2020). In the present study, bacterial stimulus resulted in a lower Ht value, RBC count, and MCH value showing that bacterial stimulus-induced hypochromic microcytic anaemia (Alsaid et al. 2015) and decreased the Hb content in the RBCs. Nevertheless, the increase of MCHC value detected in stimulated fish could be due to the lower RBC count, as a compensatory mechanism to improve the oxygen-carrying capacity in the bloodstream, particularly evident in the fish fed the AW supplemented diets. In this regard, Passos et al. (2021) had described a modulatory effect of raw *Gracilaria gracilis* supplementation at 2.5% on both Ht and MCV values of gilthead seabream, improving growth, health, and resistance against *Phdp*. Also, *Pangasius hypophthalmus* fed for 12 weeks a diet containing hot-water extracts of

Sargassum oligocystum have shown increased haematological parameters, such as WBC and RBC, Hb, Ht, and platelet (Baleta et al. 2019).

In the current study, the inoculation of PBS or *Phdp* in placebo and stimulated groups, respectively, led to the recruitment of leukocytes from circulation to the site of injection. This induced inflammatory response was more pronounced in stimulated fish as expected, and it was characterised by a higher phagocyte response than the placebo group by revealing higher neutrophil and monocyte counts in their blood. In addition, the number of peripheral neutrophils increased in *Phdp* stimulated fish fed supplemented diets (NT and BL) compared to the CTR group, despite no differences were detected in the number of total peritoneal leukocytes between both dietary groups. This suggests that the NT and BL diets boosted the immune response of gilthead seabream following stimulation, particularly those related to the proteolytic and anti-microbial activities, which may also involve a burst in reactive oxygen species (ROS) generation at the local of the inflammation by the neutrophils (Charlie-Silva et al. 2019). A similar pattern was revealed by the thrombocytes whose number increased in response to the bacterial stimulus, related to their role in the innate immune response. In addition, the increased number of thrombocytes in the blood of fish fed the AW supplemented diets supports the idea that bioactive compounds may be involved in the modulatory effect of the innate immune response. Similarly, an improvement in the immune response of fish was detected in *Oreochromis niloticus* fed for 12 weeks a diet containing Spirulina, *Arthrospira platensis*, resulting in increased phagocytic activity in blood in response to *Aeromonas hydrophila* infection (Abdel-Tawwab et al. 2009).

In the present study, the observed lymphopenia in fish exposed to the bacterial stimulus might be explained due to the migration of these cells from the bloodstream to the inflamed peritoneal cavity in response to the inflammatory processes. Additionally, the short duration of the feeding trial period applied in the current study provided important information on modulation of the innate immune response by the dietary supplementation, which is relevant during an inflammatory response (Falco et al. 2012; Pionnier et al. 2013). In this line, the results showed an improvement of the humoral immune response of gilthead seabream mediated by the AW supplementation. This is supported by the increased proteases activity detected in the plasma of fish fed AW supplemented diets compared to fish fed the CTR diet, regardless of the sampling time and the stimulus, suggesting an improvement in the capacity to contain pathogens, as this enzyme activity is responsible for cleaving bacterial proteins. Proteases may also

indirectly participate in activating and enhancing the production of various components of the immune response including the complement system (Subramanian et al. 2007; Vasta et al. 2011). Despite that additional proposed role for proteases activity in plasma, no significant changes were detected regarding the haemolytic complement activity of fish fed the AW supplemented diets in this study. Also, no significant changes were detected in antiprotease activity in the plasma of gilthead seabream following the incorporation of AW in the diet. However, lower antiprotease activity detected at 24h post-stimulus in fish fed the BL diet indicates a modulatory effect in response to a bacterial stimulus which was time-depend. Results in this study are similar to those previously reported for European sea bass (*Dicentrarchus labrax*) fed diets supplemented with raw *Gracilaria* sp. (at 5%) (Peixoto et al. 2016) or its aqueous (at 5%) and methanolic (at 0.5%) extracts (Peixoto et al. 2019a; Peixoto et al. 2019b), by showing beneficial effects on the humoral immune status in fish fed these diets. Similarly, Xu et al. (2011) evaluated the immune response of the *Siganus canaliculatus* fed a diet with raw *Gracilaria lemaneiformis* at 33%, showing an improvement of innate immune response by increasing the lysozyme and the ACH_{50} activities. Also, dietary supplementation with laminarin at 1.0 and 1.5% increased plasma lysozyme activity of *Epinephelus coioides* (Yin et al. 2014). Increased plasma haemolytic complement, phagocytic and respiratory burst activities were detected in gilthead seabream fed for 4 weeks diets supplemented with whole biomass of *Phaeodactylum tricornutum at* 5 and 10%, which has been associated with the immunostimulant effects of β-1,3-glucans present in this diatom (Cerezuela et al. 2012). In this regard, the presence of complex carbohydrates in seaweed could be linked to the immunostimulant effect detected in the current study. Interestingly, several studies have shown a positive modulation of fish immune response when carbohydrates and crude extracts from SW are supplemented in diets (Hindu et al. 2018). For instance, dietary supplementation of low molecular weight agar (0.2%) resulted in increased lysozyme activity, phagocytosis, respiratory burst, alternative complement activities, as well as a higher survival rate in Basa fish (*Pangasius bocourti*) infected with *Aeromonas hydrophila* (Van Doan et al. 2014). Similarly, Rajendran et al. (2016) have shown increased antibody production improving the resistance to a pathogen in common carp (*Cyprinus carpio*) fed diets supplemented with increasing levels of polysaccharide.

The immune response may be limited by oxidative stress (Biller-Takahashi et al. 2018), since its response decrease at the expense of the attempt to avoid the excessive ROS

production (Biller-Takahashi et al. 2018). Therefore, the balance between prooxidants and antioxidants is essential to minimize oxidative stress and the subsequent negative effects on immune status. Earlier studies reported an increase in the efficiency of antioxidant defense mediating host benefits (Magnoni et al. 2017; Gora et al. 2018; Sotoudeh et al. 2018; Kamunde et al. 2019). In the present study, gilthead seabream fed both NT and BL diets have shown an improved response in the hepatic antioxidant system displayed by an increase of CAT activity compared to the CTR group. CAT enzyme has a key role in the defense of organisms against oxidative stress (Ighodaro et al. 2018), as it is required to decompose hydrogen peroxide (H_2O_2) generated in abundance by phagocytes during an immune response (Biller-Takahashi et al. 2018). Also, GR activity in the liver of fish fed the BL diet increased compared to the CTR group, which along with the diet-modulated effect on GSH levels suggests that this diet is modulating the antioxidant system by inducing changes in the glutathione system. Dietary supplementation with AW differently modulated the antioxidant response in the anterior and posterior portions of the intestine of gilthead seabream as well. The most remarkable result to emerge in this organ is related to the GST activity, which was increased in fish fed the BL diet in the anterior intestine, while an opposite pattern was detected in the posterior intestine. The enzyme GST is involved in the detoxification process and the mitigation of cellular toxicity of several endogenous and environmental chemicals. The decreased GST activity observed in the posterior intestine in fish fed the BL diet could be linked to the higher LPO detected in this tissue section. The GR activity, enzyme reducing GSSG and maintaining the level of antioxidant molecules of GSH (Abd El 2012), was positively modulated by the BL diet leading to an increase of GSH levels in the anterior intestine in that group. Higher GSH levels may be associated with a change in cellular redox status even without a change in GSH/GSSG ratio (Miller et al. 2002). The results provided in the present study support previous findings showing that the antioxidant defense mechanism in gilthead seabream juveniles is differentially modulated between tissues and location, as it was shown in the liver and different parts of the intestine (Coutinho et al. 2016; Magalhães et al. 2020).

Conclusions

This study indicated an improvement of haematological parameters of gilthead seabream fed both supplemented diets with AW at 2.5% (BL and NT), suggesting a higher carrying-oxygen capacity in these groups. The inflammatory response was enhanced in fish fed the

NT diet, revealed by higher neutrophil counts compared to the CTR group. Fish fed both AW supplemented diets exhibited an improvement on the humoral innate immune response, displayed to an enhancement of plasma protease activity. Finally, the antioxidant response was differently modulated in the liver and the intestine of fish fed the experimental diets.

This work provided evidence that the dietary supplementation with AW from *Gracilaria gracilis* cultured under NT and BL conditions might be used as a sustainable prophylactic strategy to increase the immune and antioxidant response of gilthead seabream, suggesting a beneficial effects on fish health and welfare.

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CHAPTER 7

General Discussion, conclusions and future approaches

7.1. General discussion

An overview

A novel application for the residue of agar extraction (AW) obtained from the seaweed *Gracilaria gracilis* as a dietary supplement was demonstrated in this thesis, aiming to boost the antioxidant capacity, improve the stress response and enhance the immune status in farmed gilthead seabream. First, the antioxidant properties of the *G. gracilis* and the by-products obtained, including AW, were confirmed *in vitro* (Chapter 2). Then, the *in vivo* trials revealed a positive modulation of the antioxidant system in the liver and intestine of gilthead seabream-fed diets supplemented with *G. gracilis* by-products (Chapters 4 and 6). Moreover, an improved immune response in fish fed diets supplemented with AW was demonstrated in this study (Chapter 6), including an increased number of neutrophils and lymphocytes in tissues and blood, as well as an enhancement in haematological parameters when compared to fish fed a basal (control) diet. In crowded seabream, a condition which usually occurs during fish farming operations, the levels of several acute stress markers in the plasma were lower in fish fed AW supplemented diets, suggesting improved welfare (Chapter 4). In addition, the number of beneficial bacteria belonging to the *Clostridium* genera was increased in the intestine of fish fed AW supplemented diets, which could enhance the digestive function, immune response, disease resistance, and oxidative balance in fish. The dietary supplementation with AW in seabream also increased the yellowness of the skin, an attribute appreciated by the consumers (Chapter 3). Finally, Chapter 5 revealed that the use of the different light spectrum to grow *G. gracilis* in an IMTA-system may be an effective method to improve the characteristics and commercial value of the agar produced without altering the antioxidant capacity, which could be relevant for the potential use as a supplement in fish diets.

Agar waste (AW) displays antioxidant capacity and could be used as a dietary supplement to improve the antioxidant system in farmed gilthead seabream

Previous studies revealed that dietary supplementation with seaweeds had positive effects on the antioxidant system of farmed fish species, including sea bass, meagre, and sea

bream (Peixoto et al., 2016b; Peixoto et al., 2019b; Magnoni et al., 2017). A variety of bioactive compounds present in the seaweeds are linked to a modulatory response detected in the antioxidant system of fish. Spite the bioactive compounds present in seaweeds, the potential application of AW generated during the agar extraction of IMTA-farmed *G. gracilis* has never been assessed, aiming to its use as a dietary supplement in aquafeeds. In **Chapter 2** of this thesis, the antioxidant properties of the AW obtained from IMTA-farmed *G. gracilis* were investigated by employing several *in vitro* assays measuring the antioxidant capacity of the raw seaweed as well as the residues obtained. The antioxidant capacity of ethanolic extracts obtained from *G. gracilis* was compared to that measured in the AW, the by-product obtained from aqueous agar extractions. The results revealed that the AW showed similar or twice as much high antioxidant activity as those displayed by the different ethanolic extracts obtained from raw *G. gracilis*. These results corroborate previous studies showing low solubility of antioxidant compounds in water, like some polyphenols, even when high temperatures were applied during the agar extraction process (Chan et al., 2015), and could be related to the antioxidant activity that is present in AW showed in **Chapter 2**. In the present study, the raw *G. gracilis* extracted at 60 ºC induced a decrease in the antioxidant activity of these extracts in comparison to those obtained at 25 ºC (room temperature), supporting other studies establishing a negative correlation between the extraction temperature and the bioactivity of the extracts (Dahmoune et al., 2015; Boi et al., 2017). Nevertheless, the higher total phenol content (TPC) and free radical scavenging activity detected in the AW in comparison to those obtained from raw *G. gracilis* at 60 ºC indicated that considerable amounts of compounds with antioxidant activity remained into the AW after the agar extraction. Since the antioxidant potential of both raw *G. gracilis* and AW was validated by the *in vitro* assays, the next trials in this thesis aimed to evaluate the modulatory effect that feeding gilthead seabream with an AW supplemented diet may have on its antioxidant system. In accordance, C**hapter 4** demonstrated that the antioxidant system of the gilthead seabream was positively modulated after a long-term feeding trial (59 days) with diets supplemented with aqueous and ethanolic by-products obtained from *G. gracilis* (AW and EEW, respectively). The most remarkable results to emerge from the study is a decrease of the hepatic GPx and GR activities in the gilthead seabream fed AW and EEW supplemented diets without lipid oxidative damage within this tissue, as the hepatic LPO levels remained similar in fish fed the different experimental diets. In compliance,

Chapter 6 showed that feeding gilthead seabream for 15 days diets supplemented with AW obtained from *G. gracilis* cultured under blue spectral light condition (BL) improved the hepatic GR activity compared to the fish fed a basal diet, which along with the diet-modulated effect on GSH levels showed the dietary modulation of the antioxidant system. Together, the *in vitro* (**Chapter 2**) and *in vivo* (**Chapters 4** and **6**) experiments showed that *G. gracilis* by-products improve the antioxidant capacity, more likely by the presence of bioactive compounds which directly act against the formation and/or propagation of free radicals, or indirectly by stimulating the antioxidant non-enzymatic and enzymatic systems of the fish. Some of these compounds have been associated with pigments, polysaccharides, and polyphenols included in the seaweeds, which may act as electron scavengers during the free radical formation (Gomez-Zavaglia et al., 2019).

Long-term dietary AW supplementation in gilthead seabream does not have negative effects on growth performance or the digestive function

Previous studies have reported that the dietary supplementation of seaweed or their by-products, which are rich in polysaccharides (Cheong et al., 2018; Saleh, 2020), contributes to the nutrient and energy content of the diets, also impacting the digestive process and altering the plasma metabolite levels (Gonçalves and Gallardo-Escalate, 2017). However, the relative low supplementation levels of AW in the diets (5% or less) presented in this thesis along with the composition of the experimental diets (**Chapter 3**) revealed that the addition of by-products from seaweed did not change by macronutrients levels nor the total energy of the diets. In agreement with this, **Chapter 3** also indicated that adding 2.5% - 5% AW in gilthead seabream diets did not affect feed intake, digestive enzyme activities, or growth performance, more likely not altering as well diet palatability or digestibility. These results were supported by the lack of differences detected in the intestinal amylase and lipase activities in seabream fed the different experimental diets, which is relevant as these digestive enzymes are key for the digestion of starch and lipids, respectively (Moreau et al., 2001; Kurtovic et al., 2009). In addition, the lack of differences in the plasma metabolites, including glucose, triglycerides, and cholesterol levels measured 24h after feeding, suggests that dietary AW supplementation is not altering the digestibility of complex carbohydrates and lipids. These results are following a previous study by Magnoni et al. (2017) showing that dietary supplementation with heat-treated *Gracilaria* sp. at 5% had no significant impacts on the growth performance

when gilthead seabream was fed for 34 days at apparent satiation. Also, the growth performance of European seabass after 80 days of feeding a diet supplemented with *Gracilaria* sp. aqueous extract at 5% was similar to that of fish fed a control diet (Peixoto et al. (2019a).

Dietary AW supplementation in gilthead seabream have beneficial effects by modulating the intestinal microbiota community along with the antioxidant system

Chapter 3 evaluated the effects that dietary supplementation of *G. gracilis* ethanolic extracts and by-products (EEW and AW) has on the intestinal bacterial community of gilthead seabream. This is highly relevant as this fish species is an opportunistic feeder that consumes seaweeds as part of its natural diet (Arias, 1980; Pita et al., 2002), including *Gracilaria* species (Pita et al., 2002).

The results indicated that the dietary supplementation with AW at 5% increased the relative abundance of the Firmicutes phylum in the intestine of gilthead seabream when compared to fish fed the other experimental diets. This result suggested an improved intestinal bacteria conformation, as several genera of this phylum are recognized by their capacity to improve digestion, immune response, disease resistance, and oxidative balance in fish (Mathur et al., 2020; Ringø et al., 2018; Vieco-Saiz et al., 2019). In accordance, the analyses of the antioxidant system in the anterior and posterior intestines of fish, assessed and discussed in **Chapter 6**, suggested an improved functionality of this organ with dietary AW supplementation. In this regard, higher GR activity and GSH level measured in the anterior intestine of fish fed the AW supplemented diet with seaweed cultured under the BL condition suggest a change in the cellular redox status, although the GSH/GSSG ratio was not altered by this diet. In addition, the increase of GST activity in the anterior intestine of fish fed the AW supplemented diets suggest an improvement of the detoxification process, which could be beneficial for the mitigation of cellular toxicity of several endogenous and environmental chemicals. Despite the positive effects of the AW supplemented diets on the redox status of the anterior intestine of fish, these results should be carefully interpreted as these diets increased the LPO levels in the posterior intestine, reducing the GST activity in this intestine section. The results provided in the present study support previous findings showing that the antioxidant response is dependent on tissue and location, displaying a differential regulation in the

liver and the different sections of the intestine (Coutinho et al., 2016; Magalhães et al., 2020).

Feeding gilthead seabream AW supplemented diets resulted in improved welfare when exposed to an acute stressor

Dietary supplementation in gilthead seabream with *G. gracilis* by-products (EEW and AW) has displayed its role in the attenuation of the acute stress response when fish were subjected to crowding for 1h (**Chapter 4**). This was supported by the lower plasma cortisol levels in fish fed the diets supplemented with AW and EEW compared to the control diet. Dietary supplementation with *G. gracilis* and other seaweeds has been considered to attenuate the negative effects of stressors in several farmed fish species due to the antioxidant effect (Magnoni et al., 2017) and immunostimulant properties (Peixoto et al., 2016b; Peixoto et al., 2016a). Some beneficial effects of dietary seaweed supplementation could be attributed to the presence of bioactive compounds such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Awad and Awaad, 2017), which may act by decreasing the negative outcomes of stressors. Furthermore, as the bioactivity of seaweeds and their by-products appear to be associated with the antioxidant compounds, the stress attenuation response reported in **Chapter 4** could be also linked to the improvement of the antioxidant system response detected in both **Chapters 4 and 6**.

Dietary AW supplementation in gilthead seabream resulted in an improved immune status

Seaweed can modulate the immune response allowing fish to improve their physiological responses to challenging conditions. Recent studies have shown the beneficial effects of the dietary supplementation of *Gracilaria* sp. and their extracts in the gilthead seabream (Magnoni et al., 2017) as well as other farmed fish species (Peixoto et al., 2019a; Peixoto et al., 2019b; Peixoto et al., 2016b). In **Chapter 6** of this thesis, the effects of the AW supplementation on the immune response against a bacterial stimulus in gilthead seabream were investigated. Two weeks after feeding gilthead seabream AW supplemented diets, the haematological parameters, and the immune response were improved. In particular, the AW supplemented diets enhanced the oxygenation capacity, by decreasing the red blood cells volume (MCV) and increasing the haemoglobin

concentration (MCHC) in the blood of fish, based on the principle that larger surface area to volume ratios allows a more rapid oxygenation/deoxygenation of haemoglobin due to shorter diffusion distances (Dal'Bó et al., 2015). In addition, the AW supplementation altered the inflammatory response of gilthead seabream by increasing the number of the peripheral neutrophils which are involved in reactive oxygen species (ROS) generation, as well as in both proteolytic and anti-microbial activities (Charlie-Silva et al., 2019). Also, gilthead seabream exposed to a challenging condition (crowding) described in **Chapter 4** revealed that feeding fish for 59 days a diet supplemented with AW at 5% increased plasma peroxidase activity, suggesting an improvement in the immune response. The increase of neutrophils numbers together with the higher peroxidase activity reported in **Chapters 4** and **6**, respectively, suggest that both cellular and humoral components of the immune system in the gilthead seabream are boosted by the supplementation of AW into the diets. In addition, fish-fed AW supplemented diets showed higher plasma antiprotease and protease activities after being subjected to a crowing condition (1h) (**Chapter 4**) or bacterial stimulus (**Chapter 6**), respectively. Together, these results indicated that AW supplementation triggered mechanisms involving the bloking of proteolytic enzymes activity (Rebl and Goldammer, 2018) and the cleaving of the bacterial proteins. In contrast, the haemolytic complement activity, involved in both initiation of the innate immune response and mounting of an adaptive immune response (Secombes and Wang, 2012; Smith et al., 2019), did not seem to be modified by the supplementation of the AW in the diet, as this parameter remained unchanged after crowding (**Chapter 4**) and bacterial stimulus (**Chapter 6**).

Different light conditions applied in an IMTA production system can be used to modulate the agar yield and properties of the seaweed *Gracilaria gracilis*

Chapter 5 investigated the effects that different spectral light conditions in cultured *G. gracilis* had on growth, the agar yield and gel strength, pigments content, and antioxidant capacity. The study found evidence for a modulatory effect of blue spectrum light (BL condition) on the carbohydrate metabolism of this seaweed. This effect was demonstrated by the improvement of the agar yield and gel strength in relation to that obtained from a seaweed cultured under standard conditions (NT condition) in an IMTA-system at the ALGAplus company. These are promising results as the yield and the gel strength are both key factors to make feasible the business of farming *Gracilaria* for agar extraction

in Europe. Unfortunately, despite the promising results regarding agar features, the BL condition induces a significant reduction in the growth rate and productivity of this seaweed, which may raise some concerns to the seaweed production sector. Given a trade-off between the seaweed productivity and the agar properties, we suggested that the BL treatment could be applied at later stages in the seaweed production cycle in IMTA-systems. Beyond the effects that the spectral light had on the agar characteristics (**Chapter 5**), the results also showed that the BL treatment did not change the pigment content of seaweed nor the antioxidant compounds content and its antioxidant capacity. The results were also interesting taking into account the potential of the use of *G. gracilis* for future applications by extracting pigments (Pereira et al., 2020) or antioxidant compounds (Reboleira et al., 2020).

The AW obtained from *Gracilaria gracilis* cultured under different light conditions modulates the immune and antioxidant systems of seabream when supplemented in diets

The findings presented and discussed in **Chapter 5** led to the question of the alterations induced by the BL condition, regarding carbohydrate content, which could reflect differences in terms of the immune and antioxidant properties of the AW. To address this question, **Chapter 6** was designed to test whether dietary supplementation with AW obtained from a seaweed cultured under BL condition or NT condition in contrast to a control diet may have a different protective effect against a biotic stimulus (inactivated bacteria). The results indicated that the supplementation of the AW, regardless of light treatment, produced an improvement in the haematological parameters of gilthead seabream in comparison to fish fed the CTR diet. The inflammatory response was also improved in fish fed both AW supplemented diets (BL and NT) in relation to the CTR diet, by increasing the number of peripheral neutrophils, which are involved in the proteolytic and anti-microbial activities by ROS generation at the local of the inflammation (Charlie-Silva et al., 2019).

Both BL and NT diets produced an enhancement of the hepatic antioxidant system of gilthead seabream by increasing the CAT activity, important to break down the hydrogen peroxide (H_2O_2) generated in abundance by phagocytes during the immune response (Biller-Takahashi and Takahashi, 2018). In addition, the BL diet induced an increase in the hepatic GR activity, which along with the diet-modulated effect on GSH levels

suggests that this diet modulates the antioxidant system by inducing changes in the glutathione system. Similarly, the higher GR activity and the increase of GSH levels measured in the anterior intestine of fish fed the BL diet suggest an improvement in cellular redox status.

Overall, the AW obtained from the seaweed *G. gracilis* cultured in the BL condition seems to present better results than that in the NT condition when supplemented in gilthead seabream diets in terms of immune and antioxidant responses.

7.2. Conclusions

1) AW is an undervalued by-product with demonstrated bioactive properties either *in vitro* and *in vivo* (fish);
2) AW may be used as a supplement in aquafeeds for gilthead seabream up to 5% (w/w) level without compromising growth performance or digestion;
3) Dietary AW supplementation positively modulated the intestinal microbiota of gilthead seabream.
4) Dietary AW supplementation enhanced the skin yellowness of gilthead seabream;
5) Dietary AW supplementation improved the immune status of gilthead seabream by allowing better inflammatory response to inactivated *Phdp* (a bacterial pathogen stimulus), with higher cellular and humoral responses, which may improve disease resistance;
6) Dietary AW supplementation improved the redox status of fish by increasing the activities of some of the key enzymes involved in the antioxidant system, as well as by increasing the non-enzymatic compounds of the antioxidant system (GSH) in the liver and the anterior intestine of gilthead seabream;
7) Filtered sunlight in the BL condition (Blue light filter) may be used as an economical and effective method in the *G. gracilis* production cycle in IMTA-systems to improve both the yield and gel strength of the agar when the biomass production reaches target levels, without compromising the antioxidant capacity of the seaweed or their by-products;
8) The AW obtained from *G. gracilis* cultured under the BL condition showed more beneficial effects on both immune and antioxidant responses of gilthead seabream than that seaweed cultured under the NT condition (Neutral light filter).

Overall, the results presented in this thesis validated the use of AW as a sustainable dietary supplement to be applied by the aquafeed industry to increase the economic and ecological sustainability of this activity. This thesis also showed that dietary AW supplementation modulated the antioxidant and immune systems of gilthead seabream without compromising its growth. Finally, this thesis showed that by using filtered sunlight (BL condition) it is possible to increase the agar yield and quality, opening the door for upscaling *G. gracilis* biomass production towards polysaccharide extraction under a biorefinery approach. Therefore, the studies included in this thesis present novel data providing both basic and applied scientific knowledge towards the development of the aquaculture sector in view to find sustainable practices of both fish and seaweed cultivation.

7.3. Future approaches

Further research should address how AW supplementation modulates specific pathways involved in fish defence mechanisms, antioxidant responses, and stress responses. Such knowledge would allow the application of functional diets, for a short period before stressful events, enhancing fish resistance to bacterial infections and diminishing the recovery time allowing to maximize growth performance. A proteomic approach would be key since it would give more precise information regarding the protein profile involved in response to inflammation/infection as well as in response to a stressful event. Moreover, an infection trial with activated bacteria to assess the survival rate and the immune response will be also important to provide a more detailed view on mechanisms involving the role of dietary AW supplementation as a nutraceutical in fish by including other several proteomics approaches (transcriptomics and metabolomics).

In this thesis, the effect of the different filtered sunlight in the growth and composition of *G. gracilis* was assessed during winter. Therefore, it would be important to test the effects of the different filtered sunlight on the agar properties obtained from this seaweed in several production years/cycles to understand how agar production may be affected by the different time/seasons. Moreover, in this thesis, the seaweed growth trial lasted 4 weeks, however, shorter assays will be useful to test if the shorter time could result in an

equivalent modulatory effect regarding the improvement on agar properties extracted from *G. gracilis* detected in this study.

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