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ABSTRACT

The aim of this study was to evaluate the effect of *Gracilaria gracilis* inclusion in European seabass (*Dicentrarchus labrax*) diets and to analyse if the application of technological processes and additives in these diets can enhance their benefits to fish. The fish parameters evaluated were growth performance, feed efficiency, body composition, apparent digestibility of nutrients, innate immune response, and intestinal morphology. Six experimental diets were formulated to be isoproteic (53.4% Dry matter), isolipidic (14.6% Dry matter) and isoenergetic (22.3 kJg^{-1} Dry matter). A commercial-based diet was used as control diet (CTRL) and compared to other five diets with 8% inclusion of *G. gracilis*, namely: entire *G. gracilis* (GRA), after physically process (PHY), supplemented with 0.02% of phytogenic compounds (PFA), 2.5% of oligo-alginates (OLIGO), or 0.08% of nucleotides (NUCL). Dietary treatments had no effect on growth performance, voluntary feed intake, feed conversion ratio, protein efficiency, and final whole body composition. There was a decrease of muscle dry matter with the inclusion of phytogenic compounds and oligo-alginates comparing with the CTRL group. Dietary treatments with entire *G. gracilis*, phytogenic compounds and oligo-alginates had decreased the hepatosomatic index comparing to the CTRL group. The physical process of *G. gracilis* improved the apparent digestibility coefficient and nutrient balance of diets with *G. gracilis* inclusion. Furthermore, the oligo-alginates and nucleotides increased the protein digestibility of these algae. The physical process and oligo-alginate increased the nitrogen gain and decreased the nitrogen faecal losses. In relation to phosphorus digestibility, there was only a significantly decrease in fish fed with NUCL, which was not reflected in phosphorus faecal losses. The innate immune system was not affected by the dietary seaweed treatments. There was a decrease of *villus* width in anterior intestine with GRA diet, which was improved by physical process (Patent WO/2019/171293), oligo-alginates and nucleotides. There was a positive correlation between *villus* width and the protein and energy digestibility. Our findings suggest that physical process can improve the digestibility and nutrient absorption of diets with 8% *G. gracilis* without compromise growth performance, immune response and intestinal health. In addition, oligo-alginates and nucleotides could also improve *G. gracilis* application with advantages on protein digestibility and intestinal health.

Keywords: *Dicentrarchus labrax*; *Gracilaria gracilis*; Algae technological process; Phytogenic compounds; Oligo-alginates; Dietary nucleotide.

RESUMO

Na aquacultura intensiva, a proteína representa o componente alimentar mais dispendioso. Por um longo período de tempo, a farinha de peixe tem sido a principal fonte proteica usada em peixes carnívoros devido à sua elevada quantidade proteica, digestibilidade e palatabilidade. Contudo, o limite de captura e sobreexploração de stocks selvagens de peixe restringiram a disponibilidade mundial de farinha de peixe e tornaram a sua produção insustentável, aumentando o seu preço comercial. Para além disso, a produção de farinha de peixe causa um elevado impacto ambiental devido ao combustível e energia requeridos pelos barcos de pesca, pela necessidade energética associada à produção dos pellets, e pelos efluentes ricos em partículas orgânicas.

Deste modo, surgiram alternativas mais sustentáveis e menos dispendiosas como as proteínas vegetais como por exemplo cereais, soja e legumes. Porém, o elevado requerimento proteico de peixes carnívoros na sua dieta, limita a inclusão de proteínas vegetais como a principal fonte proteica. Para além disso, esta fonte pode estar associada a fatores anti nutricionais que prejudicam a sua digestibilidade, absorção de nutrientes e palatabilidade. Contudo, o aumento da procura das fontes vegetais como uma fonte proteica alternativa tem vindo a aumentar o seu preço.

A ausência de água doce nem de terra arável para a sua produção, o seu elevado conteúdo proteico e perfil balanceado de aminoácidos foram algumas das vantagens para o aumento do uso de algas marinhas como uma fonte proteica alternativa na aquacultura. O género *Gracilaria* tem um elevado conteúdo proteico, compostos bioativos, carotenoides, atividade antiviral, antioxidante e anti-inflamatória que podem potenciar o crescimento, resposta imune e resposta intestinal dos peixes. Contudo, a sua digestão e disponibilidade de nutrientes pode ser dificultada pela presença de compostos anti nutricionais e ausência de celulases endógenas e de enzimas necessárias nos peixes carnívoros. O elevado teor de fibra nas paredes celulares das algas também pode reduzir o seu uso nas dietas de aquacultura.

A elevada quantidade de fibras presentes nas paredes celulares das algas e a sua rigidez dificultam a sua digestibilidade, limitando o seu uso como fonte proteica nas aquaculturas. A biodisponibilidade, digestibilidade dos nutrientes e extração da proteína presentes nas algas pode ser potenciada pela remoção de compostos não-proteicos como a fibra. A extração da proteína é influenciada também pela elevada viscosidade e pela

presença de polissacáridos, como o agar, nas paredes celulares das algas vermelhas. A eficiência da extração da proteína das algas pode ser potenciada pela rutura das paredes celulares. Para isso, processos químicos e físicos são usados nas dietas com algas para aumentar a sua digestibilidade. Processos químicos como os enzimáticos limitam a ligação entre os polissacáridos e a proteína aumentando a digestibilidade das algas. Por sua vez, os processos físicos como a moagem e o tratamento ultrassónico rompem as paredes celulares das algas melhorando a extração da proteína.

Do mesmo modo, a suplementação de dietas com algas com aditivos comerciais, tais como os compostos fitogénicos, oligo-alginatos ou nucleótidos, pode melhorar a digestibilidade, palatabilidade e a resposta imune dos peixes. Os aditivos fitogénicos são compostos bioativos que derivam de plantas naturais cuja composição inclui óleos essenciais que melhoram o desempenho do peixe, a palatabilidade e o sabor da dieta. Estes atuam como suplementos naturais profiláticos que resulta numa atividade antiviral, anti microbial e anti-inflamatória, beneficiando a resposta imune. Os oligossacáridos alginatos são outro exemplo de suplementos usados na aquacultura. Estes têm uma elevada capacidade de aglutinação que reduz a lixiviação e otimiza a textura, melhorando a digestibilidade e crescimento dos peixes. Outro exemplo de suplemento são os nucleótidos, usados como uma fonte externa para potenciar processos fisiológicos como um rápido crescimento face a situações de *stress*. Para além disso, estes suplementos aumentam o comprimento das vilosidades intestinais, estimulam a produção enzimática, melhorando a digestão e disponibilidade de nutrientes.

Deste modo, este trabalho visou avaliar o efeito da inclusão de *Gracilaria gracilis* em dietas de robalos (European seabass, *Dicentrarchus labrax*), bem como avaliar se a aplicação de processos tecnológicos e da suplementação com aditivos potenciam estas dietas. Os parâmetros avaliados foram o crescimento, eficiência alimentar, composição corporal, digestibilidade aparente dos nutrientes, resposta imune inata e da morfologia do intestino. Foram formuladas seis dietas experimentais para serem isoproteicas (53.4% Matéria seca), isolipídicas (14.6% Matéria seca) e isoenergéticas (22.3 kJg^{-1} Matéria seca). A dieta controlo (CTRL) foi comparada com cinco dietas teste aonde foram incluídos 8% da espécie *Gracilaria*, nomeadamente: alga inteira (GRA), após processo físico (PHY), suplementado com 0.02% de compostos fitogénicos (PFA), 2.5% de oligo-alginatos (OLIGO), ou 0.08% de nucleótidos (NUCL).

Como resultado, não houve efeitos no crescimento, consumo voluntário, índice de conversão alimentar, eficiência proteica e composição corporal do robalo alimentado com alga processada, não processada e suplementada com aditivos. A matéria seca do músculo do robalo diminuiu com a inclusão de aditivos fitogénicos e oligo-alginatos. O índice hepatosomático diminuiu significativamente nos peixes alimentados com GRA, PFA e OLIGO. O processo físico melhorou a digestibilidade aparente e o balanço de nutrientes da *Gracilaria* sp. A digestibilidade da proteína da *G. gracilis* foi potenciada com a adição de oligo-alginatos e nucleótidos. Os processos físicos e os oligo-alginatos aumentaram o ganho de nitrogénio e diminuíram as perdas fecais do mesmo. Os peixes alimentados com a dieta GRA teve maior digestibilidade do fósforo e menor perda fecal. Em relação ao sistema imune inato, não foi verificado nenhuma alteração neste parâmetro em nenhum dos tratamentos. Quanto à morfologia do intestino, a largura das vilosidades diminuiu no intestino anterior com a dieta GRA mas foram melhorados com os processos físicos e a suplementação com oligo-alginatos e nucleótidos. Houve uma correlação positiva entre a largura das vilosidades e digestibilidade da proteína e da energia.

Em conclusão, apesar da inclusão de *G. gracilis* a 8% não ter comprometido o consumo alimentar nem o crescimento do robalo, foi verificada a sua fraca digestibilidade. Os processos físicos aplicados na dieta com algas conseguiram melhorar a sua digestibilidade que associada com o aumento do tamanho das vilosidades intestinais do intestino anterior pode promover um aumento da absorção de nutrientes, acessibilidade e digestibilidade. A digestibilidade da *G. gracilis* foi também melhorada com a suplementação destas dietas com oligo-alginatos e nucleótidos possivelmente devido ao aumento do tamanho das vilosidades intestinais do intestino anterior.

A digestibilidade do fósforo não foi afetada pelas dietas, excepto nos peixes alimentados com nucleótidos aonde se verificou uma diminuição significativa em relação ao controlo, não se tendo refletido nas perdas fecais. Por outro lado, a inclusão de *G. gracilis* e de compostos fitogénicos pode aumentar a descarga de azoto no ambiente sendo uma fonte de poluição, requerendo um tratamento de efluentes.

Palavras-chave: *Dicentrarchus labrax*; *Gracilaria gracilis*; Processos tecnológicos; Compostos fitogénicos; Oligo-alginatos; Nucleótido.

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ABBREVIATIONS

ABW	Average body weight
ACH50	Alternative complement pathway
ADC	Apparent digestibility coefficient
AOAC	Association of Official Analytical Chemists
Cr_2O_3	Chromium (III) oxide
DE	Digestible Energy intake
DGI	Daily growth index
DHA	Docosahexaenoic acid
D_{ing}	% nutrient (or kJ. g^{-1} gross energy) of the test ingredient
DL	Digestible Lipid intake
DM	Dry matter
DN	Digestible Nitrogen intake
DP	Digestible Phosphorus intake
D_{ref}	% nutrient (or kJ. g^{-1} gross energy) of the reference diet
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol Bis (2-Aminoethyl Ether)-N,N,N',N' Tetraacetic Acid
EPA	Eicosapentaenoic acid
EU	Enzyme unit
FAO	Food and Agriculture Organization (of the United Nations)
FCR	Feed conversion ratio
FM	Fishmeal
GRA	Diet with entire *Gracilaria* sp.
H_2O_2	Hydrogen peroxidase
$H_2PO_4^-$	Dihydrogen phosphate
H_2SO_4	Sulphuric acid
HBSS	Hanks Balanced Salt Solution
HIS	Hepatosomatic index
HPO_4^{2-}	Hydrogen phosphate
KH_2PO_4	Potassium hydrogen phosphate
NDF	Neutral detergent fiber
NUCL	Diet with entire *Gracilaria gracilis* supplemented with nucleotides
OLIGO	Diet with entire *Gracilaria gracilis* supplemented with oligo-alginates

PER	Protein efficiency ratio
PFA	Diet with entire *Gracilaria gracilis* supplemented with phytogenic compounds
PHY	Diet with physically processed *Gracilaria gracilis*
RaRBC	Rabbit red blood cells
RAS	Recirculating aquaculture system
TMB	3,3’,5,5’-tetramethylbenzidine hydrochloride
VFI	Voluntary feed intake
W_0	Initial weight gain
W_1	Final weight gain
ΔT	Variation of temperature

CHAPTER 1

GENERAL INTRODUCTION

1.1. World Fish Aquaculture Production

In 2016, global fish production (capture and aquaculture) reached 171 million tonnes, of which about 88% was used for human consumption with aquaculture contributing 46.8% to this tonnage (FAO, 2018).

In 2016, inland aquaculture contributed 64.2% to food produced worldwide in terms of aquaculture as compared to mariculture and costal aquaculture (35.9%; FAO, 2019). In 2017, freshwater species was the most produced aquaculture species (39.9%), where carps, barbels and other cyprinids accounted 63.5% to this tonnage (FAO, 2019). The marine fish reached 2.8% of the annual global production (FAO, 2019).

Over the last two decades, Asia has been responsible for 89% of world aquaculture production (FAO, 2018), China in particular, contributing significantly to the world food stock and nutrition due to their large population and fish farmed domestically (Tacon et al., 2009). By 2030, the capture fisheries and aquaculture production are expected to grow up to 201 million tonnes, an 18% increase in relation to 2016 (Klinger and Naylor, 2012). Furthermore, with the expected increase of world population to 9 billion in 2050, aquaculture could be a potential source to meet this increased demand for human consumption, since it is one of the fastest growing food-producing sectors (Béné et al., 2015).

1.2. European seabass (*Dicentrarchus labrax*)

European seabass belong to the genus *Dicentrarchus*, Moronidae family and Perciformes order (Linnaeus, 1758).

This specie have a long shaped body (*Figure 1.1*). The body coloration varies with the origin of the fish, ranging from silvery grey to bluish on the back, silvery on the side, white or pale yellow in the belly with silver-blue, pale gold or bronze in flanks, with a dark sideline very visible. The young seabass is paler and have dark spots on the back and the upper sides of the head are pointed, becoming blunter in older fish. This species is covered by small scales can reach 1 meter of length and weight up to 12 kg. The opercle has two flat spines, the preopercle is large, with forward-directed spines on the lower margin and have a terminal mostly protractile mouth. They have two dorsal fins, with the first fin being constituted with 8 to 10

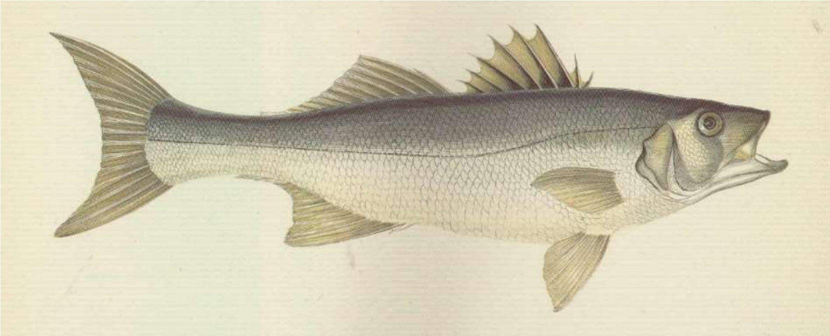

Figure 1.1 – European seabass (*Dicentrarchus labrax*) (Couch, 1865).

spines and the second with 1 spine and 12 or 13 soft rays. The anal fin has 3 spines and 10 to 12 soft rays. The caudal fin is moderately forked (FAO, 2005).

1.2.1. Habitat and Biology

European seabass inhabits coastal inshore waters, estuaries and brackish water lagoons in shallow coastal waters up to 100 m of depth (Wheeler 1975), since they are eurythermal and euryhaline (Pickett et al., 1994). This species can be found in the Eastern Atlantic from Norway to the coast of Morocco, in the Mediterranean and in Black Sea (Wheeler 1975). They are migratory in British waters approaching inshore in spring and summer and move to deep waters in late autumn (Wheeler 1975).

This species is also known as “sea wolf” due to their aggressive feeding behavior, being an opportunistic predator. They are carnivorous feeding on crustaceans, molluscs and small pelagic fish, such as sardines, sprats and sand smelts (Pickett et al., 1994).

European seabass is gonochoristic and temperature-dependent for sexual determination (Piferrer et al., 2005). This species reaches sexual maturity at the third year in males and fourth in females in the Mediterranean, and in the fourth and seventh, respectively in Atlantic.

The spawning season occurs between December and March in the Mediterranean and up to June in Atlantic (FAO, 2019), spawning once a year in inshore areas with salinities lower than 35‰ (Wheeler 1975). The female spawns small pelagic eggs with dimensions of 1.0 to 1.5 millimeters in diameter, reaching 300 000 eggs per one kg of body weight (Vázquez and Muñoz-Cueto, 2014).

1.2.2. Production

European seabass is an economically important cultured fish species in Mediterranean coastal waters (Alasalvar et al., 2002).

The major world producer of seabass is Asia, followed by Europe. Turkey is the country with the largest production of this species, followed by Greece (FAO, 2019).

In terms of nutrition, European seabass requires a diet with a very high protein content, ranging from 40 to 50% (Lupatsch 2005); an energy content of 19% (Lupatsch 2005); a lipid content between 15 and 18% (Webster and Lim 2002); fibre content between 3 and 4% (NRC, 2011); and phosphorus content at 0.8% (Oliva-Teles and Pimentel-Rodrigues 2004).

1.3. World seaweed production

The world seaweed production reached 30.4 million tonnes in 2015, in which 96.7% corresponded to culture production (Ferdouse et al., 2018). This sector had doubled their production between 2000 and 2010, while wild seaweeds harvested had declined from 1.2 to 0.9 million tonnes (Nayar and Bott, 2014). From the 221 species of commercial seaweeds globally produced, about 10 species are intensively cultivated, such as brown seaweeds (*Saccharina japonica*, *Undaria pinnatifida*), red seaweeds (*Porphyra* spp., *Euchema* spp., *Kappaphycus alvarezii*, *Gracilaria* spp.), and green seaweeds (*Enteromorpha clathrate, Monostroma nitidum* and *Caulerpa* spp.) (Wikfors and Ohno 2001). China is the largest producer of seaweeds, contributing 60% to the global volume, followed by Indonesia, Republic of Korea and Philippines (Ferdouse et al., 2018). The genus *Euchema*, Japanese kelp, *Gracilaria* and *Undaria* are the most produced seaweed worldwide (Ferdouse et al., 2018). Between 1950 and 2017, Norway was the major producer of seaweeds in Europe, followed by France and United Kingdom (FAO, 2019). Portugal had a seaweed production of 506 847 kg, between 1950 and 2017 (FAO, 2019).

1.3.1. *Gracilaria* sp. production

The genus *Gracilaria* has 150 species and is the third largest red algal group of its taxonomical rank (Baweja et al., 2016) and is one of the highly valued seaweed species (Ferdouse et al., 2018).

Gracilaria spp. have the ability to achieve high growth rates, high regeneration capacity, high yields (Kain and Destombe, 1995), high crop productivities per unit area (Bolton et al., 2009) and do not require arable land or freshwater availability (Angell et al., 2016). These advantages have turned them into one of the highest valuable and exploited worldwide seaweed genus (Buschmann et al., 2017). They represented 13.6% of the annual world production in 2016 (Ferdouse et al., 2018), providing more than 66% of agar supply to the phycocolloid industry (Pereira and Yarish, 2008). This genus is also used in human consumption and in Integrated Multi-Trophic Aquaculture to have sustainable and cleaner fish aquaculture systems (Zhou et al., 2006).

Asia is the major producer of *Gracilaria* sp. in world, in which China and Indonesia are responsible for 98% of the global production (Kim et al., 2017). Italy is the major producer of Europe, where Portugal produce this species mainly using Integrated Multi-Trophic Aquaculture concept (Troell et al., 2009).

1.4. Fish meal as ingredient in aquafeed industry

In intensive aquaculture, fish feeding represents a large proportion of the overall costs, ranging from 30 to 60% (Azaza et al., 2009), with protein being the most expensive dietary component (Lovell, 2002).

According to feeding habits, there are several differences related to the nutritional requirements of freshwater and marine fish species (Oliva-Teles et al., 2015). Marine fish species require essential fatty acids in their diets, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), while freshwater species have no such need (Silva et al., 2010). Omnivorous and herbivorous fish require diets with low protein content, between 25 and 35% in the diet, whereas diets with higher protein content are required for carnivorous fish, from 40 to 55% in the diet (NRC, 2011).

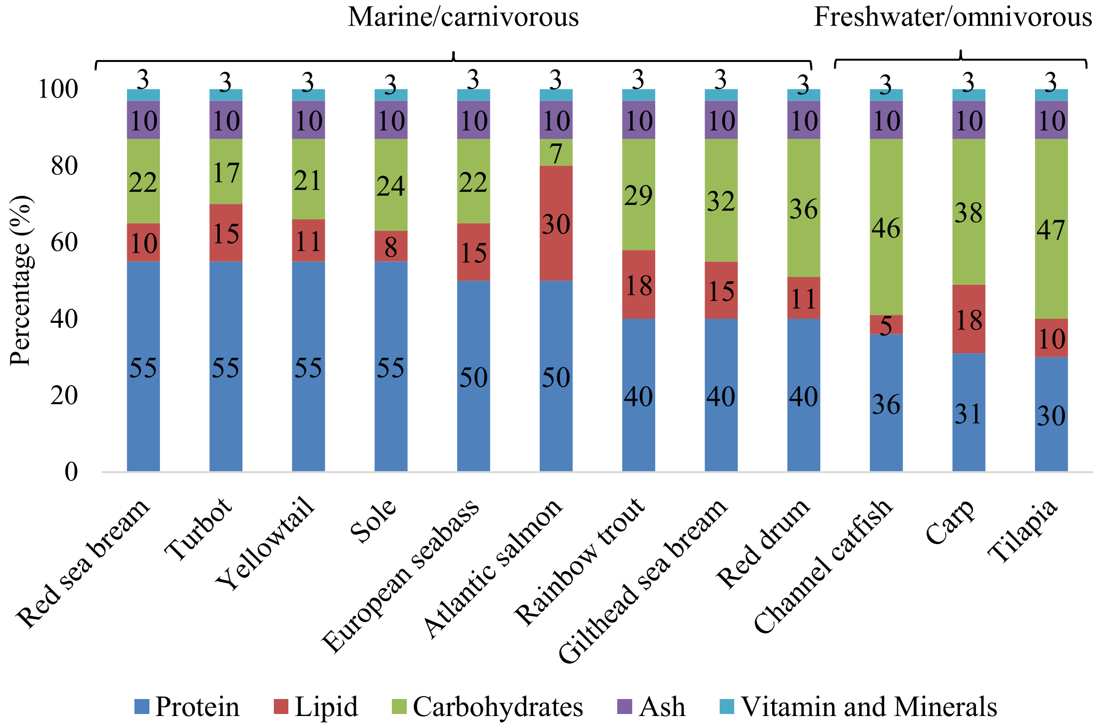

Figure 1.2 - Dietary composition of marine and freshwater fish species (Oliva-Teles et al., 2015)

Fishmeal (FM) has been used for a long time in aquafeeds as the main protein source, especially for feeding carnivorous fish, due to their high protein content, ranging between 60 and 72% (Shepherd and Jackson 2013), high digestibility and palatability, and adequate amino acid content (Hardy 2010). However, the growth of aquaculture production has increased the price ratio of fish and soybean meal, an alternative protein source, between 1990s and 2010 from 2:1 to 4:1, respectively (Shepherd and Jackson, 2013). Furthermore, the limitation of world capture fisheries and the overfishing of wild stocks has limited the world availability of FM and made their production unsustainable (Nordahl, 2011).

Regarding the environmental impact, the production of FM contributes largely to greenhouse gas emissions and carbon footprint, mainly due to the fuel and energy used by fishing vessels and processing energy inputs required to grind, mix, make and dry the pellets (Pelletier and Tyedmers, 2007). In addition, the effluents derived from aquaculture systems can cause serious impacts on environment due to the production of dissolved inorganic and particulate organic matter from uneaten food, faeces, dead fish and excretion products (Marinho et al., 2013).

The search for less expensive and more sustainable alternative protein sources is being studied for a partial or total replacement of FM (Shpigel et al., 2017).

1.5. Vegetable sources as alternative protein source

The main alternative protein sources to FM used in aquafeeds are plant feedstuffs, such as oilseeds (soybean, rapeseed, cottonseed, sunflower), legumes (lupin, peas, alfalfa, fava bean), cereals (maize, wheat, sorghum) and tubers, due to their high availability, low price and potential increase in production (Tacon et al., 2011).

These ingredients are the major protein source in omnivorous and herbivorous fish diets; however, carnivorous fish requires a high protein content that limits the use of plant feedstuffs as the main protein feedstocks (Oliva-Teles et al., 2015). The total replacement of FM by soybean meal could not affect carnivorous fish, such as European sea bass (Couto et al., 2015), cobia (Romarheim et al., 2008) or Atlantic cod (Colihueque et al., 2010).

The sensitivity of FM replacement by plant feedstuffs could be species-specific (Oliva-Teles et al., 2015), but it is recognized that these compounds have anti-nutritional factors that can decrease digestion and absorption of nutrients, neutralize vitamin functions, and induce toxicity (Oliva-Teles, 2012). It can cause intestinal inflammation or decrease immune response in Atlantic salmon (Krogdahl et al., 2010) and gilthead sea bream (Kokou et al., 2012). Furthermore, it is deficient in lysine and methionine (Oliva-Teles et al., 2015), has low palatability, low digestibility and high carbohydrate content (Olsen and Hasan 2012).

Additionally, the price of plant meals had also increased due to the increase in global demand for food (FAO 2009).

The aforementioned disadvantages of fish and vegetable meal use requires a search for sustainable alternative protein sources such as marine macroalgae (Al-Asgah et al., 2016).

1.6. Seaweeds used as alternative protein source

Seaweeds have a higher production yield per unit area and do not require freshwater or arable land to be cultivated (Van Krimpen et al., 2013), when compared to fish or soybean meal. These characteristics, together with their high protein content, between 8 to 50% of dry weight

(Fleurence 1999), and balanced amino acid profile, could turn seaweeds into a potential source of protein in aquaculture (Wong and Cheung 2000).

Furthermore, the natural exposure of algae to high oxidative levels and stress has led to a development of protective compounds, such as pigments and polyphenols that enhance the health of consumers (Wang et al., 2009).

The ingestion of algae by carnivorous fish is generally not an important source of nutrients and energy, since they do not have endogenous cellulases necessary to digest plant or macroalgal biomass (Stickney and Shumway 1974), and do not have secreting enzymes able to degrade complex algal polysaccharides (Sera et al., 1974). Despite this, a 10% inclusion of *Gracilaria bursa-pastoris* and *Ulva rigida* on European seabass (Valente et al., 2006) and 10% *Porphyra* sp. on rainbow trout (Soler-Vila et al., 2009) did not have a negative effect on growth performance, nutrient utilization and body composition.

1.6.1. *Gracilaria* sp. as feed ingredient

Among the different seaweeds, the red algae (*Rhodophyta*), where *Gracilaria* sp. is included, provide a better result due to their chemical composition, their high protein content (Angell et al., 2016) and high diversity of bioactive compounds, such as polysaccharides and pigments (Wells et al., 2017). The characteristic of these algae is the presence of phycobilins, responsible for their red colour (Kadam et al., 2013) and the richness of carotenoids, such as fucoxanthin, lutein, and *β*-carotene (Ortiz et al., 2009), which enhance their use as replacement or reduction of artificial colourants in aquafeeds (Nickell and Bromage 1998).

Wild *Gracilaria* sp. has a protein content that varies between 5.6 and 24%, the ash content ranges from 22.7 to 53.4% and the lipid content is lower than 4% (Gressler et al., 2010). The high protein variation in seaweeds could be due to environmental conditions, geographic areas and seasons (Ito and Hori 1989). However, under controlled conditions, the production of seaweed can balance their nutritional values in order to improve their utilization.

1.6.1.1 *Gracilaria* sp. effect on fish growth performance

An inclusion level of seaweed at a maximum of 10% is recommended in aquafeeds (Evans and Critchley 2014) to not compromise growth, feed utilization and digestibility in commercial fish, both herbivores and carnivores.

Even so, the use of seaweed as dietary supplementation has disadvantages. A reduction on growth performance (27%), protein utilization (17%) and protein retention (6%) was observed in Nile tilapia with inclusion levels of *Gracilaria* sp. above 10% (Marinho et al., 2013). Even with 10% inclusion level of *Gracilaria* sp., feed conversion ratio (FCR), growth performance and protein efficiency ratio (PER) were affected in Nile tilapia (Araújo et al., 2015), rainbow trout (Valente et al., 2015) and African catfish (Al-Asgah et al., 2016). The same pattern related with growth performance was reported in European seabass with the inclusion of *Gracilaria* bursa-pastoris at 10% and *Gracilaria cornea* at 5% (Valente et al., 2006). The inclusion of 10% *Gracilaria vermiculophylla* had also a negative effect on feed intake and growth performance of Nile tilapia (Silva et al., 2015).

1.6.1.2 *Gracilaria* sp. effect on fish immunity

The innate immune response in fish is highly important as the first line of host defence acting by recognizing pathogen-specific molecules, buying time until the animal is able to develop an efficient immune response (Saurabh and Sahoo, 2008).

Alternative complement pathway (ACH50), lysozyme and peroxidase activities plays an important role in fish immune defence against pathogens. These parameters are essential in non-specific immune response (Tort et al., 2004) acting by lyse of pathogens, or by directly disruption of bacteria cell walls, or by the production of oxidative chemicals, respectively (Nayak 2010). These indexes are positively related with disease resistance (Fevolden et al., 1994).

Gracilaria sp. has antiviral, antifungal, anticancer, antioxidant, anticoagulant, anti-inflammatory, neuroprotective and antibacterial compounds (Kumala et al., 2018) that can improve ACH50, lysozyme and peroxidase activities of Nile tilapia, with an increase of 54%, 61% and 69%, respectively, at 5% inclusion levels (Araújo et al., 2015).

The bioactive compounds, such as β-glucans (Díaz-Rosales et al., 2005) present in red seaweeds could be enhance immune system and improve production in several fish species as meagre (Peixoto et al., 2017), clownfish (Karuppiah 2014) and European seabass (Peixoto et al., 2019).

The stimulation of non-specific immune response by *Gracilaria* sp. can be a potential sustainable alternative to reduce antibiotic use (Radhika and Ameer, 2016) and minimize stress conditions in aquaculture production (Scapigliati et al., 2002).

1.6.1.3 *Gracilaria* sp. effect on fish histomorphology of intestinal tract

The intestine is an important and complex multifunctional organ that acts as a barrier against pathogens and involves digestion, nutrient absorption and osmoregulation (Buddington et al., 1997). Morphological and functional damages in intestine occur when there is external aggression and the barrier disrupt, affecting the intestinal epithelial permeability, which increase the passage of pathogens, protein, and toxins (Berkes et al., 2003).

The presence of antinutrional compounds (Sangeetha et al., 2014) may reduce bioavailability and digestibility of nutrients (Al-Asgah et al., 2016), which affects gastrointestinal tract and fish growth performance (Déléris et al., 2016). In addition, the concentration of fibre and ash is often high in seaweeds, which need to be kept low to maintain the proper amino acid contents, decreasing the digestible energy content (Al-Harthi and El-Deek 2012).

Seaweeds can also affect negatively the intestinal morphology of fish, such as Nile tilapia with 10% inclusion of *Gracilaria*, *Porphyra* and *Ulva* (Silva et al., 2015), in terms of *villus* length, and thickness, affecting digestion and absorption of nutrients. Furthermore, in rainbow trout, the inclusion of 10% *Gracilaria* sp. had also reduced intestine diameter and *villus* length (Araújo et al., 2016).

1.6.2. Use of technological processes in seaweeds

The poor protein digestibility, mainly caused by the high fibre content in cell walls and their rigidity, reduce the use of seaweed in aquafeeds (Bleakley and Hayes 2017). In order to improve the bioavailability of seaweed, the removal of non-protein components, such as ash and fibre, is important to extract the protein and increase the concentration of essential amino acids (Angell et al., 2016). The extraction of algal protein is determined by the availability of protein molecules present in their composition, which is influenced by the high viscosity and anionic cell wall polysaccharides, such as carrageenan and agar in red seaweed (Fleurence 1999). Cell wall disruption methods can be a way of enhancing the efficiency of algal protein extraction through the increase of nutrient accessibility and digestibility, by breaking the anionic cell wall maintaining the nutritive quality of bioactive compounds inside the cell (Teuling et al., 2019).

Enzymatic pre-treatment is one method used to cleavage or limit the linkage between polysaccharides and proteins (Fleurence 1995a) that can enhance the protein digestibility of *Ulva pertusa*, *Undaria pinnatifida* (Amano and Noda 1992), *Palmaria palmata* (Lahaye and Vigouroux 1992), *Porphyra yezoensis* (Amano and Noda 1992), *Chodrus crispus* and *Gracilaria verrucosa* (Fleurence 1995b), *Ulva rotundata* and *Ulva rigida* (Fleurence 1995a).

Another methods used to disrupt cell walls of seaweeds are physical and mechanical treatments, such as mechanical grinding, bead milling, ultrasonic treatment and osmotic shock (Harnedy and FitzGerald 2013). Physical grinding applied to *Porphyra acanthophora*, *Sargassum vulgare* and *Ulva fasciata* improved their protein extraction (Barbarino and Lourenço 2005). Furthermore, the inclusion of autoclaved *Ulva* sp. in rainbow trout had increased the protein ADC (Güroy et al., 2013). The bead milling method applied in microalgae, such as *Nannochloropsis* sp. and *Scenedesmus* sp., have increased FCR in 11% and improved digestibility at 16.3% in African catfish (Agboola et al., 2019).

1.7. Use of feed additives in aquafeeds

With the purpose of improving and optimizing the ability of fish to efficiently digest seaweed as ingredient, a supplementation with dietary additives could be a potential solution (Wong and Cheung 2001). Additives can improve the fish growth performance (Sanchez et al., 2016), reduce the low palatability and increase the feed intake of alternative protein sources (Glencross et al., 2007).

Furthermore, dietary supplements can act as a natural immunostimulants that could increase the resistance against pathogens (Bricknell and Dalmo 2005) under stress conditions (Bagni et al., 2000), and improve survival by enhancing non-specific defence mechanisms before pathogen exposure, stimulating growth (Sakai 1999). In this way, it can also reduce economic and environmental costs in aquaculture production related with antibiotics and vaccines (Peterson et al., 2015).

Some examples of dietary additives are phytogenic compounds, oligo-alginates and dietary nucleotides, which will be studied in this work.

1.7.1. Phytogenic compounds

The phytogenic compounds, a natural plant-derived bioactive substances, have in their composition organic acids, acidifiers and essential oils (Abo-State et al., 2017). The addition of essential oils, such as carvacrol and thymol into the diets, can improve fish growth performance in channel catfish (Peterson et al., 2014), palatability of weaned piglets (Windisch et al., 2008) and feed flavour (Knowles et al., 2005). It can also enhance shelf-life of fillets on common carp (Mahmoud et al., 2006) and had effects on fillet and muscle content of channel catfish, decreasing fat and increasing protein content (Peterson et al., 2014).

These feed additives are a natural prophylactic supplements used to replace antibiotics in aquaculture (Heidarieh et al., 2012) due to their antiviral, antimicrobial, antifungal, anti-inflammatory and anti-oxidative responses (Brenes and Roura 2010). It acts through lectin-mediated pathways (LaFrentz et al., 2012) which recognize, agglutinate and opsonize microbial pathogens, activate the complement system (LaFrentz et al., 2012), increase serum lysozyme levels (Abo-State et al., 2017), improving immune resistance (Peterson et al., 2015) of channel catfish (Zheng et al., 2009) and rainbow trout (Ahmadifar et al., 2011).

Regarding the gastrointestinal tract, the phytogenic compounds can improve the morphology of small intestinal tissues of broiler chickens (Murugesan et al., 2014), poultry (Applegate et al., 2010), and weaned pigs (Namkung et al., 2004) by increasing the *villus* height, and decreasing crypt depth and mucosal thickness (Yang et al., 2015). These compounds stimulate the production of digestive secretions (Syahidah et al., 2015) and enzymatic activity (Windisch et al., 2008), which allied to a reduction in mucosal thickness, contribute to a more efficient digestion and availability of nutrients (Abo-State et al., 2017). This can lead to a pressure reduction in the immune system (Murugesan et al., 2014), improving feed utilization efficiency and growth performance (Panserat and Kaushik 2010).

However, high levels of phytogenic compounds, above 0.2%, showed no improvement of growth performance and disease resistance in channel catfish (Peterson et al., 2014), with a negative effect on growth and resistance to pathogens in rainbow trout (Sönmez et al., 2015). In addition, their inclusion levels at 0.05% could have beneficial effects on growth, antioxidant activity, and resistance to *Aeromonas hydrophila* in channel catfish (Zheng et al., 2009).

1.7.2. Oligo-alginates

Alginates are indigestible polysaccharides used in fish diets as a stabilizer, thick or emulsifier agent and dietary fibre (Brownlee et al., 2005). They are produced by brown algae as structural polysaccharides and by bacteria as extracellular polysaccharides (Mahmoud et al., 2006). Oligo-alginates, generated via hydrolysis from polymeric alginates, are a growth promoter and species-specific (Yokose et al., 2010) that have in their constitution sodium, ammonium, potassium, and calcium salts of alginic acids (Igbinosun and Roberts, 1988). Because of the extrusion process, these compounds have agglutination capacity that reduces lixiviation of water-soluble nutrients and optimizes feed texture, which improves digestibility, growth rate and develops high energetical feeds (Rodriguez-Miranda et al., 2012). These additives improved growth performance of orange-spotted grouper, *Epinephelus coioides*, at 1% inclusion (Yeh et al., 2008) and in Nile tilapia at 2% inclusion (Igbinosun and Roberts, 1988).

In relation to immune defence, oligo-alginates improved the non-specific immune response and disease resistance of blue-and-yellow grouper (Balaraman and Subramanian, 2017), tiger grouper (Harikrishnan et al., 2011), rainbow trout (Ballesteros et al., 2015), common carp (Liu et al., 2006), kelp grouper (Harikrishnan et al., 2011), snakehead (Miles et al., 2001), and European seabass (Bagni et al., 2005).

Furthermore, oligo-alginates can improve intestinal morphological structure of pigs with an increase in *villus* height in duodenum and jejunum, promoting a higher absorption and digestibility of nutrients, improving growth performance (Wan et al., 2018). Their supplementation can also enhance the intestinal barrier function of pigs (Wan et al., 2017).

1.7.3. Dietary nucleotides

Dietary nucleotides are highly used in aquaculture as feed attractants in crayfish diet (Safari et al., 2015), as coenzymes by increasing the activity of digestive enzymes (Murthy et al., 2009) and as a prebiotic (Carver, 1994).

These compounds are an additional source of exogenous nucleotides that can optimize physiological processes during stress conditions such as rapid growth, reproduction, environmental change and disease (Hoffmann, 2008).

Nucleotides can improve growth performance in Atlantic salmon (Burrells et al., 2001b), white shrimp (Li et al., 2007), largemouth bass (Kubitza et al., 1997), rainbow trout (Adamek et al.,

1996) and meagre (Rodrigáñez et al., 2013), but their use needs to be balanced with the nutrient retention efficiency. This is an important requirement once high levels of dietary nucleotides increase voluntary feed intake (VFI) but decrease digestive enzyme activities affecting growth performance and immune responses due to the limited capacity of absorption of its gastrointestinal tract (Safari et al., 2015).

Nucleotides also improved the immune response of crayfish (Safari et al., 2015), salmonids (Burrells et al., 2001a) and Nile tilapia (Ramadan et al., 1994), through the increase of serum complement and lysozyme activities (Sakai et al., 2001), and stimulation of B and T lymphocytes (Safari et al., 2015).

The nucleotides may reverse the intestinal pathology caused by inflammatory reaction (Rodrigáñez et al., 2013). For example, dietary inclusion of nucleotides minimized diarrhea-causing secretor pathway in the intestine of seabream, with a consequent improvement of growth (Rodrigáñez et al., 2013). These compounds increased *villus* height in weaning rats recovered from diarrhoea (Bueno et al., 1994).

1.8. Research objectives

The main goal of the present dissertation is to analyse the effect of *Gracilaria gracilis* inclusion in European seabass diets, evaluating fish growth performance, digestibility, nutrient utilization, immunology and intestinal morphology. Moreover, it intends to answer the question whether these fish parameters can be enhanced by the application of physical processes and supplementation with phytogenic compounds, oligo-alginates or nucleotides in *G. gracilis* diets.

CHAPTER 2

MATERIALS AND METHODS

2.1. Experimental diets

Six isoproteic (53.5% dry matter, DM), isolipidic (14.9% DM) and isoenergetic (22 kJg^{-1} DM) diets were formulated. A commercial-based diet for European seabass was used as control diet and compared to other five diets with 8% inclusion of *Gracilaria gracilis* namely: after physically processed (PHY), entire (GRA), supplemented with 0.02% of phytogenic compounds (PFA), 2.5% of oligo-alginates (OLIGO) or 0.08% of nucleotides (NUCL) (Table 2.2).

Gracilaria gracilis was produced by ALGAplus and the seaweed was introduced in the diet at expense of FM and wheat meal. Tested ingredients and additives were kindly provided by Marinalgae4aqua project that also supported all experimental work of this thesis. In relation to the application of physical process, there is a patent that limits the explanation of the entire process (Patente WO/2019/171293).

SPAROS, Lda extruded all the experimental diets with a pellet size of 2 mm to assure better ingestion rate in the growth trial.

At the end of growth trial, the remaining diets were used to evaluate the ADC diets, by the addition of 1% chromic oxide (CR_2O_3, Merck KGaA, Germany) as an inert marker.

2.2. Experimental animals

European seabass from a commercial fish farm in Spain (Sonrionansa S.L., Cantabria) were transported to CIIMAR, located in Matosinhos. Fish were maintained in a closed saltwater recirculating aquaculture system (RAS) and were kept in quarantine for 2 weeks and fed with a commercial diet, before experimental trial.

2.3. Growth Trial: Experimental conditions

After acclimation the fish were fasted for 24 h and were individually weighed (g) and measured (total length, cm). Fish were randomly distributed among fibreglass tanks (*n* = 18, 50 L) in homogeneous groups of 19 fish per tank (average of body weight of 29.7 ± 0.02 g, total length of 13.7 ± 0.08 cm, density of 11.3 kg/m^3). This RAS had a flow rate of 4 L/min and maintained at a constant water temperature, salinity and dissolved oxygen at 21 ± 1.8 °C, 35 ± 1 ‰ and 90 % saturation level, respectively. Physiological parameters were measured every day (salinity and pH) and twice per week (ammonium (NH_4^+) and nitrite (NO_2^-)) using a commercial kits

(Sera) and maintained at recommended levels for marine species, such as European seabass (FAO, 2005) and gilthead seabream (Table *2.1*). The photoperiod established was 12 h of light and 12 h of dark.

Table 2.1 – Recommended parameter levels for marine species in a RAS system (FAO, 2005).

	Parameters evaluated			
	Salinity	pH	Ammonium (NH_4^+)	Nitrite (NO_2^-)
Recommended levels	35 ppm	8.0 to 8.5	≤ 0.05 mg/L	≤ 0.5 mg/L

The 6 experimental diets were randomly distributed among tanks, with triplicates per treatment. For 2 days, the fish were adapted to the new conditions and fed until apparent satiety 3 times per day (9:00, 13:00 and 17:00) with temporized automatic feeders. All feed events were monitored in order to assure that the quantity of feed given was eaten. Adjustments in the automatic feeders were done according to the observation of acceptance and refusal of food by the fish, being the initial amount of diet provided of 2% total body weight per day. In the case of food losses from unfed fish, the amount of feed given decreased by 5 % in the next feed event until this occurrence was not observed. Conversely, the feed amount increased by 10% when no losses were recorded for 2 days.

The growth trial had a duration of 106 days. During the trial, one tank from the NUCL treatment was lost by accidental mortality, due to a problem related to the aeration system of the tank where the animals were maintained.

2.4. Sampling Procedures

All fish were individually weighed and measured at the beginning and at the end of the experiment. Two intermediate sampling procedures were done at 39 and 75 days where each tank were bulk weighed. Feed consumption per tank was registered during all trial. Once the fish more than duplicated their initial body weight, the final sampling procedures was performed. After a 24h fasting period, 4 fish per tank were collected, except for NUCL where 6 fish per tank were sample in order to have 12 fish/treatment. Fish were anesthetized with a lethal dose 150 mgL^{-1} of MS222 prior to tissues collection. For each fish, blood was collected through the caudal vein with heparinized 2 mL syringes, placed in heparinized microcentrifuge tubes and centrifuged (10 minutes, 2 935 RPM, 4°C) in order to obtain fish plasma (supernatant). Plasma (200 µL) were placed in microcentrifuge tubes and stored at -80°C for analysis of innate immune parameters.

Liver and intestine were weighed to obtain HSI and VSI, respectively. For histomorphological evaluation, approximately 0.5 cm of the anterior (after the pyloric caeca) and posterior (before the rectum sphincter) intestine were collected (*Figure 2.1*), washed, fixed in 10% neutral-buffered formalin for 24h.

Fish muscle was collected, placed in an aluminium foil and immediately frozen in liquid nitrogen and stored at -80°C for analysis of nutritional value (DM, crude protein and crude fat).

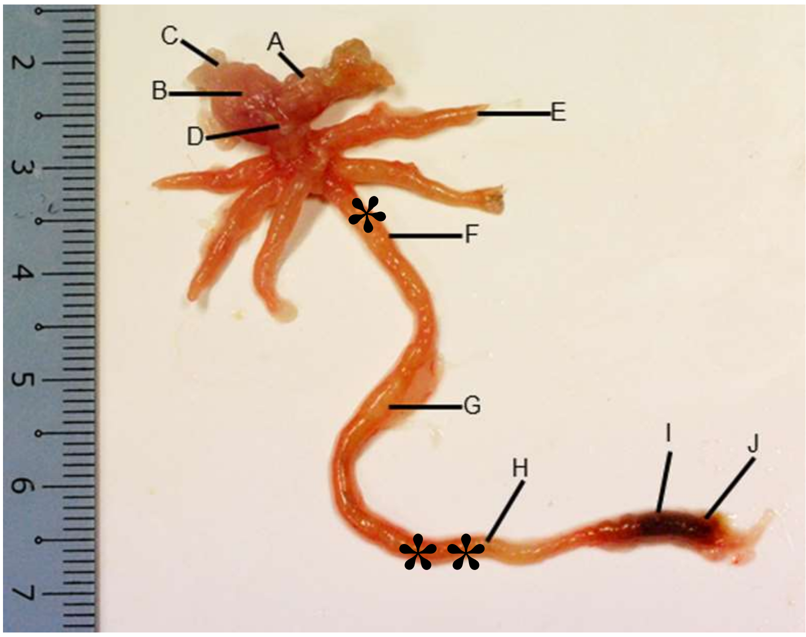

Figure 2.1 – Representation of seabass intestine sections removed in the sampling day from the anterior (*) and posterior part (**). A – Esophagus; B – Cardia stomach; C – Fundic stomach; D – Pyloric stomach; E – Pyloric caeca; F – Anterior intestine; G – Mid intestine; H – Posterior intestine; I – Anterior rectum; J – Posterior rectum. (adapted from Purushothaman et al., 2016)

For whole body composition analysis, 15 fish per treatment (10 fish in NUCL) were collected after a 24 h fasting period and sacrificed by overdose of MS222 and kept at -20°C until further analyses.

2.5. Digestibility Trial

The remaining fish from the growth trial, around 11 fish per treatment, were transferred and maintained in tanks with individual faeces sedimentation columns (Guelph system). Fish were fed twice a day (10:00 and 17:00) until apparent satiation for 10 days to adapt to the experimental diets incorporated with 1% of chromic oxide, performed by UTAD, before the collection of faeces. The experimental diets were tested in triplicates, with the exception of NUCL, tested in duplicate. During the period of collection, the fish continued to be fed ad libitum twice a day. About 30 minutes after feeding, the tanks were cleaned to assure no remain of uneaten feed in tanks and in the sedimentation column. Faeces were always collected twice a day (9:00 and 16:00) from the sedimentation column before feeding, and centrifuged (7200 rpm, 5 minutes, 4°C) to eliminate water excess before freezing at -20°C. Daily collection of the faeces was performed until reached enough amount to perform chemical analysis (1 month). At the end of the trial, the faeces were freeze-drier prior to analysis.

2.6. Evaluation of innate humoral immune parameters

Total peroxidase activity in plasma was measured in accordance to Costas et al. (2011) for *Solea senegalensis*. Sulphuric acid (H_2SO_4), 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and hydrogen peroxidase (H_2O_2) were prepared for a final volume of 100 mL each one. Plasma samples (25 µL) were diluted in 25 µL of Hanks Balanced Salt Solution (HBSS) without Ca^{+2} and Mg^{+2}. Then, 15 µL of diluted plasma was put in flat-bottomed 96-well plates in triplicates and was added 135 µL of HBSS. Three wells were used as blank with 150 µL of HBSS. To each well was added 50 µL of 2.5mM TMB and 50 µL of 5 mM H_2O_2. After 2 minutes, the reaction was stopped by adding 50 µL of 4 M H_2SO_4. The optical density was measured at 450 nm.

Lysozyme activity were performed in accordance to Parry et al. (1965) with adaptation for microplates of 96 wells of Hutchinson and Manning (1996). A solution of *Micrococcus lysodeikticus* (0.05 mg mL^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. In a flat-bottomed 96-well plate the controls: the buffer solution (265 µL/well) and the bacterial suspension (265 µL/well) were added. A pattern solution of hen egg white lysozyme (Sigma) was diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to performe the standard curve. This solution (15 µL), the plasma samples (15 µL) and the bacterial suspension (250 µL) were added to the microplate. Then, the absorbance at 450 nm was measured after 0.5 and 4.5

minutes. The formula of the standard curve was used to calculate the amount of lysozyme in the sample. All the analyses were performed in triplicates.

The ACH50 was performed as described by Sunyer and Tort (1995). It was prepared a saline solution (0.9%), a test buffer at pH 7.3 and a stop buffer at pH 7.3. For both buffers was added 0.1% of gelatine. After cooling down, was added 250 mL of distilled water, 5mM of sodium barbiturate and 0.13mM of sodium chloride. For the test buffer was added manganese (10 mM) and ethyleneglycol-*bis*-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 10 mM). For the stop buffer was added ethylenediaminetetraacetic acid (EDTA, 20 mM). Rabbit red blood cells (RaRBC; Probiológica, Belas, Portugal) were used to determine ACH50. RaRBC (10 to 15 mL) were washed 3 to 4 times by centrifugation (3500 rpm, 4°C, 5 minutes) with a dilution of 1:4 in the saline solution. In the last wash, after rejecting the supernatant, was added test buffer until reached 25 mL. Then, 10 µL of RaRBC was added to 1990 µL of the saline solution to adjust the cells concentration to 2.8×10^8 cells mL^{-1} using a Neubauer chamber. Afterwards it was prepared two dilutions: 1:16 dilution (10 µL plasma + 150 µL test buffer) and 1:24 dilution (10 µL plasma + 230 µL test buffer). In the first row of a conical bottom 96 well plates were added, in duplicate, 80 µL from 1:16 dilution of each plasma sample with a posterior dilution using the test buffer (1:32, 1:64 and 1:128). To the second row was added 80 µL from 1:24 dilution of each sample and serial dilutions (1:48, 1:96 and 1:192) were done using test buffer. To each well was added 10 µL of RaRBC. Then, in two different columns of the microplate, 10 µL of RaRBC suspension were added to 40 µL of test buffer and 40 µL distilled water, representing a spontaneous lysis and 100% lysis. The microplates were incubated at room temperature with shaking for 100 minutes. After that, stop buffer (150 µL) was added to each well to stop the reaction, and samples were then centrifuged (1000 rpm, 2.5 minutes). The supernatant (150 µL) was collected and transferred to a flat bottom microplate. The extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The concentration of plasma that gave 50% haemolysis of RaRBC defined the ACH50 units.

2.7. Intestine histomorphology evaluation

After the 24 hours fixation, histology samples were preserved in ethanol 70% until processing according to standard histological procedures. Briefly, transversal sections (3 µm) of the samples were performed in a semi-automated rotary microtome (Leica RM 2245), with posterior dewax and were stained with specific Alcian Blue/PAS staining (pH 2.5). Then, the micrographs were examined under a light microscope (Olympus BX51, cell^B software, GmbH, Hamburg, Germany) and a camera (Olympus DP50). An imaging software (Olympus cellSens Dimension Desktop) was used to measure the following parameters of the intestinal section: area (mm^2), *villus* length and width (µm). The measure of *villus* length and width was performed in the eight highest folds, with the measure of width being performed at the base of the fold and the measure of the length performed from the fold tip to the bottom, following the curves of the fold. All measurements were done as described by Batista et al., (2016).

2.8. Chemical analysis performed in ingredients, diets, carcass, faeces and muscle

Proximate composition analyses were performed according to AOAC (2006) procedures.

2.8.1. Samples preparation for chemical analysis

Algae and diets were individually minced and homogenised prior to analysis.

Frozen carcass from initial stock and from each tank of the growth trial were transversally cut without thawing and the pieces were minced and homogenised using a meat grinder. The pieces were placed into the crusher meat and the final product was recrushed three times to have a better homogenisation. Part of the minced sample was immediately used to calculate moisture content. Other part of the mixture was laid in an aluminium form, and prepared to lyophilisation. After that, samples were stored at -20°C until future analysis.

The fish muscle stored at -80°C was also lyophilised (Labconco, Freezone6) and stored at -20°C until future analysis.

Faeces from digestibility trial were collected in 50 mL falcon tubes and stored at -20°C until lyophilisation. After this, faeces samples were sifted, homogenised and stored at -20°C until future analysis.

2.8.2. Dry matter determination

In order to quantify DM, 1g of each diet, ingredient and muscle, and 0.5g of faeces were individually placed into crucibles in duplicate and kept into a stove at 105°C for 24h.

For wet carcass moisture determination, 3g of minced carcass sample were weighed in triplicate and kept into a stove at 105°C for 24h. After this period, samples were weighed, and the moisture value was obtained.

2.8.3. Ash determination

After the DM determination and using the same sample and crucibles, ash content was determined by incineration of the samples in a muffle furnace (Nabertherm Industrial Furnace Manufacturing) at 550°C for 6h and then weighed again to obtain the inorganic residue.

2.8.4. Crude Protein determination

The crude protein was evaluated in duplicates by the combustion method (AOAC Official Method 990.03) of a small amount of sample (80 mg of carcass, muscle and diets, 25 mg of faeces) in pure oxygen at high temperatures using a nitrogen microprocessor (FP-528, LECO, St. Joseph, USA) where the nitrogen is converted into equivalent protein.

2.8.5. Crude fat determination

The quantification of crude fats of diets, ingredients, carcass, faeces and muscle were analysed by the Soxtherm method (AOAC Official Method 2003.05 to Animal Feed) through lipid extraction by an organic solvent (petroleum ether), utilizing a Soxtec^{TM} 2500 model (Höganäs, Sweden; FOSS). Approximately, 1 of faeces, 1.5g of diets and ingredients, 0.5g of carcass samples were weighed in duplicate into a cellulose cartridge, covered by cotton, placed a magnet and positioned in the extraction unit. Then, 50 to 100 mL of petroleum ether were added to the extraction cups, previously rested from the oven at 150°C and weighed and placed in the extraction unit. Samples were boiled for 20 minutes in petroleum ether, rinsed for 45 minutes to the extraction of lipids and rinsed 10 minutes for evaporation of solvent for 10 minutes. After 1 minute of cooling, the cups were dried in an oven at 105°C for 1h. The difference in weight of extraction cups before and after the extraction process allowed the estimation of the lipid content.

2.8.6. Gross energy determination

An adiabatic bomb calorimeter (IKA-Werke GMBH & CO.KG, Staugen, Germany) determined the gross energy of sample by direct combustion of oxygen. The heat released by the combustion is absorbed by a calorimeter that causes the rise of water temperature, which is measured by a precision thermometer of Beckmann, before and after the end of the reaction when the temperature inside the calorimeter is constant. The variation of temperature (ΔT), registered by the calorimeter is proportional to the heat that the reaction release or absorb and depending of the calorific capacity of the calorimeter. By direct measurement of ΔT, the quantity of the gross energy in function of the sample weight was determined. Samples (500 mg) of diets, ingredients and carcass were weighed and pelletized. Approximately, 100 to 150 mg of faeces were weighed in capsules, taking into account the weight of the capsule.

2.8.7. Phosphorus determination

The first step to determine total phosphorus is the mineralization and oxidation of the humid sample, performed in a digester (Kjeldatherm Block digestion units, Gerhardt, Königswinter, Germany). The ash sample (40 mg) was weighed in duplicate into a 100 mL Kjeldahl tube and placed in the digester. It was added hydrochloric acid (HCL, 10 mL, 6M) to the tubes with sample and blanks (without sample) for 60 minutes. The digested solution was filtered into volumetric balloons of 100 mL and filled with distilled water. To determine mineral orthophosphates, such as hydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate ($H_2PO_4^-$), was added molybdenum acid (10 mL) and the previous diluted solution (100 µL) to a 15mL plastic tube, homogeneized and placed in water bath (75°C, 30 minutes). The reaction between inorganic orthophosphate and ammonium molybdate, in the presence of the ascorbic acid, create a blue coloration, whose concentration was determined by spectrophotometry at 820 nm (Shimadzu, UV – 1603), after a rest period of 30 minutes in the dark at natural temperature. A pattern solution was prepared with potassium hydrogen phosphate (KH_2PO_4, 0.5624g) dissolved in distilled water to a final volume of 100 mL. This solution was diluted into a 50 mL ballons to have a final concentration of 0, 10, 20, 40, 60, 80 and 100 mg/L of phosphorus. Then, was added H_2SO_4 to the solution. The absorbance of the pattern solutions was read in the spectrophotometer at 820 nm.

2.8.8. Chromium oxide (Cr_2O_3) determination

Approximately, 200 mg of diets and faeces and 50 mg of a pattern solution of chromium (III) oxide (Cr_2O_3) were weighed to a 100 mL Kjeldahl tube. The tubes were put in a digester (Kjeldatherm Block digestion units, Gerhardt, Konigswinter, Germany) at 230ºC, where was added 5 mL of the oxidant reagent. This reagent was prepared in a cold-water bath by dissolving sodium molybdate (10 g) in distilled water (150mL), and posterior addition of concentrated sulphuric acid (150 mL) and perchloric acid (200 mL). Upon 90 minutes of digestion, the total oxidation of Cr^{3+} into Cr^{6+} was assured by yellowish and clear appearance. Then, the tubes were kept rest for 30 min. The addition of perchloric acid (2mL) was performed for all samples and pattern solutions, and the tubes were reheated for about 15 min. Afterwards, the tubes were cooled by 30 minutes, filtered into volumetric balloons of 50 mL and 100 mL for samples and pattern solutions, respectively, and diluted with distilled water. From the pattern solution, it was performed different aliquots of 10 to 140 µg/mL with a final volume of 25 mL. The absorbance of solution samples was read in a spectrophotometer (Shimadzu, UV – 1603) at 440nm. All the analyses were performed in duplicates.

2.9. Calculations

2.9.1. Determination of growth performance and nutrient retention

The moisture content was calculated as 100 – Dry Matter average (%). FCR was calculated as dry feed intake (g) × wet weight gain $^{-1}$ (g). The daily growth index (DGI; g kg^{-1} BW day^{-1}) was calculated as DGI = $100 \times [(W_1)^{1/3} - (W_0)^{1/3}]$/trial duration in days, where W_0 and W_1 are the initial and the final fish mean weights in grams. VFI (g kg^{-1} BW day^{-1}) was calculated as VFI = (dry feed intake /ABW/trial duration), where average body weight (ABW) was calculated as $(W_1+W_0)/2$. The PER was calculated as PER = weight gain (g) /protein ingested (g). Daily nutrient intake (g kg^{-1} ABW day^{-1}) was calculated as nutrient intake/ABW/trial duration. Daily nutrient gain (g kg^{-1} ABW day^{-1}) was calculated as (final body nutrient content − initial body nutrient content)/ ABW/ trial duration. Nutrient retention (g kg^{-1} intake) was calculated as (nutrient W_1 – nutrient W_0)/ nutrient intake. The HSI and VSI indexes (g kg^{-1}, wet weight basis) was calculated as HSI = liver weight/ whole body weight, and VSI = 100 × viscera weight / whole body weight.

2.9.2. Digestibility calculations

The ADCs were calculated according to Maynard et al. (1979) as ADC (%) = 100 × (1- ((dietary Cr_2O_3 level/faeces Cr_2O_3 level)) × ((Faeces nutrient or energy level)/(dietary nutrient or level))). The ADC of DM were calculated as ADC (%) = 100 × (1-(dietary Cr_2O_3 level/faeces Cr_2O_3 level)). The ADC of nutrients and energy of the test ingredients were estimated according to NRC (2011): ADC_{ing} (%) = $ADC_{test} + ((ADC_{test}-ADC_{ref}) \times ((0.7 \times D_{ref})/(0.3 \times D_{ing})))$, where ADC_{test} = ADC (%) of the experimental diet; ADC_{ref} = ADC (%) of the reference diet, D_{ref} =

% nutrient (or kJ. g^{-1} gross energy) of the reference diet (DM basis), D_{ing} = % nutrient (or kJ. g^{-1} gross energy) of the test ingredient (DM basis).

2.10. Statistical Analysis

Statistical analyses were performed with the software SPSS (IBM SPSS STATISTICS, 25.0 package, IBM Corporation, New York, USA). Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were transformed whenever necessary. Data were analysed by a one-way ANOVA and when significant differences were obtained, Tukey's post hoc test were performed to identify significant differences between groups. When data did not meet the assumptions of ANOVA, Krustal-Wallis test was performed for each factor and the pairwise multiple comparison of mean ranks were done to identify significant differences between groups. Pearson's correlation coefficient was used between nutrients ADC and intestinal morphology of European seabass fed with all dietary treatments. The minimum level of significance of all tests was $p < 0.05$.

CHAPTER 3

RESULTS

3.1. Macroalgae characterization

The entire and convection dried macroalgae *Gracilaria* sp. used in this experiment was obtained from a commercial Portuguese company, ALGA+ located at Ílhavo, Portugal.

This alga was analysed in terms of DM (in percentage, %), ash, protein, lipids, energy, fibre and phosphorus content, according to the section of chemical analysis performed for ingredients, diets and carcasses (*Table 3.1*). The ingredient composition and chemical analysis of the experimental diets is presented in *Table 3.2* and *Table 3.3*, respectively.

Table 3.1 – Proximal composition (% DM) of the entire and processed *Gracilaria* sp. biomass (mean ± sd).

	***Gracilaria* sp.**	***Gracilaria* sp. physically processed**
Dry matter (DM, %)	91.0	94.6
Crude protein (% DM)	30.0	31.3
Crude fat (% DM)	0.9	0.5
Gross Energy (kJ g^{-1} DM)	14.5	13.4
Carbohydrates*	32.2	31.9
Neutral detergent fibre (NDF, % DM)	29.7	12.4
Ash (% DM)	28.3	31
Phosphorus (% DM)	0.5	0.5

*Calculated by estimation: 100 - (ash + crude protein + crude fat + moisture)

Table 3.2 – Formulation and proximate composition of the experimental diets.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***
Ingredients (%)						
FM 70	4.13	4.13	4.13	4.13	4.13	4.13
FM 60	16.50	13.37	13.37	13.37	13.37	13.37
Soy protein concentrate	8.25	8.25	8.25	8.25	8.25	8.25
Pea protein concentrate	1.90	1.90	1.90	1.90	1.90	1.90
Wheat gluten	12.13	12.13	12.13	12.13	12.13	12.13
Corn gluten	6.60	6.60	6.60	6.60	6.60	6.60
Soybean meal	11.14	11.14	11.14	11.14	11.14	11.14
Rapeseed meal	4.13	4.13	4.13	4.13	4.13	4.13
Wheat meal	13.86	8.37	8.37	8.36	6.31	8.31
Sardine oil	11.98	12.22	12.22	12.22	12.22	12.22
Vit & Min Premix	0.83	0.83	0.83	0.83	0.83	0.83
Binder	0.17	0.17	0.17	0.17	0.17	0.17
L-Tryptophan	0.05	0.09	0.09	0.09	0.09	0.09
DL-Methionine	0.37	0.45	0.45	0.45	0.45	0.45
Haemoblobin powder	5.00	5.00	5.00	5.00	5.00	5.00
Porcine gelatin	3.00	3.00	3.00	3.00	3.00	3.00
Gracilaria gracilis		8.25		8.25	8.25	8.25
G. gracilis physically processed			8.25			
Phytogenic feed additives				0.02		
Oligo-alginate					2.50	
Dietary nucleotides						0.08

Table 3.3 – Chemical composition of the experimental diets.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***
Chemical composition						
Dry matter (DM, %)	89.45	92.22	91.04	91.38	91.40	93.05
Crude protein (% DM)	53.68	53.26	53.69	53.26	53.04	53.44
Crude fat (% DM)	13.69	14.91	15.56	15.00	14.80	13.69
Carbohydrates (% DM) *	13.84	15.10	13.30	14.45	14.16	15.48
Neutral Detergent Fibre (%DM)	25.29	12.92	15.27	12.36	14.03	16.57
Gross Energy (kJ g^{-1} DM)	22.67	22.28	22.28	22.25	22.01	22.22
Phosphorus (% DM)	0.91	0.96	0.87	0.95	0.88	0.88
Ash (% DM)	7.29	8.93	9.11	8.84	9.95	9.01

*Calculated by estimation, 100 - (ash + crude protein + crude fat by Soxhlet method + moisture)

3.2. Growth Performance

As can be seen in *Table 3.4*, there was no significant differences among the treatments in relation to growth parameters, in particularly focusing on VFI which shows that the experimental diets were well accepted by European seabass. The final body weight varied between 77.7 (CTRL group) and 85.3 (OLIGO group). The FCR values ranged between 1.5 (PHY and PFA groups) and 1.7 (NUCL group). The NUCL group had the lowest value of PER, which varied between 1.1 and 1.2.

HSI ranged from 1.7 to 2.1, having the fish fed with GRA, PFA and OLIGO diets a significantly lower value ($p<0.05$) than the fish fed with CTRL diet. The VSI ranged from 4.5 (NUCL group) to 5.0 (CTRL group), with no significant statistical differences among treatments.

The dietary inclusion of *Gracilaria* sp., additives and physical process did not significantly affect final whole body composition (*Table 3.5*). However, there was significant differences in DM of the muscle composition, where fish fed with PFA and OLIGO diets had a significantly lower value than CTRL group ($p<0.05$).

Table 3.4 – Growth performance and somatic indexes of European seabass fed with the experimental diets for 106 days.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***	***p-value***
Growth performance							
Initial body weight (g)[1]	29.7 ± 3.6	29.7 ± 3.3	29.7 ± 3.3	29.7 ± 3.7	29.7 ± 3.6	29.7 ± 3.7	1.00
Final body weight (g)[2]	77.7 ± 13.8	80.8 ± 12.9	84.4 ± 12.2	81.5 ± 16.4	85.3 ± 14.1	78.5 ± 13.2	0.03*
DGI[3]	1.1 ± 0.04	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.01	0.13
VFI[3] (g/kg ABW/day)	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	0.28
FCR[3]	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.51
PER[3]	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.52
Somatic indexes							
Hepatosomatic Index[4]	2.1 ± 0.3^{a}	1.7 ± 0.1^{b}	1.9 ± 0.1^{ab}	1.7 ± 0.1^{b}	1.7 ± 0.1^{b}	1.8 ± 0.2^{ab}	0.02
Viscerosomatic Index[4]	5.0 ± 0.2	4.8 ± 0.3	4.9 ± 0.2	4.7 ± 0.3	4.6 ± 0.2	4.5 ± 0.1	0.32

Values are means ± standard deviation. Means in rows without a common superscript letter differ significantly ($p<0.05$). Absence of superscript indicates no significant difference between treatments. [1]n=57 except for NUCL with n=38; [2]n=50 for CTRL, n=55 for GRA, n=56 for PHY and OLIGO, n=54 for PFA, n=37 for NUCL, n=57, [3]n=3, except for NUCL with n=2; [4] n=24. * No differences were present in the post-hoc test.

Table 3.5 – Whole body and muscle compositions of European seabass fed with the experimental diets for 106 days.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***	***p-value***
Final whole body composition (% WW)[1]							
Dry matter (DM)	31.7 ± 0.7	32.0 ± 0.8	32.0 ± 0.6	30.9 ± 1.0	31.2 ± 0.6	30.8 ± 1.3	0.35
Crude protein	17.3 ± 0.3	17.7 ± 0.2	17.8 ± 0.3	17.8 ± 0.1	17.6 ± 0.3	17.5 ± 0.004	0.10
Crude fat	10.5 ± 1.0	11.0 ± 1.3	10.4 ± 1.2	8.7 ± 1.7	9.8 ± 1.0	9.4 ± 0.8	0.34
Gross energy (kJ g^{-1})	7.8 ± 0.3	7.9 ± 0.4	8.0 ± 0.2	7.5 ± 0.4	7.6 ± 0.2	7.5 ± 0.4	0.32
Ash	4.1 ± 0.4	3.9 ± 0.4	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.1	4.2 ± 0.4	0.96
Phosphorus	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0 3	0.7 ± 0.1	0.7 ± 0.04	0.7 ± 0.1	0.86
Muscle body composition (% WW)							
DM	25.4 ± 1.2^{a}	24.3 ± 0.7^{ab}	24.3 ± 0.9^{ab}	23.8 ± 0.5^{b}	23.8 ± 1.1^{b}	24.4 ± 1.1^{ab}	0.001
Crude protein	21.5 ± 0.3	20.9 ± 1.7	21.2 ± 0.3	21.3 ± 0.1	21.3 ± 0.4	21.3 ± 0.7	0.95
Crude fat	3.5 ± 0.1	2.9 ± 0.5	3.1 ± 0.6	2.3 ± 0.2	2.7 ± 0.3	2.8 ± 0.7	0.10

Values are means ± standard deviation. Means in rows without a common superscript letter differ significantly ($p<0.05$). Absence of superscript indicates no significant difference between treatments. [1]Initial body composition: dry matter, 35.6%; protein, 18.8%; lipids, 12.0%; energy, 8.2 kJ g^{-1}; ash, 4.5%, phosphorus, 0.7%.

3.3. Apparent Digestibility Coefficient and Nutrients Balance

ADC of European seabass fed with the experimental diets are presented in *Table 3.6*. Fish fed with GRA diets had a significantly (*p*<0.05) lower dry matter, protein, lipid and energy digestibility compared to CTRL and PHY groups. In relation to lipid ADC, the fish fed with PHY diet had the highest value (95%), being significantly (*p*<0.05) different from GRA group (86%). The OLIGO group had a significantly lower dry matter and energy digestibility comparing to CTRL group (*p*<0.05). Regarding phosphorus ADC, there was no statistical differences between the dietary treatments, except in NUCL group (39%) where there was a significantly decrease compared to the CTRL group (57%) (*p*<0.05).

The results of nitrogen and phosphorus balance are presented in *Table 3.7*. The fact that no differences on digestible N intake (DN) among the treatments were found, the N gain was significantly higher (*p*<0.05) in fish fed with PHY and OLIGO diet (0.25 g/kg/ABW/day) compared to CTRL group (0.22 g/kg/ABW/day). However, this was not reflected in N retention efficiency where there was no statistical differences between the dietary treatments. This resulted in a significantly higher value (*p*<0.05) of faecal N losses from fish fed with GRA (0.38 g/kg/ACW/day) and PFA (0.31 g/kg/ABW/day) diets compared to CTRL group (0.17 g/kg/ABW/day). There was no significant differences among dietary treatments in relation to non-faecal N losses, which varied between 0.6 g/kg/ABW/day in fish fed with GRA and PFA diet and 0.8 g/kg/ABW/day in CTRL group.

Regarding the phosphorus balance, there was no significant differences in relation to digestible P intake, P gain, P retention efficiency among treatments, faecal P losses and non-faecal P losses among the dietary treatments.

Regarding lipid balance (*Table 3.8*), the digestible L intake (DL) had a significant higher value (*p*<0.05) in fish fed with PHY and OLIGO diets (2.0 g/kg/ABW/day) compared to CTRL group (1.6 g/kg/ABW/day). However, there were no significant differences in L gain and L retention efficiency. There was a significant higher value (*p*<0.05) of faecal L losses in fish fed with GRA diet (0.3 g/kg/ABW/day) compared to PHY group (0.1 g/kg/ABW/day). However, no significant differences in non-faecal L losses, which ranged between 0.8 g/kg/ABW/day (CTRL group) and 1.3 g/kg/ABW/day (PFA group), were observed.

Concerning digestible energy intake (DE), fish fed with GRA (115.8 g/kg/ABW/day) and PFA

(128.9 g/kg/ABW/day) diets had a significant lower value (*p*<0.05) than CTRL (194.1 g/kg/ABW/day) and PHY (194.8 g/kg/ABW/day) groups. However, E gain and E retention efficiency displayed no significant differences among dietary treatments. There was a significant higher value (*p*<0.05) of faecal E losses in fish fed with GRA (191.5 g/kg/ABW/day), PFA (173.6 g/kg/ABW/day), OLIGO (163.4 g/kg/ABW/day) and NUCL (163.9 g/kg/ABW/day) diets compared to CTRL (109.9 g/kg/ABW/day) and PHY (113.8 g/kg/ABW/day). There were no significant differences in non-faecal E losses, which varied between 14 g/kg/ABW/day (GRA group) and 19.1 g/kg/ABW/day (CTRL group). The metabolized energy had a significant lower value (p<0.05) in fish fed with GRA (101.9 g/kg/ABW/day) and PFA (113.5 g/kg/ABW/day) diets compared to CTRL (174.6 g/kg/ABW/day) and PHY (176.1 g/kg/ABW/day). This pattern was also shown in total heat loss, where the fish fed with GRA (101.3 g/kg/ABW/day) and PFA (112.9 g/kg/ABW/day) diets had a significant higher value (p<0.05) than CTRL (174 g/kg/ABW/day) and PHY (175.4 g/kg/ABW/day) groups.

Table 3.6 – Apparent digestibility coefficient (ADC) of nutrients and energy of European seabass fed with the experimental diets.

	CTRL	**GRA**	**PHY**	**PFA**	**OLIGO**	**NUCL**	***p-value***
ADC (%)							
Dry matter (DM)	50.1 ± 5.6^{a}	16.0 ± 2.1^{b}	42.9 ± 5.3^{a}	12.4 ± 3.7^{b}	24.8 ± 8.7^{b}	24.8 ± 0.9^{b}	<0.001
Protein	85.4 ± 0.4^{a}	67.7 ± 8.1^{c}	83.7 ± 2.0^{ab}	73.0 ± 1.0^{bc}	77.2 ± 3.3^{abc}	76.4 ± 3.3^{abc}	0.002
Lipids	89.4 ± 1.0^{ab}	86.3 ± 6.7^{b}	94.6 ± 0.6^{a}	92.4 ± 0.6^{ab}	93.2 ± 0.6^{ab}	90.6 ± 2.8^{ab}	0.02
Energy	63.8 ± 2.0^{a}	37.6 ± 5.2^{b}	63.1 ± 3.6^{a}	42.5 ± 5.6^{b}	47.4 ± 8.1^{b}	49.0 ± 3.9^{ab}	<0.001
Phosphorus	57.1 ± 7.5^{a}	42.9 ± 3.4^{ab}	52.9 ± 9.4^{ab}	51.6 ± 4.6^{ab}	50.3 ± 4.7^{ab}	38.5 ± 1.9^{b}	0.046

Values are means ± standard deviation: n = 3, except for NUCL with n = 2. Means in rows without a common superscript letter (a, b, c) differ significantly ($p<0.05$), whereas absence of superscript indicates no significant difference between treatments.

Table 3.7 – Nitrogen and Phosphorus balance of European seabass fed with the experimental diets.

	CTRL	**GRA**	**PHY**	**PFA**	**OLIGO**	**NUCL**	***p*-value**
Nitrogen (N) balance (g kg ABW^{-1} day^{-1})							
Digestible N intake (DN)	1.0 ± 0.04	0.8 ± 0.1	1.0 ± 0.04	0.8 ± 0.03	0.9 ± 0.1	0.9 ± 0.1	0.04*
N gain	0.22 ± 0.003^{b}	0.24 ± 0.01^{ab}	0.25 ± 0.01^{a}	0.24 ± 0.01^{ab}	0.25 ± 0.01^{a}	0.23 ± 0.001^{ab}	0.01
N retention efficiency (% feed intake)	19.2 ± 1.0	20.3 ± 1.3	20.9 ± 1.1	21.0 ± 1.1	20.6 ± 1.2	20.6 ± 1.2	0.13
Faecal N losses	0.17 ± 0.01^{c}	0.38 ± 0.1^{a}	0.19 ± 0.02^{bc}	0.31 ± 0.01^{a}	0.27 ± 0.03^{abc}	0.29 ± 0.03^{ab}	0.001
Non-faecal N losses	0.8 ± 0.04	0.6 ± 0.1	0.7 ± 0.04	0.6 ± 0.04	0.7 ± 0.1	0.7 ± 0.1	0.04*
Phosphorus (P) balance (g kgABW^{-1} day^{-1})							
Digestible P intake (DP)	0.07 ± 0.01	0.05 ± 0.003	0.06± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.09
P gain	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.004	0.1 ± 0.01	0.1 ± 0.004	0.1 ± 0.01	0.77
P retention efficiency (% feed intake)	49.5 ± 7.2	43.8 ± 2.9	50.8 ± 4.5	52.3 ± 13.7	50.7 ± 5.4	50.1 ± 14.1	0.70
Faecal P losses	0.05 ± 0.01	0.07 ± 0.002	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.31
Non-faecal P losses	0.01 ± 0.01	0.002 ± 0.002	0.005 ± 0.004	0.01 ± 0.01	0.003 ± 0.01	0.00 ± 0.00	0.51

Values are means ± standard deviation: n = 3, except for NUCL with n = 2. Means in rows without a common superscript letter (a, b, c) differ significantly ($p<0.05$), whereas absence of superscript indicates no significant difference between treatments. *No differences were present in the post-hoc test.

Table 3.8 – Lipid and Energy balance of European seabass fed with the experimental diets.

	CTRL	**GRA**	**PHY**	**PFA**	**OLIGO**	**NUCL**	***p*-value**
Lipid (L) balance (g kg ABW $^{-1}$ ***day*** $^{-1}$***)***							
Digestible L intake (DL)	1.6 ± 0.1^{b}	1.8 ± 0.2^{ab}	2.0 ± 0.1^{a}	1.9 ± 0.1^{ab}	2.0 ± 0.1^{a}	1.8 ± 0.04^{ab}	0.01
L gain	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.43
L retention efficiency (% feed intake)	49.3 ± 11.8	51.8 ± 15.0	42.1 ± 9.5	31.7 ± 11.1	40.2 ± 6.6	37.3 ± 6.9	0.30
Faecal L losses	0.2 ± 0.02^{ab}	0.3 ± 0.1^{a}	0.1 ± 0.01^{b}	0.2 ± 0.02^{ab}	0.1 ± 0.01^{ab}	0.2 ± 0.1^{ab}	0.04
Non-faecal L losses	0.8 ± 0.2	0.9 ± 0.4	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	0.15
Energy (E) balance (kJ kg ABW $^{-1}$ ***day*** $^{-1}$***)***							
Digestible E intake (DE)	194.1 ± 10.4^{a}	115.8 ± 20.3^{b}	194.8 ± 14.2^{a}	128.9 ± 17.4^{b}	148.0 ± 28.3^{ab}	158.0 ± 20.4^{ab}	0.001
E gain	0.6 ± 0.05	0.7 ± 0.1	0.7 ± 0.05	0.6 ± 0.04	0.7 ± 0.05	0.6 ± 0.05	0.31
E retention efficiency (% feed intake)	21.0 ± 2.3	21.8 ± 3.3	22.9 ± 1.1	20.5 ± 1.2	21.3 ± 1.0	18.7 ± 2.4	0.37
Faecal E losses	109.9 ± 6.9^{b}	191.5 ± 9.8^{a}	113.8 ± 9.8^{b}	173.6 ± 17.2^{a}	163.4 ± 22.2^{a}	163.9 ± 4.1^{a}	<0.001
Non-faecal E losses	19.1 ± 1.1	14.0 ± 3.2	18.7 ± 0.9	15.1 ± 0.9	17.0 ± 1.9	18.0 ± 2.2	0.04*
Metabolizable E	174.6 ± 9.5^{a}	101.9 ± 17.2^{b}	176.1 ± 13.6^{a}	113.5 ± 16.9^{b}	131.0 ± 26.7^{ab}	140.0 ± 18.2^{ab}	0.001
Total heat loss	174.0 ± 9.5^{a}	101.3 ± 17.2^{b}	175.4 ± 13.5^{a}	112.9 ± 16.9^{b}	130.3 ± 26.7^{ab}	139.4 ± 18.3^{ab}	0.001

Values are means ± standard deviation: n = 3, except for NUCL with n = 2. Means in rows without a common superscript letter (a, b, c) differ significantly ($p<0.05$), whereas absence of superscript indicates no significant difference between treatments. *No differences were present in the post-hoc test.

3.4. Humoral Innate Immune Parameters

Humoral immune parameters evaluated (peroxidase, lysozyme and ACH50) were not significantly affected by inclusion of *G. gracilis*, additives and physical process (*Table 3.9*). The lysozyme values of fish varied between 823.7 enzyme units (EU)/mL (NUCL group) and 974.7 EU/mL (PFA group). In relation to peroxidase values, the values ranged from 324.4 EU/mL in PFA group and 182.3 EU/mL in NUCL group. The ACH50 values varied between 153.9 EU/mL (GRA group) and 238.7 EU/mL (CTRL group).

Table 3.9 – Humoral innate immune parameters of European seabass fed with the experimental diets.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***	***p-value***
Lysozyme (EU mL^{-1})	834.7 ± 283.4	903.7 ± 362.3	956.1 ± 181.2	974.7 ± 225.1	836.6 ± 347.2	823.7 ± 388.7	0.76
Peroxidase (EU mL^{-1})	301.4 ± 106.8	279.9 ± 123.3	322.1 ± 131.7	324.4 ± 161.9	283.8 ± 139.5	182.3 ± 67.2	0.10
ACH50 (Units mL^{-1})	238.7 ± 101.4	153.9 ± 61.3	197.5 ± 51.5	188.9 ± 96.2	179.0 ± 75.2	219.8 ± 92.6	0.20

Values are means ± standard deviation. Absence of superscript letters indicates no significant difference between treatments. ACH50 – Alternative complement pathway; EU - enzyme unit.

3.5. Intestine histomorphological evaluation

In spite of no significant differences among the dietary treatments in relation to anterior intestine section area (*Table 3.10*), this parameters varied between 4.6 (OLIGO group) and 7.1 (PHY group) mm^2. The *villus* length had also no significant differences between treatments, ranging 1.1 mm for all treatments, except in PFA group (1.0 mm). There were only significant differences in *villus* width of anterior intestine, where fish fed with PHY (150.6 µm) had a significant higher value ($p<0.05$) compared to GRA (113.2 µm) and PFA (117.6 µm) groups.

The posterior intestine had no significant differences among dietary treatments in all parameters evaluated. The posterior intestine section area varied between 3.1 (CTRL group) and 4.9 (OLIGO group) mm^2. The *villus* length ranged from 0.5 mm in fish fed with OLIGO and CTRL diets and 0.6 mm in GRA, PHY, PFA and NUCL groups. The *villus* width varied between 97.2 (GRA group) and 112.6 (NUCL group) µm.

There was only a positive correlation ($p<0.05$) between the intestine *villus* width and ADCs of protein, energy and phosphorus (*Table 3.11*), despite no correlations were between the other parameters evaluated.

Table 3.10 – Effects of the experimental diets on intestinal morphology of European seabass after 106 days of feeding.

	CTRL	***GRA***	***PHY***	***PFA***	***OLIGO***	***NUCL***	***p-value***
Anterior intestine							
Intestine section area (mm²)	5.7 ± 1.0	5.6 ± 1.4	7.1 ± 1.3	6.4 ± 1.4	4.6 ± 1.1	5.9 ± 1.8	0.08
Villus length (mm)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	0.91
Villus width (µm)	131.6 ± 16.5^{ab}	113.2 ± 9.4^{b}	150.6 ± 6.2^{a}	117.6 ± 20.4^{b}	131.9 ± 18.5^{ab}	127.8 ± 20.2^{ab}	0.01
Posterior intestine							
Intestine section area (mm²)	3.1 ± 0.7	4.2 ± 0.6	4.5 ± 1.5	4.2 ± 1.1	4.9 ± 2.2	4.8 ± 1.4	0.32
Villus length (mm)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.27
Villus Width (µm)	107.1 ± 22.0	97.2 ± 9.4	107.9 ± 10.7	109.8 ± 3.0	104.8 ± 15.0	112.6 ± 18.3	0.58

Values are means ± standard deviation: n = 6; Means in rows without a common superscript letter differ significantly ($p<0.05$). Absence of superscript indicates no significant difference between treatments. *No differences were present in the post-hoc test.

Table 3.11 – Correlation between nutrients ADC and intestinal morphology of European seabass fed with all dietary treatments.

	ADC									
	DM		**Protein**		**Lipids**		**Energy**		**Phosphorus**	
	r-value	*p*-value	*r*-value	*p*-value	*r*-value	*p*-value	*r*-value	*p*-value	*r*-value	*p*-value
Anterior intestine										
Intestine section area (mm²)	0,080	0,759	0,276	0,284	0,323	0,206	0,224	0,387	-0,302	0,238
Villus length (µm)	0,065	0,804	0,161	0,536	0,125	0,633	0,049	0,851	-0,252	0,329
Villus width (µm)	0.448	0.071	0.587*	0.013	0.374	0.139	0.528*	0.030	-0.568*	0.017
Posterior intestine										
Intestine section area (mm²)	-0,182	0,485	-0,043	0,868	0,301	0,241	-0,054	0,836	0,133	0,610
Villus length (µm)	-0,234	0,366	-0,224	0,387	-0,032	0,904	-0,197	0,447	0,027	0,917
Villus width (µm)	0,204	0,432	0,195	0,453	0,192	0,461	0,251	0,331	-0,239	0,356

A significate correlation is represented by "*" with $p<0.05$.

CHAPTER 4

DISCUSSION

Seaweeds are being used in aquafeeds as a more sustainable alternative to common protein sources as fish and vegetable meals. One of the advantages of seaweed use is the high production yield per unit area, no requirement to freshwater or arable land (Van Krimpen et al., 2013), high protein content and a balanced amino acid profile (Wong and Cheung 2000).

In particular, *Gracilaria* sp. is receiving more attention due to their high protein digestibility (88%) (Pereira et al., 2012), high diversity of bioactive compounds (Wells et al., 2017), anti-viral, antioxidant, antibacterial, anti-inflammatory compounds, and the presence of carotenoid that can modulate humoral immune response (Araújo et al., 2016).

However, their low digestible energy value (Davies et al., 1997), anti-nutritional factors (Francis et al., 2001), high fibre content (Azaza et al., 2008) and undigestable non-starch polysaccharide (Zhu et al., 2016) can have a negative effect on bioavailability of nutrients (Francis et al., 2001), palatability (Lobo 2018), gastrointestinal tract and growth performance (Thiessen et al., 2003). Furthermore, carnivorous fish, such as European seabass, are not able to digest well algae, due to their low presence of amylase activity (Hidalgo et al., 1999) and limited capacity to hydrolyse complex polysaccharides present (Montgomery and Gerking, 1980).

These disadvantages can be improved by supplementation of the diets with algae with additives and using physical processes on the algae, such as grinding. Physical processes can improve the bioavailabilty of nutrients through the removal of non-protein components present in seaweeds, such as polysaccharides and fibre that reduce protein digestibility (Angell et al., 2016). On the other hand, additives such as phytogenic compounds containing essential oils are able to enhance digestibility and absorption of nutrients (Michiels et al., 2010), improve palatability (Lee and Gao 2012), muscle protein content, and growth performance of channel catfish (Zheng et al., 2009), shrimp (Cheng 2008) and Nile tilapia (Seden et al., 2009). Other additives as oligo-alginates with agglutination capacity can improve feed texture, digestibility and growth rate of orange-spotted grouper (Yeh et al., 2008) and Nile tilapia (Igbinosum and Roberts 1988). Moreover, an additional source of exogenous nucleotides in diet could optimize and accelerate functions such as dividing tissues, being important at rapid growth rates, during reproduction, stress conditions, recovery from injury or health challenges (Burrells et al., 2001a).

In the present study, the inclusion of *Gracilaria gracilis*, additives and physical process showed similar growth performance, palatability and feed efficiency in European seabass when compared to the CTRL group. This effect was also observed in African catfish (Al-Asgah et al., 2016), Nile tilapia (Kumala et al., 2018), Atlantic salmon (Lozano et al., 2016), catfish (Al-Asgah et al., 2016), snakehead (Hashim and Saat, 1992) and black seabream (Xuan et al., 2013) fed with 10% *Gracilaria* sp. Another experiment with a seaweed (*Ulva rigida* and *Cystoseira barbata*) inclusion of 10% displayed the same pattern in Nile tilapia (Güroy et al., 2007), in European seabass with *U. rigida* (Valente et al., 2006), and in rainbow trout with *Porphyra dioica* (Soler-Vila et al., 2009). However, lower inclusions (5%) of *Pterocladia capillacea* in European seabass (Wassef et al., 2013) and *Porphyra spheroplasts* in red seabream (Kalla et al., 2008), leaded to an improvement of growth performance. In relation to phytogenic compounds, it was observed the same pattern on growth performance and feed intake of European seabass (Volpatti et al., 2013) and channel catfish (Peterson et al., 2014) with inclusions up to 0.05%. Inclusion levels between 0.1 and 0.5% can led to an improvement of growth performance, PER and protein retention digestibility of Nile Tilapia (Mohamed and Megahad, 2014). However, higher inclusions might have no effect on growth performance and feed utilization in European seabass (Yilmaz et al., 2015), rainbow trout (Giannenas et al., 2012), and Nile Tilapia (Petkam et al., 2008). The absence of effect on growth performance with oligo-alginate supplementation up to 10% was also reported in rainbow trout (Storebakken, 1985) and orange spotted grouper (Yeh et al., 2008). Regarding the dietary nucleotides, lower inclusion than our study, up to 2%, improved the growth performance of largemouth bass (Kubitza et al., 1997), rainbow trout (Burrells et al., 2001b), Atlantic salmon (Burrells et al., 2001a) and white shrimp (Andrino et al., 2012). As happen in the present thesis (*Table 3.4*), Agboola et al. (2018) also observed similar growth performance in African catfish fed with physical process applied to microalgae diets.

The absence of influence of dietary treatments on whole body composition was also reported in Nile tilapia when fed diets with *Ulva* sp., *Gracilaria* sp. and *Porphyra* sp. at 10% inclusion level (Silva et al. 2015), in red seabream fed with *P. dioica* diets at 5, 10 and 15% inclusion level (Soler-Vila et al., 2009), in African catfish fed with *U. lactuca* diets at 10, 20 and 30% inclusion level (Abdel-Warith et al. 2016), and in European seabass when fed with *Gracilaria bursa-pastoris*, *U. rigida* and *G. cornea* at 5 and 10% inclusion level (Valente et al., 2006). However, an increase in protein content on body composition was reported in *Labeo rohita* with inclusion of G*racilaria* sp.at 10, 20 and 30% inclusion level (Vadher et al., 2016), and in rainbow trout fed with *P. dioica* included at 5, 10 and 15% inclusion level (Soler-Vila et al. 2009). Furthermore, this pattern was reported in channel catfish for phytogenic compounds included in the diet at 0.2% (Peterson et al., 2014), and in rainbow trout with alginates included at 10% into the diet (Heidarieh et al., 2012).

Regarding the muscle body composition, there was only a decrease in DM with the addition of phytogenic compounds and oligo-alginates. The supplementation with phytogenic compounds had increased the DM of broiler chickens (Marcincak et al., 2011), increased the protein and decrease the lipid content of channel catfish (Peterson et al., 2014). Although no differences on GRA group in relation to muscle crude protein and lipid, in another studies it was reported an increase in protein content in red seabream fed with *Ascophyllum nodosum*, *P. yezoensis* and *U. pertusa* at 5% inclusion (Mustafa et al. 1995). In addition, a reduction of muscle and carcass lipid was reported in African catfish fed with *Gracilaria* sp. (Al-Asgah et al., 2016), and in European seabass fed with 10% inclusion of *G. cornea* (Valente et al., 2006).

Another parameter used to evaluate the metabolic capacity and health condition of fish was the HSI. The present study reported a significantly decrease of HSI in fish fed with GRA, PFA and OLIGO diets comparing to CTRL fish groups. This suggests that the energy located in organ or tissue to growth change direction to combat stress factors (Al-Asgah et al., 2016), being this relevant, since indications of stress may have long term negative consequences. This could be due to the efficient lipid metabolism, accumulation and mobilization supported by algae and additives (Al-Asgah et al., 2016). Azaza et al. (2008) suggested that this decrease could be due to the reduction of fat deposition and consequent reduction of liver size. The decrease in HSI levels was also reported in African catfish fed diets with inclusion of 10% *Gracilaria sp.* (Al-Asgah et al., 2016), in black seabream fed diets with 20% *G. lemaneiformis* inclusion (Xuan et al., 2013), and in rainbow trout fed diets with 15% and 10% inclusion of *P. dioica* (Soler-Vila et al., 2009). With phytogenic additives supplementation, it was reported a decrease in HSI in channel catfish with 5% inclusion of essential oil (Zheng et al., 2009), in European seabass with 1% inclusion of thyme (Yilmaz et al., 2013) and in olive flounder with 1% inclusion of green tea (Cho et al., 2007).

The improvement of growth performance depends also on the digestion of feed ingredients and nutrient composition available to fish. These parameters can be defined by the nutrients ADC

(Eusebio et al. 2004). The ADC DM represents the digestibility of all nutrients digested by the fish, in which a high digestibility means a high quality of feed ingredient (Suryaningrum et al., 2017). In the present study, the DM ADC was affected negatively by the GRA, PFA, OLIGO and NUCL diets. However, the physical process had similar results to the reference diet, improving the utilization of seaweed.

The present study reported a significant decrease of protein digestibility in fish fed with GRA and PFA diet comparing to the CTRL group, which led to a significantly higher nitrogen faecal loss in fish fed with these diets. Another study focused in European seabass reported no differences in protein ADC in the fish fed with 5 and 10% *G. bursa-pastoris*, *U. rigida* and *G. cornea* (Valente et al., 2006). However, the low protein digestibility was reported for other seaweed species such as *Ulva* sp. included at 10 and 20% in Nile tilapia diets (Azaza et al., 2008), and *Laminaria japonica* included in rats diets (Suzuki et al., 1993). These negative effects of seaweeds on protein digestibility could be due to the presence of anti-nutritional factors that can suppress digestive enzyme activities (Araújo et al., 2015), or the presence of polysaccharides that act as a barrier (Marrion et al., 2003) affecting the efficiency of nutrient assimilation in the fish gut (Sinha et al., 2011). At higher inclusion levels, the supplementation of diets with phytogenic compounds improved the nutrient digestibility of apparent protein content in Nile tilapia up to 4% inclusion levels (Mohamed and Megahad 2014). Regarding other diets tested, the fish fed with PHY, OLIGO and NUCL diets had similar protein digestibility to the CTRL group demonstrating a possible beneficial effect of these dietary supplements and physical processes applied to GRA diets. In another study, the inclusion of autoclaved *Ulva* sp. at 5 and 10% improved the protein ADC of rainbow trout (Güroy et al., 2013).

In relation to phosphorus ADC, there was only a significantly decrease in NUCL group comparing to the other diets. However, this negative influence was not reflected in a higher release of P into the environment, resulting in a similar pattern to the CTRL diet. Otherwise, the inclusion of GRA and PFA resulted in higher N discharge into water, which can result in pollution of aquaculture effluents if no treatment is applied (Campos et al., 2017).

The energy digestibility decreased with GRA diets. The low energy digestibility of seaweed is caused by the presence of low carbohydrate digestibility, high ash content (Suryaningrum et al., 2017) and high crude fibre (Jung and Allen 1995). One way to decrease the impact of low energy digestibility of seaweeds is their inclusion at low levels (Hintz and Heitman 1967). Studies with the inclusion of microalgae (*Nannochloropsis* sp.) reported the reduction of energy and lipid digestibility in Nile tilapia (Sarker et al., 2018). Also in Nile tilapia, the inclusion of *U. lactuca* resulted in lower energy digestibility as compared to plant feedstocks such as wheat bran and corn (Suryaningrum et al., 2017). As observed in this study, another possible solution to improve the energy digestibility of *Gracilaria* sp. could be the application of a physical processing step and the addition of nucleotides.

Physical processing applied to *Gracilaria* sp. had similar results of nutrient digestibility compared to those of the CTRL group. Processing treatment can improve the nutrient digestibility and accessibility, through the disruption of cell walls, which enhance growth performance (Agboola et al., 2019). The application of these processes in microalgae improved nutrient digestibility up to 84%, in particularly protein and lipid, enhancing the growth performance in African catfish (Agboola et al., 2019) and Nile tilapia (Teuling et al., 2019).

The inclusion of nucleotides in GRA diet had also similar digestibility results with CTRL. Furthermore, in another studies, the supplementation with nucleotides increased lipid and energy ADC of crayfish (Safari et al., 2015), Asian tiger shrimp (Hertrampf and Mishra 2006) and white shrimp (Oujifard et al., 2008).

Regarding the innate immune system, there were no differences with the inclusion of *Gracilaria* sp., additives and physical processing. The supplementation with *G. vermiculophylla* had also no effect on the innate immune system of European seabass (Lobo 2018) and with *Ulva* sp.in Nile Tilapia diets (Valente et al., 2006). However, the presence of polysaccharides, complex carbohydrates and bioactive compounds present in seaweeds (Vadher et al., 2016) can enhance the immunological response, such as lysozyme activity and ACH50, in olive flounder (Choi et al. 2015), red seabream (Satoh et al., 1987), Nile tilapia (Kazemi et al., 2018), white-spotted spinefoot (Xu et al., 2011), rainbow trout (Araújo et al. 2016), and European seabass (Peixoto et al., 2017). Physical processes can cleave the polysaccharides present in seaweeds that stimulate the immune response (Lee and Gao 2012).

In relation to phytogenic feed additives supplementation, absence of a significant effect in innate immune response of European seabass (Volpatti et al., 2013) and catfish (Zheng et al., 2009) has been reported. However, other studies described an enhancement of immune competence (Yang et al., 2015) through their ability of activating the complement system (LaFrentz et al., 2012) and increasing serum lysozyme levels (Abo-State et al., 2017) in rainbow trout (Diler et al., 2017), European seabass (Volpatti et al., 2013) and catfish (Peterson et al., 2014).

Despite the results of oligo-alginate supplementation being similar to those of the CTRL diet, other studies reported an increase in innate immune parameters, such as the alternative complement and lysozyme activity of European seabass (Bagni et al., 2005), and orange-spotted grouper (Cheng et al., 2008). In relation with nucleotide supplementation, it can enhance the immune response and resistance of carp (Sakai et al., 2001), Atlantic salmon, coho salmon (Burrells et al., 2001a), rainbow trout (Leonardi et al., 2003), pacific white shrimp (Safari et al., 2015), and whiteleg shrimp (Cheng et al., 2004).

Antinutritional compounds present in seaweed can cause histological changes in intestine, such as shorter villi and deeper crypts, reduce intestinal absorption and immune response in salmonids (Krogdahl et al., 2000) and sea bream (Sitjà-Bobadilla et al., 2005). In particular, the inclusion of *Gracilaria* sp. can cause a decrease of *villus* height and width, which is associated with a reduction of absorption surface in intestinal cells, lowering growth and nutrient retention (Moldal et al., 2014) in rainbow trout (Araújo et al. 2015) and Nile tilapia (Silva et al., 2015). In our study, the inclusion of *G. gracilis* did not altered the *villus* height and width, but when applied the physical process to the seaweed, an increase on *villus* width was observed, being significantly different from GRA diet. Furthermore, there was a positive correlation between the *villus* width of anterior intestine and the protein and energy digestibility, which can indicate that the inclusion of these alga can have an influence in *villus* width, which might be responsible by the reduction in nutrient accessibility, decreasing ADC of protein and energy. This could be related with the presence of antinutritional compounds that increase the permeability of the intestinal mucosa cells and damage pyloric caecum with a consequent reduction of mineral bioavailability and protein digestibility (Francis et al., 2001). Furthermore, the fibre structures present in seaweed could act as a physical barrier between nutrients and digestive enzymes, reducing the accessibility and availability of intestinal enzymes to nutrients (Potty, 1996). However, the physical process applied to *G. gracilis* increased the *villus* width in anterior intestine of European seabass, which can be associated with an increase in the absorption surface in intestinal cells, with a consequent improvement of growth and nutrient retention (Moldal et al., 2014).

The dietary treatment of PFA also decreased the *villus* width in anterior intestine of European seabass compared to the CTRL group. The acidifiers present in phytogenic compounds reduce the pH of stomach and upper gut, stimulating pepsin activity, which enhance nutrient protein digestibility, nitrogen retention and mineral absorption (Muhl and Liebert, 2007). Furthermore, Murugesan et al. (2015) reported a positive correlation between growth and the apparent digestibility of nutrients through the increase of *villus* height of broilers chicken.

The other additives had similar *villus* width compared to reference diet, which can reveal a potential improvement of oligo-alginates and nucleotides in *G. gracilis* diets. The supplementation with oligo-alginate can have a positive effect on gastrointestinal morphology increasing *villus* length in European seabass diets (Heidarieh et al., 2012) and Nile tilapia (Merrifield et al., 2011). On the order hand, dietary nucleotides can increase the *villus* height of rainbow trout (Burrells et al., 2001b) and in weanling rats (Uauy et al., 1990), promoting a rapid intestinal repair and elongation of intestinal tract, reducing stress and absorbing chemicals (Li and Gatlin III, 2006).

CHAPTER 5

CONCLUSION

The results obtained in this study showed than an inclusion of *Gracilaria gracilis* at 8% did not compromise feed intake neither growth performance of European seabass. However, it was detected the poor digestibility of *Gracilaria* sp., which might be improved by the application of the physical processing of the algal biomass. This improvement linked with the increase of *villus* width could promote an increase in nutrient absorption, accessibility and digestibility. The supplementation with oligo-alginates and dietary nucleotides further improved the protein digestibility of *G. gracilis*, an effect that may be due to the increase in the *villus* width of the anterior intestine.

The inclusion of *G. gracilis* and phytogenic compounds should be taken into account, since it can increase the nitrogen discharge into the environment, becoming a potential source of pollution requiring the removal of excess N from aquaculture effluents.

Further studies are needed to determinate the potential use of algae biomass, as *Gracilaria* sp., in European sea bass diets as well to better understand the effect of some additives in their growth and health performance. The physical processes and additives could be applied in other genus of seaweed or could be incorporated in diets for other fish species such as omnivorous or other carnivorous fish to evaluate their physiological parameters. Despite of the high cost of *Gracilaria* sp., their inclusion in fishmeal diets could be analysed due to the presence of antioxidant compounds that can improve the immune resistance and growth performance. Furthermore, it should also be evaluated the alteration of fillet colour due to the pigments present in red seaweeds.

CHAPTER 6

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