Improvement of macroalgae utilization as a valuable ingredient for European seabass aquafeeds

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Abstract

The world's human population is increasing to numbers that have never been recorded before. As such, food demand is increasing, pressuring food industries to keep up with the pace. Regarding the aquatic organism food industry, wild fisheries production has stagnated in recent decades, surpassed by seafood production from aquaculture. Thus, aquaculture presents itself as a solution to the human population food demand issue, being a fast-growing food sector. Although aquaculture is a complex and profitable industry, aquatic organisms production, especially fish, requires tonnes of aquafeeds to optimize their growth performance and profitability. Hence, the two predominant ingredients in this industry are fish meal and fish oil, particularly carnivorous fish diets. Despite being excellent protein and lipidic sources, respectively, these two ingredients' production often leads to the overexploitation of wild fish stocks, which is not ecologically sustainable. Moreover, the inclusion of other ingredients such as plant feedstuffs is also increasing, to replace fish meal. However, these vegetal ingredients lead to greater competition with the human food market, leading to higher prices and demand. Thus, this work seeks to use low inclusions levels of fish meal and replace vegetal feedstuffs inclusions in aquafeeds, looking for more eco-friendly and cheap alternatives such as macroalgae and their by-products, and understanding how their inclusion in fish diets can impact fish growth, well-being, and digestibility. Therefore, the present work aims to use undervalued feedstuffs or industrial residues as valuable ingredients for aquafeeds, reuse its nutrients, and respect the idea of a circular economy, seeking the most sustainable path possible, from a biological, environmental, and economic point of view. Two different trials were performed, one growth trial and the other, a digestibility trial of diets with these alternative ingredients. The first one tested the effect of different inclusion levels (0%, 5%, 10%, and 15%) of *Gelidium* by-products in diets for European seabass juveniles on fish growth performance, feed utilization, and oxidative status. The second trial assessed the digestibility of diets for the same species with different *Palmaria* and *Codium* inclusions (0%, 7.5%, and 15%). Both trials had diets with

the tested ingredient treated with alkaline plus thermal treatment (NaOH + autoclave), to determine if those pre-treatments were effective in the decrease of non-digestible fiber content, thus allowing better fish growth performance and diet digestibility coefficients.

Regarding the growth trial, three hundred European seabass juveniles (initial body weight =38g) accepted the experimental diets, with no significative differences in growth performance and feed utilization,feed intake (of fish fedG15 + treatment diet was higher compared to fish fed G5 and G15 untreated diets), but a negative trend was found with increasing Gelidium by-products dietary inclusion levels. Whole-body composition of fish was similar irrespectively between diets. Moreover, Glutathione peroxidase and Glutathione reductase activity increased in treated 15% Gelidium by-products diet in comparision with 0, 5%, and 10% diets.

The digestibility trial showed no significant differences between all experimental diets. Therefore, inclusions of *P. palmata* e *C. tomentosum* had no negative effect on ADC values and digestibility performance. However, alkaline and thermal treatments performed did not improve nutrient digestibility. Thus, treatments were not effective in decreasing non-digestible fiber content. In conclusion, *Gelidium* by-product can be included up to 15% without affecting fish growth and oxidative stress. Also, *Codium tomentosum* and *Palmaria palmata* inclusions levels up to 15% can be added in fish aquafeeds without influencing negatively digestibility performance.

Keywords: Aquafeeds; Alternative ingredients; Macroalgae; *Gelidium* by-product; *Codium Tomentosum, Palmaria Palmata, Dicentrarchus labrax*, European seabass.

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Abbreviation list

ADC - Apparent digestion coefficient

ADP - Adenosine diphosphate

ANOVA - Analysis of variance

ARA - Arachidonic Acid

ATP - Adenosine triphosphate

BHT - Butylated hydroxytoluene

CAT - Catalase

CL - Crude Lipid

CP - Crude protein

CT - *Codium tomentosum*

DGI - Daily growth index

DHA - Docosahexaenoic acid

DW – Dry Weight

EFA - Essential fatty acids

EPA - Eicosapentaenoic acid

FAO - Food and agriculture organization

FBW - Final body weight

FCR - Feed conversion ratio

FE - Feed efficiency

FI - Feed intake

FM - Fish meal

FO - Fish oil

GPx - Glutathione peroxidase

GR - Glutathione reductase

GSH - Reduced glutathione

GSH / **GSSG** - Reduced glutathione to oxidized glutathione ratio.

GST - Glutathione s-transferase

IBW - Initial body weight

IMTA - Integrated Multi-trophic aquaculture

LC-PUFAs - Long chain-polyunsaturated fatty acids

LPO - Lipid peroxidation

MDA - Malondialdehyde

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

PER - Protein efficiency ratio

PF - Plant feedstuffs

PP- *Palmaria palmata*

RAS - Recirculation aquaculture system

ROS - Reactive oxygen species

SOD - Superoxide dismutase

TBARS - Thiobarbituric acid reactive substances

US$ - United States Dollar

WG - Weight gain

WM - Wheat meal

1.Introduction

1.1. State of art

1.1.1. Aquaculture

Aquaculture is the farming of aquatic organisms with human intervention during the process to enhance production (Lucas et al., 2019). Aquaculture dates back thousands of years, whose origin is believed to have begun in China, with carp production (Tidwell, 2012). Not surprisingly, these rearing traditions that started in China and progressed to other Asian countries still dominate today's market. Therefore, according to FAO (2022), the Asian continent's production corresponds to about 91.61% of total aquaculture production worldwide, of which China represents 62.77%. The

implementation and consolidation of aquaculture as a large and complex industry was accomplished only at the end of the second half of the 20th century, encompassing, at that time, more than 200 different species of aquatic organisms (Lucas et al., 2019). Today, the sector produces more than 650 species (FAO, 2022). This delayed development was due to several reasons, for instance, the fact that humans are terrestrial inhabitants made the production conditions of terrestrial animals more intuitive compared to aquatic organisms. The biological and environmental requirements of aquatic organisms are naturally different, making aquaculture a more complex practice. Therefore, it is mandatory to have greater control of the aquatic environmental physicochemical conditions. Aquatic organisms are quite sensitive to these environmental variables, such as i) oxygen, which is less abundant in water compared to air; ii) CO_2, which is very soluble in water; iii) water pollution; iv) the presence of heavy metals and other toxic substances to aquatic organisms, such as ammonia; v) the pH and salinity of the water and vi) the presence of organisms that affect their development or can lead to their death, such as parasites, bacteria, and others (Lucas et al., 2019). Thus, due to all these variables, aquaculture is the food production industry that, despite currently expanding the most, has the programs and projects that take the longest to develop and establish (Lucas et al., 2019). Although its industrialization only began in the mid-twentieth century, it was only in the 90s that equipment and cultivation methods became more sophisticated, accompanied by more significant interest in investment and economic expansion. Being a natural competitor of fishing, it was only from this time onwards that its growth began to be noticed, although still small. Nonetheless, this sector was acknowledged as a viable and consistent alternative to fisheries in the following decades.

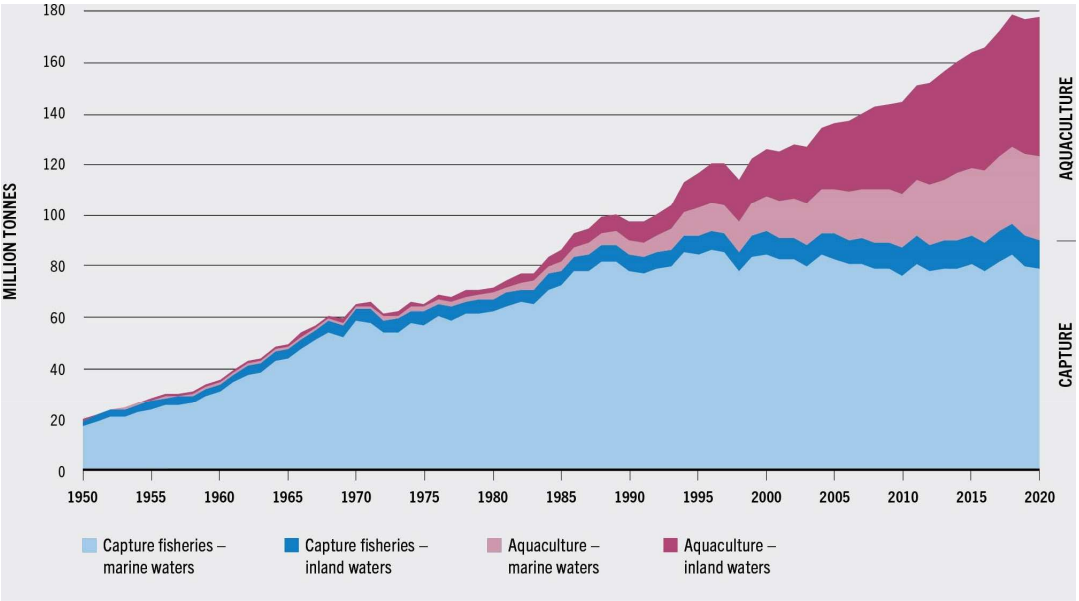


Figure 1 - Comparative graph between aquaculture production and capture from 1950 to 2020 (FAO, 2022).

Presently, it is clear to notice the gradual growth of aquaculture production, in contrast with the stagnation of fisheries (Figure 1). With the ever-increasing human population growth and fish consumption, the demand for higher fish production through wild fisheries will not be enough. Moreover, there will be an increase in overfishing and exploitation of aquatic organisms stocks, making this activity suffer massive external pressures and be confronted with the sustainability issue. In this scenario, aquaculture became a more sustainable option, originating controlled production of aquatic organisms and reducing the pressure on these organisms' wild stocks. Indeed, high quality and sustainable aquaculture production, with low carbon print, is the key to continuously producing the tonnes of fish needed to feed a growing human population. In addition, in aquaculture, the organisms are not dependent and are limited by the natural productivity of aquatic ecosystems (Lucas et al., 2019). Moreover, since 2013, aquaculture production surpassed fishing, reaching, in 2020, 57% of the total production of aquatic organisms worldwide (FAO, 2022). In 2020, aquaculture production exceeded 120 million tonnes, of which 57 million tonnes corresponded to finfish production, being the most produced organism in this industry, mainly in inland aquaculture (Figure 2).

	Africa	Americas	Asia	Europe	Oceania	World	Share in world total (%)
			(tonnes, live weight)				
1. Finfish	1 857 209	1 179 727	45 526 599	551 802	5 124	49 120 461	90.2
2. Crustaceans	2	72 541	4 401 336	3 145	177	4 477 201	8.2
3. Molluscs	…	…	192 671	…	…	192 671	0.4
4. Other aquatic animals	…	370	593 161	176	…	593 707	1.1
(Aquatic animals subtotal)	*(1 857 211)*	*(1 252 638)*	*(50 713 767)*	*(555 123)*	*(5 301)*	*(54 384 040)*	*(99.9)*
5. Algae	150	1 321	62 670	349	…	64 490	0.1
Inland aquaculture	**1 857 361**	**1 253 959**	**50 776 437**	**555 472**	**5 301**	**54 448 530**	**100**
1. Finfish	379 322	1 240 969	4 502 888	2 121 867	95 587	8 340 633	12.2
2. Crustaceans	7 617	1 193 549	5 549 811	418	8 420	6 759 815	9.9
3. Molluscs	5 994	688 077	16 158 709	578 712	116 363	17 547 855	25.8
4. Other aquatic animals	60	…	459 185	6 495	2 844	468 584	0.7
(Aquatic animals subtotal)	*(392 993)*	*(3 122 595)*	*(26 670 593)*	*(2 707 492)*	*(223 214)*	*(33 116 887)*	*(48.6)*
5. Algae	103 941	23 994	34 853 646	21 443	10 065	35 013 089	51.4
Marine and coastal aquaculture	**496 934**	**3 146 589**	**61 524 239**	**2 728 935**	**233 279**	**68 129 976**	**100**
1. Finfish	2 236 531	2 420 696	50 029 487	2 673 669	100 711	57 461 094	46.9
2. Crustaceans	7 619	1 266 090	9 951 147	3 563	8 597	11 237 016	9.2
3. Molluscs	5 994	688 077	16 351 380	578 712	116 363	17 740 526	14.5
4. Other aquatic animals	60	370	1 052 346	6 671	2 844	1 062 291	0.9
(Aquatic animals subtotal)	*(2 250 204)*	*(4 375 233)*	*(77 384 360)*	*(3 262 615)*	*(228 515)*	*(87 500 927)*	*(71.5)*
5. Algae	104 091	25 315	34 916 316	21 792	10 065	35 077 579	28.6
Total aquaculture	**2 354 295**	**4 400 548**	**112 300 676**	**3 284 407**	**238 580**	**122 578 506**	**100**

Figure 2 - Total aquaculture production per continent and worldwide (FAO, 2022).

More than an alternative to wild fisheries, aquaculture proves to be one of the most efficient industries for producing protein for human consumption. For instance, aquatic organisms, using fish as an example, more specifically carp, have, in comparison with different types of livestock, better feed conversion ratios (FCR) indices (Lucas et al., 2019). Therefore, they gain more weight than land animals per kg of ingested feed, have higher protein content per unit of edible weight, have higher protein conversion efficiency, and have higher energy and protein retention (Figure 3) (Lucas et al., 2019). Also, fish have a lower carbon footprint than livestock animals (Lucas et al., 2019).

	Carp	Chicken	Pig	Beef
FCR (kg feed/kg live wt)	1.5	2.3	5.9	12.7
Feed conversion (kg feed/kg of edible wt)	2.3	4.2	10.7	31.7
Protein content (% of edible wt)	18	20	14	15
% protein conversion efficiency	30	26	13	5

Figure 3 - Different ratios comparison between carp and cattle (Lucas et al., 2019)

For the success of intensive aquaculture, it is of utmost importance to feed the proper animals using optimum diets to ensure maximum fish performance and welfare. Thus, formulating optimum aquafeeds for optimizing fish's growth and health and feed utilization based on the correct selection of the ingredients and additives is essential. Aquafeeds are still dependent on two major ingredients: fish oil (FO) and fishmeal (FM).

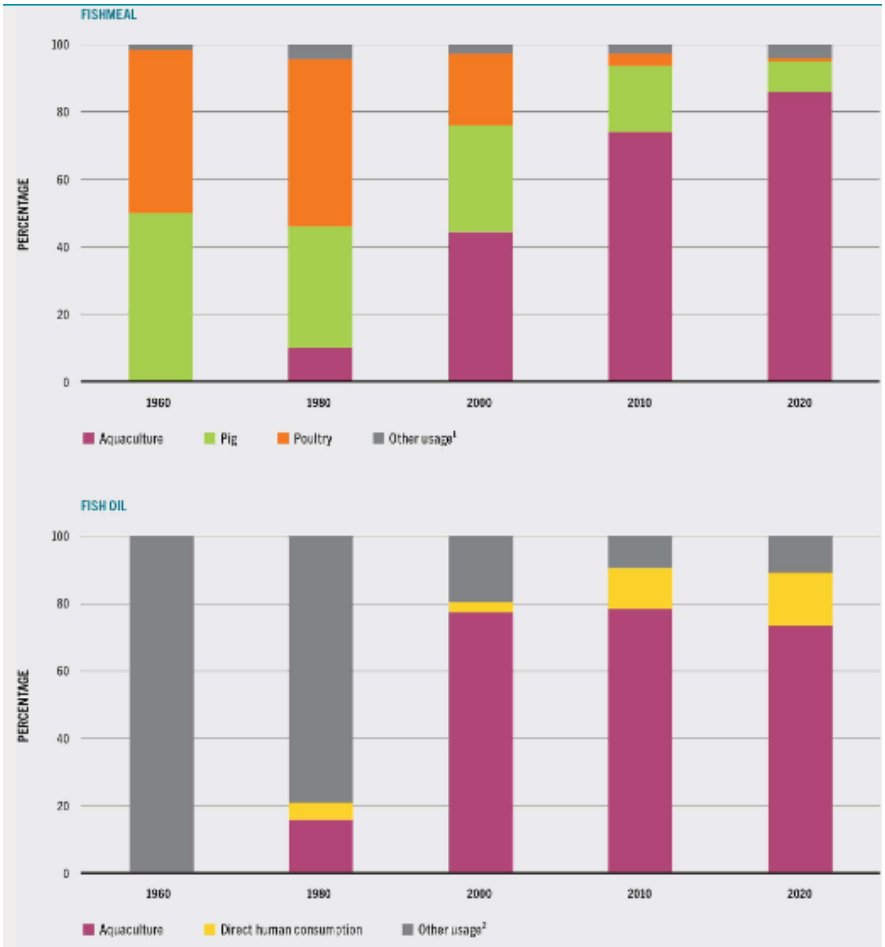


Figure 4 - Different use ratios of fish oil and fishmeal over the last 60 years (FAO 2022)

Through figure 4, it is possible to observe that the use of these ingredients directed to the aquaculture sector is increasing due to the sector production increase. Regarding FO, it is, until now, the only economically viable source of long-chain polyunsaturated fatty acids (LC-PUFAs; 20:4n-6, 20:5n-3 and 22:6n-3)(Oliva-Teles et al., 2015; Turchini et al., 2009) to be included in aquafeeds. Therefore, it is an essential ingredient for the development of marine fish, as they have little to no ability to express enzymes responsible for the desaturation and elongation of PUFAs into LC-PUFAs, compared to freshwater and diadromous fish (Council, 2020; Oliva-Teles et al., 2015; Sargent et al., 2002). Thus, it is mandatory in marine fish diets, limiting the formulation of sustainable diets using different ingredients such as vegetable oils (rich in C-18 PUFA and not in LC-PUFA).

FM is a complete ingredient for carnivorous fish species, with several nutritional properties, namely i) high protein content and a balanced amino acid profile; ii) high protein digestibility; iii) high palatability, making the food more appetizing and attractive

to the fish; iv) a high taurine concentration, which is a semi-essential non-protein amino acid that improves the animal's performance and its food intake; v) essential minerals and vitamins vi) it does not have anti-nutritional factors (Hardy, 2010; Oliva-Teles et al., 2015). Altogether, it has a series of benefits that make this ingredient suitable for carnivore fish species diets. Although FO and FM are key ingredients in carnivorous fish diets, fisheries resources are overexploited to produce these ingredients for aquafeed incorporation. Indeed, higher pressure on wild fish stocks is imposed to satisfy the needs of feed-producing companies, for example, on populations of anchovies or sardines, which are two species widely used for producing these two ingredients (Tacon, 2020) Therefore, using these ingredients threatens the biological sustainability of diverse species. With this scenario of unsustainability and their high market price, the demand for new and sustainable ingredients that use fewer natural resources and are derived from other sources, such as macroalgae, is necessary. In addition, these alternatives promote biological and environmental sustainability, being economically less expensive and, in some cases, reusing nutrients that would be neglectfully discarded. Regarding omnivorous and herbivorous fish species, the dependency on FM and FO is less evident. The real challenge is directed at marine carnivorous fish species, whose FM inclusion in diets can be reduced, but the entire replacement by other protein-rich ingredients is still hard to achieve (Oliva-Teles et al., 2015). One of the significant problems of total FM substitution is the lack of several amino acids such as lysine, methionine, and taurine, leading to health and growth problems. Moreover, with this lack of amino acids, it would be necessary to supplement the diet with several specific amino acids, leading to a higher aquafeed cost, which would prove to be yet another problem (Oliva-Teles et al., 2015). However, in the case of FO, this complete replacement is impossible, as no other viable lipid sources can satisfy the essential fatty acid needs of marine carnivorous fish. Therefore, the only currently economical and biological solution would be to, during the grow-out phase, include a small percentage of FO (5-10%), enough to satisfy the needs of marine fish in LC-PUFAs, making up the rest of the dietary lipids based on a mixture of vegetable oils, such as canola, flax, soy, sunflower, among others such as poultry and insect oils (Oliva-Teles et al., 2015).

Plant feedstuffs (PF) are the most available ingredients for aquafeeds, as they are highly produceable. Hence, since the beginning of this century, PF have gradually included fish diets as an alternative to animal-origin ingredients, regarded as a cheap

source of protein and carbohydrates (Glencross et al., 2023; Oliva-Teles et al., 2015; Olsen & Hasan, 2012). Nowadays, the market reality is different, as PF prices are increasing due to higher competition with human consumption. Hence, one of the recent factors that triggered PF prices, majorly cereals such as wheat meal (WM), was the Russian invasion of Ukraine. Since February of 2022, when the conflict began, wheat prices have been exponentially increasing, as Ukraine was one of the world's biggest wheat producers, and its availability was decreasing, as the conflict escalated. Thus, this conflict directly affects aquafeeds costs, as WM is getting expensive (Figure 5).



Figure 5 - Wheat and other cereals price variation (US$/ton) in the first 6 months of the Ukrainian war (weforum.org)

Also, PF are basic and basal ingredients in the human diet, and that tendency is increasing as the population is exponentially growing and vegetarianism is also expanding. According to the British Council (britishcouncil.org), there are 1.5 billion vegetarians, representing over 20% of the world population. Therefore, PF prices increase, especially for vegetal ingredients with high protein content, such as soybean, wheat, corn, and peas, also used as ingredients in aquafeeds. Therefore, it is necessary to substitute these plant ingredients for more unutilized ingredients with interesting nutritional profiles, such as macroalgae and by-products. Thus, this study aims to replace WM and wheat glúten in over 75% and partially reduce soybean meal inclusion without affecting fish's growth performance and welfare.

1.1.2. Marine Algae

Marine algae are a polyphyletic group divided into three taxa: *Chlorophyta* (green algae), *Rhodophyta* (red algae), and *Phaeophyta* (brown algae), whose differences are based on the different chlorophyll pigments they possess, as usually green algae have chlorophyll a and b, brown algae have chlorophyll a and c, and red algae only have chlorophyll a (Yalçın et al., 2021). They are ubiquitous organisms, inhabiting ecosystems of different temperatures, salinities, pH, light intensity, and other physical-chemical parameters, being a very diversified group whose distribution depends on all these variables.

1.1.3. Advantages and disadvantages of macroalgae, from a nutritional point of view

From a nutritional perspective, macroalgae have a relatively low to moderate protein content (usually between 10-30% dry weight (DW), but can reach nearly 50% in some cases, such as *Porphyra tenera*) (Dawczynski et al., 2007; Galland-Irmouli et al., 1999; García-Vaquero & Hayes, 2016). They have a relatively balanced amino acid profile, along with an abundant vitamin content and a combination of several different bioactive compounds, such as polyunsaturated fatty acids, phlorotannins, terpenoids, and alkaloids (Rajauria, 2015; Rengasamy et al., 2020). Also, due to their marine nature, they are rich in minerals, one of their main constituents. Macroalgae, despite having low lipids, are natural sources of omega-3 and omega-6 polyunsaturated fatty acids (Rajauria, 2015). Also, they are easily cultivated, inexpensive, and can be used as feed for many aquatic organisms, such as mollusks, crustaceans, zooplankton, and fish. However, some negative aspects come from macroalgae utilization in aquafeeds, such as the high content of non-digestible fiber, leading to lower digestibility and decreasing fish growth, especially in carnivorous species, due to the little to the non-existent presence of digestive enzymes that can degrade these polysaccharides (Holdt & Kraan, 2011; Rajauria, 2015). Another less positive aspect is the seasonal variation of seaweeds, depending on the conditions under which they were cultivated and captured or the different seasons of the year, leading to different nutritional and chemical compositions. In addition, they may also have anti-nutritional factors (e.g.: lectins, phytic acids, protease inhibitors, and ammonia)(de Oliveira et al., 2009; García-

Vaquero & Hayes, 2016; Rajauria, 2015). Despite the negative aspects, the ratio between advantages and disadvantages is rather positive, as they contribute to greater environmental sustainability and combating global warming because they are autotrophic organisms, consuming CO_2 and releasing O_2. Thus, they are one of the possible alternatives to the recurrent use of PF as FM replacers in aquafeeds seeking greater environmental sustainability. The most used macroalgae for aquafeeds are generally the genera *Ulva, Porphyra, Undaria,* and *Laminaria.* However, this work seeks to make use of other less conventional types of macroalgae in this industry, which are, in this case, the genus *Gelidium*, *Palmaria* and *Codium*.

1.1.4. Gelidium spp.

Gelidium is a red marine algae present in temperate and tropical waters, with a wide distribution across the globe, mainly in the Atlantic Ocean and the Mediterranean Sea, being present in ecosystems with strong water flows and temperatures ranging from 10ºC to 25ºC (Mouga & Fernandes, 2022). *Gelidium* spp. nutritional composition (DW) has an overall protein content of around 15-20%, 2 % lipids, 40% carbohydrates, and 20% ash (Cavaco et al., 2021; Cox's Bazar, 2013; Paiva et al., 2017). Moreover, it's remotely studied algae in animal nutrition, although some studies are addressing its use, in rats (Kang et al., 2017; Liu et al., 2017), prawns (Agarwal et al., 2021), and in marine snails (Foster et al., 1999), with overall good results, improving animals metabolism and welfare, achieving promising results, with potential anti-oxidant and anti-obesity properties. To the best of our knowledge, there are no published studies regarding using *Gelidium* spp. and its by-products as ingredient in aquafeeds. Acknowledged as a source of high-quality agar, a phycocolloid that results from the mixture of two polysaccharides, agaropectin and agarose, forming a thick and very hydrophilic gel, is widely used for its gelling abilities in food and pharmaceutical industries (Mouga & Fernandes, 2022). Therefore, it has a predominant role in this type of market, being the main seaweed used in agar production along with *Gracilaria*. Also, it is necessary to know how to use the agar industry-released *Gelidium* residues, underrating the potential that these residues may have and the variety of uses that can be given to them.

Gelidium agar-agar extraction

As mentioned before, agar is a widely used product in the food industry (as a thickening agent) and in microbiology, depending on the gel strength and properties.

The selected gelidium species and the extraction process protocol applied will influence these properties. Firstly, *Gelidium* agar-agar extraction involves alkaline pre-treatments, followed by high-temperature and high-pressure treatments, high-temperature filtration, and, lastly, numerous frost-defrost cycles, causing water evaporation, resulting solely in the agar-agar gel after drying (Figure 6) (Armisen & Galatas, 1987; Martínez-Sanz et al., 2019). Theoretically, the remnant sub-product would be wasted, giving up on the nutritional capabilities and the purpose of reusing potentially valuable resources.

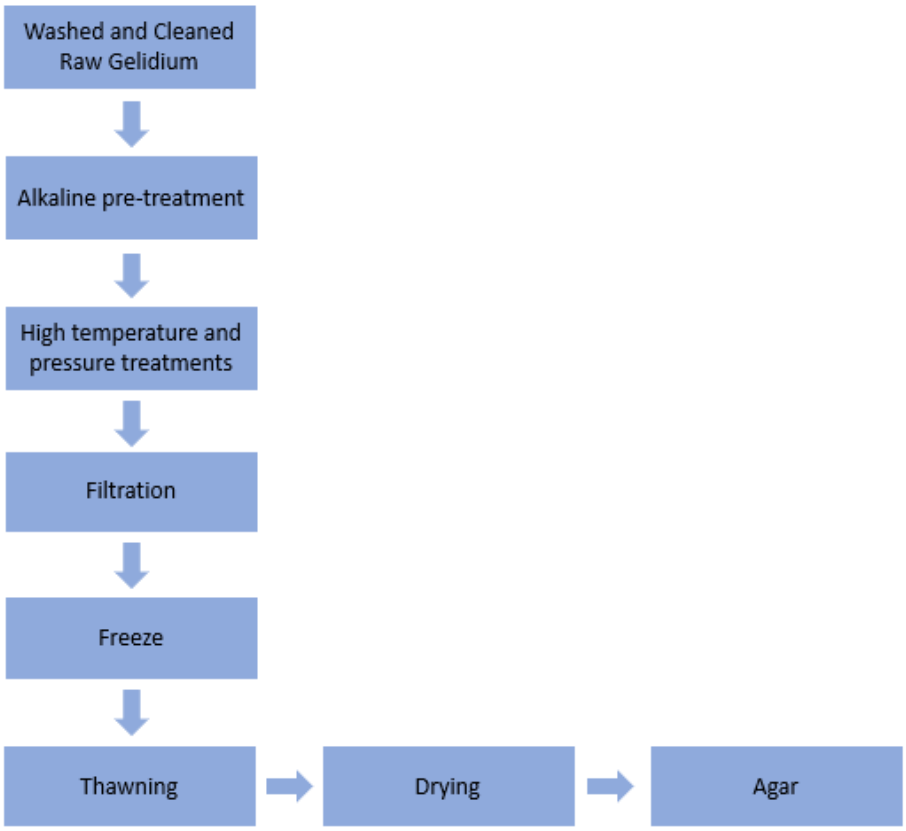


Figure 6 – Gelidium conventional method for agar-agar extraction

Gelidium by-product nutritional content

Overall, seaweed by-products's nutritional value is vaguely discussed. Nowadays, large amounts of residues are generated from industrial activities, as its quantity is worth being assessed for aquafeeds. As documented by Trigueros et al. (2021), the *Gelidium* by-product after agar extraction nutritional composition consists of 37%

carbohydrates (%DW), 21% protein (%DW), 1% lipids (%DW), and 22% ashes (%DW) (Table 1).

Table 1 - Chemical composition of *Gelidium* by-product after extraction (Trigueros et al., 2021)

Compounds	Composition (%)	SD
Protein	18.4	1.0
Lipids	0.9	0.1
Carbohydrates	37.0	2.0
Glucans	23.4	0.9
Galactans	10.9	0.5
Arabinans	2.9	0.2
Lignin	12.0	1.0
Soluble	8.7	0.1
Insoluble	3.0	1.0
Extractives*	11.5	0.9
Polyphenols	0.3	0.0
Proteins	2.6	0.3
Glucumoric acid	0.8	0.1
Ash	22.0	2.0

*Extractives were not fully described in the referred study.

According to Trigueros et al. (2021), *Gelidium* by-product is mainly constituted by carbohydrates (37%), as over 60% are glucans (cellulose), 30% galactan (agar components), and less than 10% are arabinans (cellular wall constituent). Also, lignin content is reasonably high. As a seaweed residue, protein content is still relevant and higher than in PF, such as WM (14% CP). However, WM's digestible fiber content is higher than Gelidium by-product.

It is worth noting the presence of polyphenols (0.3 %) (Table 1), as *Gelidium* spp are increasingly studied for their ability to produce phenols and other secondary metabolites, like flavonoids (Metidji et al., 2015). This polyphenolic content can have anti-oxidant properties, serving as free radicals scavengers and anti-inflammatory and antimicrobial traits (Wang et al., 2009).

Gelidium by-product is rich in minerals with a substantial ash content (22%); (Table 1) (Trigueros et al., 2021). Also, *Gelidium* by-product is rich in several minerals (Table 2): i) Mg, that plays a role in protein synthesis, cell cycle, and fish metabolism; ii) P, playing a role for skeletal tissue maintenance, molecular signaling, and a structural component of cell walls, nucleic acids and energy compounds such as ADP and ATP; iii) Ca, with a similar role to P, as a skeletal tissue essential component, playing a role in many metabolic processes, such as blood coagulation, nervous impulse transmition , muscle function, and osmoregulation ; iv) Fe, important for several biochemical processes, such as gene regulation, oxygen transport, and cell growth and differentiation; v) I, main constituent of thyroid hormones such as 3,5,3′-triiodo-L-thyronine (T3) and L-thyroxine; 3,5,3′,5′-tetraiodo-L-thyronine (T4), essential for cell growth and activity; vi) K, important for cellular osmotic equilibrium, neural impulse transmission, and acid-base equilibrium; vii) Na, necessary for fish metabolism and the absorption of nutrients in the digestive system, predominantly in the intestine (Khalili Tilami & Sampels, 2018; Lall & Kaushik, 2021; Tavares-Dias, 2022).

Table 2 – Inorganic composition of raw *Gelidium sesquipedale* and its by-product after agar extraction (Trigueros et al., 2021).

Elements	Raw seaweed	By-product
Li	1.2 ± 0.2	13.4 ± 0.6
B	260.0 ± 6	100 ± 7
Na	34.5 ± 317	875 ± 31
Mg	12,764.0 ± 187	8918 ± 214
Al	175.0 ± 59	8156 ± 217
Si	190.0 ± 62	109 ± 27
P	956.0 ± 87	5786 ± 677
S	11.2 ± 1014	4993 ± 357
K	29.4 ± 1082	863 ± 9
Ca	449.0 ± 93	6584 ± 221
Ti	0.7 ± 2	7.32 ± 0.03
V	2.1 ± 0.1	6.23 ± 0.07
Cr	1.3 ± 0.4	14 ± 1
Mn	149.0 ± 56	386 ± 115

Fe	251.0 ± 83	6330.0 ± 346
Co	0.6 ± 0.2	2.4 ± 0.1
Ni	5.0 ± 1	10.0 ± 1
Cu	6.0 ± 1	8.8 ± 0.7
Zn	246.0 ± 2	247.0 ± 26
Ga	0.3 ± 0.1	10.5 ± 0
As	5.7 ± 0.2	7.6 ± 0.1
Br	3775.0 ± 826	4871.0 ± 363
Rb	29.8 ± 0.4	10.2 ± 0.2
Sr	35.0 ± 9	267.0 ± 110
Zr	0.21 ± 0.1	2.1 ± 0
Cd	0.53 ± 0.1	1.3 ± 0.2
I	5793.0 ± 1926	5766.0 ± 1381
Ba	1.1 ± 0.3	39.5 ± 0.4
La	0.21 ± 0.1	5.0 ± 1
Ce	0.3 ± 0.1	10.0 ± 3
Nd	0.15 ± 0.1	4.0 ± 1
Pb	4.0 ± 2	10.9 ± 0.3
Total	100.629 ± 5815	54.417 + 4111

1.1.5. *Codium tomentosum*

Codium spp area very diverse green macroalgae whose distribution is predominantly Asian and located in the Northwest Atlantic and Northeast Pacific. Typically inhabits temperate climates, reasonably tolerating different salinities and temperatures. For instance, its ideal temperature for growth and development is around 24ºC (Ansell et al., 1998). Nutritional composition *Codium* spp average 14-18% protein, 30-35% carbohydrates, 1-4% lipids, and 35-45% ash (Table 3)(Echave et al., 2021; Ferreira et al., 2022; Rodrigues et al., 2015). It is widely used in the human food industry, being present in traditional Asian dishes and gourmet cuisine (Moreira et al., 2022; Pereira, 2016). In addition, it is also used in the pharmaceutical industry, due to its anti-tumor and anti-inflammatory properties (Dilshara et al., 2016; Lee et al., 2017; Moreira et al., 2022) and in the cosmetic industry (Leandro et al., 2019; Pereira, 2015).

Table 3 - *C. tomentosum* nutritional content (Ferreira et al., 2022; Rodrigues et al., 2015).

Compounds composition	Rodrigues, et al 2015	Ferreira, et al 2022
Protein	18.8 ± 0.1	14.2 ± 0.0
Lipids	3.6 ± 0.2	1.9 ± 0.3
Carbohydrates	32.8 ± 0.0	33.3 ± 1.3
Cellulose	-	10.6 ± 0.1
Xylan	-	22.7 ± 2.4
Ash	36 ± 0.5	45.5 ± 0.1

C. tomentosum is also rich in some minerals, typically containing minerals such as Mg, K and Na (Table 4).

Table 4 - Inorganic composition of *C.tomentosum* (Ferreira et al., 2022)

Elements	Ferreira, et al 2022
Zn	0.1 ± 0.0
Fe	0.3 ± 0.0
Cu	1.0 ± 0.0
Ca	8.5 ± 0.5
Mg	15.7 ± 1.1
K	64.8 ± 7.6
Na	103.0 ± 11.9

1.1.6. *Palmaria palmata*

These red macroalgae habitats from cold to temperate temperatures, being located not only in the North Atlantic but also on the Iberian coast and the North Pacific (Dumay et al., 2022). It is one of the most captured and used marine macroalgae in the human

food industry. Despite season variations, this algae nutritional composition (DW) usually contains around 20% protein, 1-2% lipids, 40-60% carbohydrates, and 15% ash (Table 5) (Galland-Irmouli et al., 1999; Grote, 2019; Morgan et al., 1980; Mouritsen et al., 2013). *Palmaria palmata* has been studied in farm animal feed, in Atlantic salmon (Wan et al., 2016), with inclusion levels up to 15% not affecting salmon growth. Moreover, is recognized for its antioxidant properties (Cornish & Garbary, 2010; Grote, 2019; Yuan et al., 2005); being also acknowledged for its anti-inflammatory capacities and for their use to prevent cardiovascular and neural diseases (Grote, 2019; Holdt & Kraan, 2011; Morgan et al., 1980; Van Ginneken et al., 2011). More recently, interestingly, has been highlighted for its potential for biofuel production (Grote, 2019), similar to *Gelidium*, being two red macroalgae with a high amount of carbohydrates, essential for the production of energy, either in the form of bioethanol, due to the hydrolysis of polysaccharides, followed by an alcoholic fermentation using glucose; or biogas, with the digestion of these carbohydrates through anaerobic bacteria (Suutari et al., 2015; Vergara-Fernández et al., 2008; Ye Lee et al., 2013; Yoza & Masutani, 2013).

Table 5 - *Palmaria palmata* nutritional composition (Mishra et al., 1993; Rødde et al., 2004).

Compounds composition	**Rødde, et al 2004**	**Mishra, et al 1993**
Protein	16.0	16.8
Lipids	-	2.0
Carbohydrates	58.8	66.4*[1]
Xylose	36.7	-
Glucose	8.1	-
Galactose	13.3	-
Ash	15.1	14.8

*[1]carbohydrate + rest content obtained by difference.

P. palmata mineral content is pourer than the other two seaweeds studied, however, contains a high amount of K (Table 6).

Table 6 – *P. Palmata* ionic composition (Rødde et al., 2004)

Elements	Rødde, et al 2004
Ca	3.8
Mg	2.3
Na	11.2
K	56.1
Br	0.2

1.1.7. Macro algae treatments to improve nutrient utilization

Algae are a source of several nutrients. However, macroalgae can undergo different types of treatment not only to extract the desired compounds, for example, fucoidan extraction using methanol, chloroform, and water pre-treatment, after drying (Ale et al., 2011; García-Vaquero et al., 2017; Lim et al., 2014) but also to eliminate anti-nutritional factors and improve nutrient digestibility.

Two types of treatment have been applied: an alkaline treatment (NaOH) and a thermal treatment (autoclave). These two types of treatments are used to promote better use of energy, protein, lipids, and carbohydrates by the animal. One of the main functions of NaOH is to decrease the non-digestible fiber content, allowing for better nutrient digestibility by the fish (Zaki et al., 1994). This alkaline treatment allows cleaving of the molecular linkages between complex sugars, removing lignin, wax, pectin, oil, and hemicellulose from natural fibers (Hernández et al., 2015; Magagula et al., 2022; Zheng et al., 2009). The autoclave treatment allows the solubilization of the lignin, cellulose, and hemicellulose wall (Hernández et al., 2015), allowing greater digestion of the food, therefore, fish can utilize nutrients more efficiently. Hence, these two types of treatments together are a possibility to increase the hydrolysis of non-digestible fibers and improve nutrient digestibility, allowing better growth performance and feed utilization by the fish.

1.1.8. European Seabass

European sea bass (*Dicentrarchus labrax*) (Figure 7) is a Mediterranean marine fish that can be found in the North East Atlantic and the Black Sea.

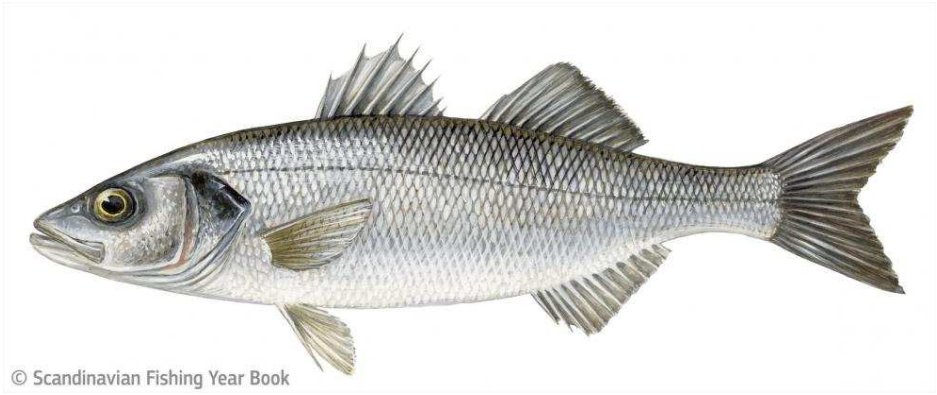


Figure 7 – European seabass (*D.labrax*), from Scandinavian Fishing Year Book.

It is a saltwater fish (>30‰), and due to being euryhaline, it can tolerate significant salinity oscillations (0-40‰), being perfectly adapted to brackish water environments. Although they can inhabit environments with different temperatures, due to their eurythermic capabilities, they can live in temperatures from 5ºC to 32ºC, but their optimal temperature is between 22 and 24ºC (Lucas et al., 2019; Vázquez & Muñoz-Cueto, 2014). European seabass is a carnivorous species with a natural diet consisting of mollusks, crustaceans, and smaller fish. Reproduction occurs between December and March in the Mediterranean, extending to June in North Atlantic waters (Vázquez & Muñoz-Cueto, 2014). This species shows sexual dimorphism, whereas females are bigger, mainly due to the high reproductive efforts, spending more energy than males. Also, females show high fecundity, spawning an average of 200,000 eggs per kg.

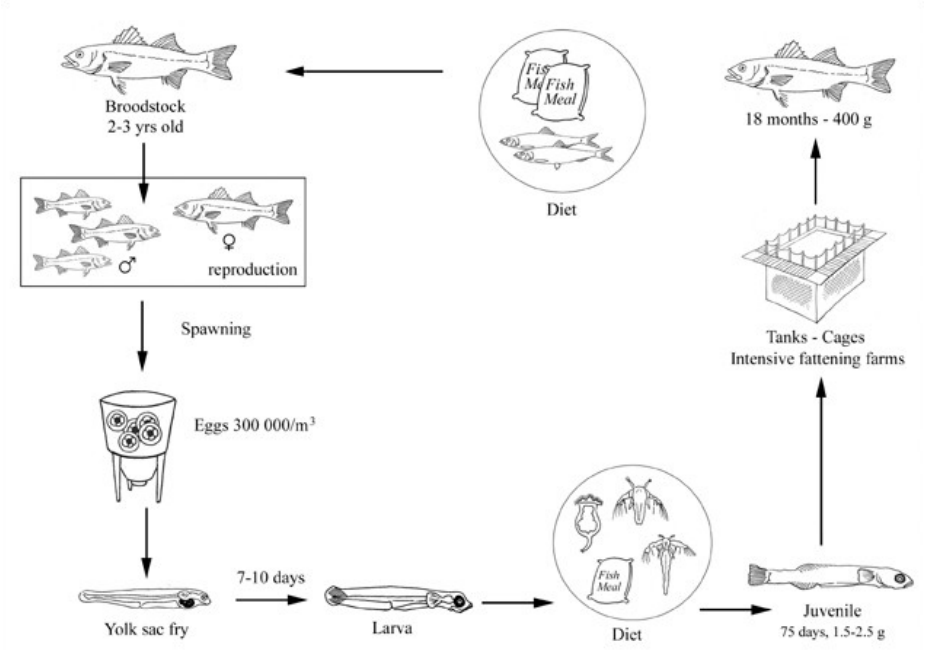


Figure 8 - European seabass production on an intensive system (FAO.org)

Overall, the ability to tolerate a wide range of physicochemical parameters, such as salinity, temperature, and low dissolved oxygen concentrations (minimum 2mg L^{-1}), made European seabass a good model for animal studies.

The production cycle of this species was fully described (Figure 8). Furthermore, European seabass is the third most produced fish species in Europe, with 200k tonnes produced in 2018, only surpassed by diadromous species such as salmon (1.5 million tonnes) and rainbow trout (400k tons) (Hough, 2022). Also, along with European seabream, it is currently the most important commercially exploited fish in Mediterranean areas(Hough, 2022), being a good source of LC-PUFAS for human consumption, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), along with a distinct aroma and fillet quality (Alasalvar et al., 2002).

Portugal is the 8th producing country (FEAP 2021), with 954 tons in 2021, +5.5% than the prior year. In Portuguese aquaculture, European seabass marine aquaculture production represents 12% of the total production, only surpassed by gilthead seabream (39%) and turbot (45%). Also, European seabass is one of the most appreciated fish species by Portuguese consumers, as the demand and market value are high.

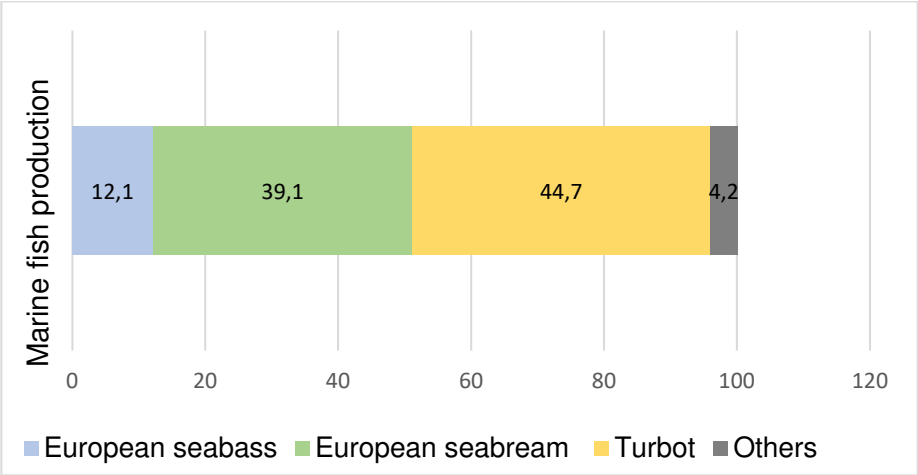


Figure 9 - Portugal's marine fish production percentage in 2021, according to INE (2022).

European seabass nutritional requirements

From a nutritional perspective, European seabass diets require around 45-55% protein (55% for juveniles and 45% for adults). As a top predator, protein and aminoacid content requirements are essential to achieve. Aminoacid limitations were compiled by Council (2020), as European seabass requires: i) ten essential aminoacids (arginine, valine, lysine, isoleucine, histidine, methionine, phenylalanine, threonine, tryptophan, and leucine); ii) seven nonessential aminoacids (alanine, asparagine, aspartate, serine, glutamate, and glycine); and iii) five semi-essential amino acids (tyrosine, taurine, proline, glutamine, and cysteine) (Li et al., 2009; Oliva-Teles, 2000). An ideal aminoacidic profile was compiled by Tibaldi and Kaushik (2005) (Table 7).

Table 7 – Ideal aminoacidic requirements for European seabass (Tibaldi & Kaushik, 2005).

Aminoacids	Tibaldi, & Kaushik, (2005)
Arg	4.6
Lys	4.8
His	1.6
Ile	2.6
Leu	4.3
Val	2.9
Trp	0.6
Thr	2.7
Phe + Tyr	2.6
Met + Cys	2.3

Lipids are an expensive nutrient in aquafeeds nowadays, as they are an indispensable source of energy, essential fatty acids, vitamins, and pigments. As a key nutrient for aquafeeds, it's the most studied macronutrient in fish nutrition (Bell & Koppe, 2010). Like the majority of marine species, *D. labrax* is unable to synthesize LC-PUFAS, such as ARA (n-6), EPA, and DHA (n-3) (Oliva-Teles, 2000). Overall, European seabass diets have 15-18% lipids for good growth results (Oliva-Teles, 2000; Peres & Oliva-Teles, 1999). EFA requirements for European seabass juveniles is about 1% EPA+DHA (Kaushik, 2002; Taşbozan & Gökçe, 2017). However, the essential fatty acid requirements depend on fish size and dietary lipid levels.

Carbohydrates are a cheap source of energy, however, European seabass, similarly to other carnivore fish, does not utilize efficiently this macronutrient. As mentioned, carnivore fish cannot degrade these carbohydrates, especially cellulose, hemicellulose, and lignin. Moreover, European seabass tolerates 25-30% of carbohydrates in diets without affecting fish growth performance (Kaushik, 2002).

Vitamin requirements data for *D. labrax* is pour, nonetheless, according to (Kaushik, 2002), dietary requirements for European seabass do not differ from salmonids. Overall, Vitamins A, C, D, and E are the most studied in fish nutrition. Vitamin A is essential for skeletal structure, influencing cell growth and development, as its optimal level in *D. labrax* is 35mg/kg^{-1} (Mazurais et al., 2009). Vitamin C and D are both critical for the prevention of skeletal abnormalities. Vitamin C is a co-substrate for enzymes involved in producing pro-collagen, carnitine, and neurotransmitters (Tolbert, 1979), and its optimal concentration is around 50 m/kg^{-1}, according to Kaushik (2002). Vitamin D is essential for intestine maturation, indirectly influencing Ca+ absorption in fish intestine. Moreover, the vitamin D requirement for *D. labrax* good growth performance is 27 mg/kg^{-1} (Darias et al., 2010). Lastly, vitamin E is important for protection against lipid peroxidation, as a supply of 500mg/kg^{-1} is required for ROS damage prevention (Messager et al., 1992).

Mineral requirements information for European seabass is scarcer, except phosphorus, which the dietary level required is around 0.65%, according to Oliva-Teles and Pimentel-Rodrigues (2004).

1.1.9. Recirculation aquaculture system

Recirculation aquaculture system (RAS) is characterized by being an almost entirely closed system, with continuous water flow due to the water pumps. Hence, it allows greater control of environmental variables, with good water quality, which is key to high-density production. Also, it requires less water volume, with little to no external influence, either environmental or biological (Lucas et al., 2019). It is equipped with i) several different filters (mechanical and biological) to remove feed and fish waste products, ii) oxygenation and aerification to monitor O_2/CO_2 levels, iii) ozonization and UV-light devices, essential for water disinfection, and iv) temperature and pH regulation equipments, maintaining optimal temperature and pH for fish growth and development (Bregnballe, 2015). RAS's main processes are shown in figure 10.

Firstly, fish are fed, manually or by feeders, producing ammonia in the liver via glutamate deamination by glutamate dehydrogenase, exiting the mitochondria and being excreted through the gills by direct diffusion from fish blood to water as NH_3 (gas) or NH_4 (ion). Moreover, apart from the ammonia excreted by fish, it can also be accumulated and converted of organic matter, feces, and feed waste by bacteria decomposition. Although excreting ammonia is the most appropriate way of nitrogenous excretion, due to ammonia's high water solubility, it is very toxic to fish. NH_3/NH_4^+ ratio increases proportionally with higher pH, leading to higher toxicity, mainly affecting the nervous system, interfering with ion transportation, displacing K^+, and causing neuron depolarization, thus leading to the increase of Ca^+ influx and, consequently, cell death (Randall & Tsui, 2002). Therefore, RAS is equipped with mechanical and biological filters to remove this issue. After feeding, water moves continuously by the water pump through mechanical filtration. This filtration allows the retention of solid organic waste particles. After that, water is pumped to a biological filter containing nitrifying aerobic bacteria (e.g.: genus *Nitrosomonas* and *Nitrobacter*) before entering again in the fish tanks. This biological filtration is key to removing dissolved and toxic waste, such as ammonia, derived from fish protein and phosphorus metabolism. These bacteria, present in the biofilter, are responsible for the nitrification process, converting ammonia into nitrite (NO_2), by NH_4 aerobic oxidation and later from nitrite to nitrate (NO_3), a lesser harmful nitrogenous compound to fishes, mainly due to fish lower membrane cell permeability to nitrate, whereas nitrite and ammonia are far more permeable and toxic (Gee et al., 1990). To maximize this biofiltration process, it is used: i) a plastic substrate (bioballs), with a high area per root meter (m^3); ii) sodium

hydroxide for pH stabilization, and iii) effective aeration, allowing bacteria growth and survival.

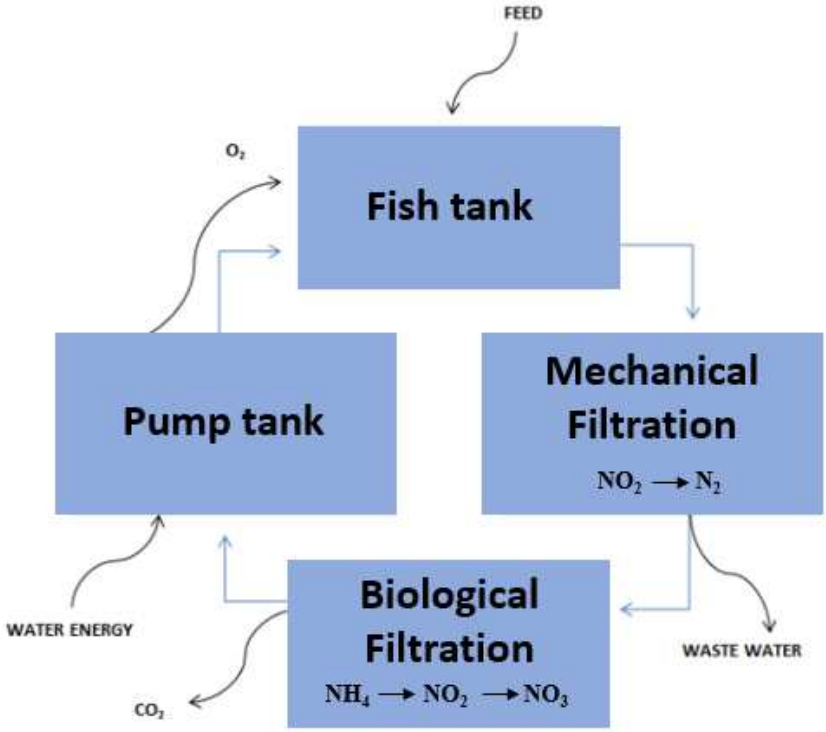


Figure 10 - Main processes in a RAS

1.1.10. Fish Tanks

Tank size, shape, and depth significantly influence fish survival, development, and overall production. Circular tanks have numerous advantages, such as i) optimum rotational velocity, which provides a uniform environment, suitable for an efficient self-cleaning and equal fish/feed distribution; ii) most efficient shape in water volume/wall area; iii) easy to equip with feeders and other equipment; iv) induce more intense fish exercise, allowing better fillet quality; and v) better O_2 diffusion and easier control of the oxygen levels (Bregnballe, 2015). Overall, provides uniform feeding, O_2, and light distribution, without leaving any “dead zones” (Figure 11), unlike square tanks (Lekang, 2020).

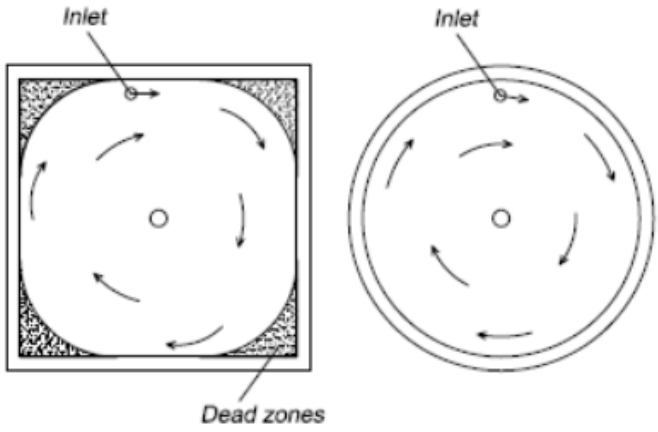


Figure 11 - Squared and circular tanks comparison (Lekang, 2020)

1.1.11. Mechanical Filters

As previously referred, mechanical filters are essential for solid waste particle removal, mainly in the form of feces and uneaten feed. Mechanical filtration can be achieved by one or more of 3 different mechanical filters: i) screen filters, which are cheap and effective in filtration of bigger particles; ii) sand filters, capable of extracting smaller particles than i); and iii) protein skimmers, expensive than the other two, but can remove, by adsorption, even smaller particles (<50 µm), forming a foam of all retained solids, later to be removed.

1.1.12. Biological Filters

As mechanical filtration is not suitable for dissolved waste removal, biological filters are essential for the nitrification process, transforming ammonia, which is very toxic, to nitrite and nitrate, the latter being a lesser harmless compound (Figure 12). This process is carried out by heterotrophic or autotrophic aerobic bacteria, with ammonia and nitrite oxidation, consuming O_2 and releasing H^+ ions (Bregnballe, 2015).

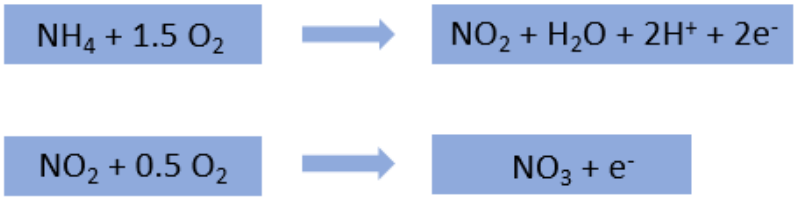


Figure 12 – Nitrification chemical process and results

Thus, this process will reduce pH levels, requiring pH stabilization (sodium hydroxide or other). Overall, pH and temperature levels must be constantly monitored (optimal conditions at 30ºC and 7.5 pH).



Figure 13 – Bioballs utilization as biological filters in CIIMAR.

1.1.13. Temperature, O_2, O_3 and aeration devices

Temperature is one of the most important aquaculture production aspects. Temperature-wise, as ectotherms, fish highly depend on system temperature. As such, fishes need to be at optimal temperature conditions to attain optimum growth and disease resistance. To maintain O_2 optimum levels, air was injected constantly, along with good aeration material. O_2, temperature, and pH levels must be monitored daily, allowing a high standard of system maintenance. Ozone is usually used in RAS as a disinfection method, with the removal of possibly pathological organisms and organic matter by heavy oxidation. Also, ozone polishes water, making it clearer, reducing suspended particles and bacteria wall adherence (Bregnballe, 2015). However, O_3 can be harmful to fish and operators when in high concentrations. As such, it is imperative to have a proper system design with correct aeration, ozone dosing, and monitoring.

1.1.14. Pumps

Water pumping usually requires electricity use, which is economically costly. Recirculation systems must run preferably by gravity, as such, all water goes from the tanks to a reservoir (sump), where mechanical and biological filtration takes place. After filtration, water is pumped back to the tanks. This lifting requires electric energy, however, the system runs by gravity most of the time, which has less electric expenses.

2. Objectives

In the presented study, three different macroalgae were used, namely, *Gelidium* by-product obtained after the agar extraction, an undervalued ingredient with potential as a novel feed ingredient; *Codium tomentosum* and *Palmaria palmata*. *Gelidium* by-product was tested in a growth trial with *Dicentrarchus labrax,* to assess fish growth performance and welfare (oxidative stress enzymes activity) with this ingredient inclusion. A digestibility trial was perfomed to evaluate *C. tomentosum* and *P. palmata* inclusion diets digestibility on *Dicentrarchus labrax.* Overall, this work aims to use low FM diets and substitute PF with marine macroalgae for *Dicentrarchus labrax* aquafeeds, seeking to respond positively to the increasing and emerging issue of biological and environmental sustainability without forgetting the economic aspect of the aquaculture sector associated with feed costs and zootechnical performance.

3. Material and Methods

3.1 Growth trial

3.1.1 Diets Composition

Five diets were formulated to be isoproteic (45% crude protein) and isolipidic (16% crude lipid) and to include graded levels (0%, 5%, 10%, and 15%) of *Gelidium* residue after the agar extraction in replacement of whole wheat meal and soybean meal of the control diet. The fifth diet had 15 % of *Gelidium* residue after the agar extraction pre-treated with 1N NaOH and autoclaved at 120ºC for 30 min. Diets contained FM (15%), CPSP (5%) shrimp hydrolyzate (1%), and vegetal feedstuffs (>60%) as protein sources, blended with fish oil as lipid source, fulfilling EPA, ARA,

and DHA requirements. Methionine was supplemented in all diets to prevent this essential amino acid deficiency.

Table 8 - Proximate composition of the experimental diets (% dry weight)

	Diets				
	G0	**G5**	**G10**	**G15**	**G15+T[1]**
Feedstuff *% dry weight basis*					
Fish meal[a]	15.0	15.0	15.0	15.0	15.0
CPSP[b]	5.0	5.0	5.0	5.0	5.0
Corn gluten[c]	17.0	17.0	17.0	17.0	17.0
Wheat gluten[d]	1.0	0.3	0.0	0.0	0.0
Soybean meal 48[e]	18.0	18.0	17.4	16.4	16.4
Pea Prot Concentrate meal[f]	10.0	10.0	10.0	10.0	10.0
Whole wheat meal[g]	17.3	12.9	8.6	4.4	4.4
Gelidium[h]	0.0	5.0	10.0	15.0	15.0
Fish Oil	12.0	12.1	12.3	12.4	12.4
Vitamin premix[i]	1.0	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5
Mineral premix[j]	1.0	1.0	1.0	1.0	1.0
Shrimp hydrolyzate[k]	1.2	1.2	1.2	1.2	1.2
Binder[l]	1.0	1.0	1.0	1.0	1.0
Methionine	0.1	0.1	0.1	0.1	0.1
Proximate analysis (% DM)					
Dry Matter (DM, %)	94.9	94.8	95.0	87.4	95.3
Protein	45.8	45.4	44.8	45.5	43.9
Lipid	15.7	14.3	14.3	14.7	16.3
Energy ($kJ.g^{-1}$)	21.4	21.3	21.6	21.2	20.9
Ash	5.5	7.6	8.0	9.5	10.8

[a] Pesquera Centinela, Steam Dried LT, Chile. Sorgal, S.A. Ovar, Portugal (CP: 70.3 %; CL: 12 %).

[b] Sopropeche G, France (CP: 80.2%; CL: 15.4%)

[c] Sorgal, S.A Ovar, Portugal (CP: 62.8 % CL:1.2%)

[d] Sorgal, S.A Ovar, Portugal (CP: 73.5 % CL: 1.7 %)

[e] Non-GMO; Cargill France SAS, St. Germain-en-Laye, France (CP: 56.4 %;CL:1.9 %)

[f] Sorgal, S.A Ovar, Portugal (CP: 54.9 %; CL: 3.3%).

[g] Sorgal, S.A Ovar, Portugal (CP: 13.8 %; CL: 3.2 %)

[h] Iberagar Sociedade Luso-Espanhola de Coloides Marinhos SA (CP: 22.5 %; CL: 0.4%)

[i] Vitamins (mg kg-1 diet): retinol, 18000 (IU kg-1 diet); calciferol, 2000 (IU kg-1 diet); alfa tocoferol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[j] Minerals (mg kg-1 diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese

[k] Sorgal, S.A Ovar, Portugal.

[l] Aquacube. Agil, UK.

[1] NaOH alkaline treatment + 30 min autoclave at 120ºC

3.1.2 Experimental design

Gelidium by-product from the agar-agar extraction process was obtained from Iberagar- sociedade Luso-Espanhola De Colóides Marinhos Sa, Spain. This by-product was triturated, with a part being posteriorly treated with 1N NaOH in a solid/liquid ratio of 4:3 and autoclaved for 30 min at 120°C. After being autoclaved and cooled, the mixture was placed in an oven at 60ºC for 48 hours, to dry and be included later in the diets. For the diet manufacture, all the ingredients were finely ground, well mixed, and pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3 mm die. Pellets were dried in an oven for 48 h at 50 °C. Then, the diets were stored in airtight bags at -18ºC until further use. Diets formulation and composition are presented in table 8. Growth trial scheme is presented in figure 14.

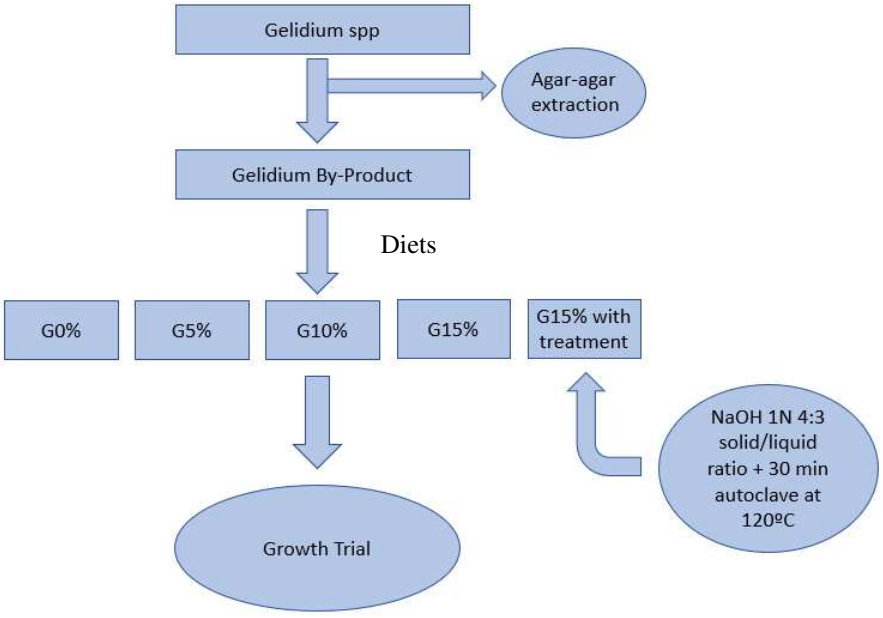


Figure 14 – Growth Trial Scheme.

The growth trial was performed at CIIMAR, Matosinhos, Portugal. European seabass (*Dicentrarchus labrax*) juveniles were purchased from a commercial fish farm (Atlantik Fish, Castro Marim, Algarve). The growth trial was carried out in a recirculation system equipped with 15 tanks, with an individual capacity of 500L, thermoregulated at 23.6 ± 0.6ºC, seawater flow of 4-5L min^{-1}, salinity at 20.6 ± 1.3 g L^{-1}, pH 6.9 ± 0.2 dissolved oxygen averaging 7.7 ± 0.3 mg L^{-1} (Table 9), and a controlled photoperiod of 12h light and 12h dark. Overall, fish were reared at similar temperatures and salinity, with no significant oscilations. The experimental diets were randomly assigned to triplicate tanks (Table 10). Three hundredfish were evenly distributed in 15 tanks in the system with an average initial body weight of 38 g, being fed by hand 6 times a week, 2 times a day (9:00 am and 4:00 pm), until apparent full satiation (defined by decreasing of the fish feeding behavior and the first three pellets reaching the tank bottom) for 49 days. Utmost care was taken to avoid feed losses. At the end of the trial, fish were lightly anesthetized with 0.3 mL L^{-1} of 2-phenoxyethanol and group-weighed, after one day of feed deprivation. For whole-body composition analyses, 10 fish from the initial stock and 3 fish from each tank at the end of the trial were randomly collected, euthanized by an excess of anesthesia, and stored at –20 °C until analysis. Wet weight, liver, and viscera weights of these fish were recorded for the determination of hepatosomatic and visceral somatic index. The remaining fish were fed for 5 more days to minimize manipulation stress and, thereafter, 4 h after the morning meal, the liver, and intestine of 3 fish per tank were randomly sampled after being euthanized by decapitation and frozen at –80 °C for oxidative stress analysis.

Table 9 – Growth trial rearing conditions through the 49 days.

RAS	Mean	SD
Temperature (ºC)	23.6	0.6
Salinity (ppt)	20.6	1.3
Oxygen	7.7	0.3
pH	6.9	0.2

Table 10– Diet distribution by the system tanks and respective bulk weights.

	Tank	10 fish (g)	10 fish (g)	Total (g)
G0	5	380.5	379.8	760.3
	9	383.6	376.3	759.9
	11	386.4	373.8	760.2
G5	4	384.1	375.6	759.7
	10	382.7	377.7	760.4
	15	380.7	379.1	759.8
G10	1	381.4	378.4	759.8
	6	377.6	382.5	760.1
	14	375.3	384.4	759.7
G15	3	380.2	379.7	759.9
	7	374.2	385.7	759.9
	12	383.5	376.2	759.7
G15+Treatment	2	374.5	385.6	760.1
	8	377.2	382.6	759.8
	13	376.0	384.1	760.1

3.1.3 Growth parameters

Growth performance was assessed using the following parameters:

$$\text{Feed efficiency (FE)} = \frac{\text{Weight gain (g)}}{\text{Feed intake (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Crude protein intake (g)}}$$

$$\text{Daily growth index (DGI)} = \frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\text{Trial duration (days)}} \times 100$$

$$\text{Thermal growth coefficient (TGC)} = \frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\text{Trial duration (days)} \times \text{Temperature (°C)}} \times 1000$$

IBW (g) - initial body weight

FBW (g) - final body weight

3.1.4 Statistical analysis

Data will be presented as the mean and pooled standard error of the mean (SEM) in growth, feed utilization and carcass composition parameters, and standard deviation (SD) in oxidative stress enzymes activity. Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. Data was normalized, if appropriate, using ln or log transformation. Statistical analysis of data was done by one-way analysis of variance (ANOVA) and an orthogonal polynomial contrasts analysis was performed with G0, G5, G10 and G15 diets data. When p-values were significant ($p < 0.05$), means were compared by Tukey´s multiple range test. All data was analyzed using the SPSS 27.0 software (IBM® SPSS® Statistics, New York, USA). All the data analysed is included as tables or figures in this thesis document.

3.2 Digestibility trial

3.2.1 Diets composition

Eight diets were formulated to be isoproteic (48% crude protein) and isolipidic (18% crude fat), and to include 3 different graded levels of *C. tomentosum* (CT) and *P. palmata* (PP) (0%, 7.5%, and 15%) (Table 11). Additionally, two diets including 7.5 % C. tomentosum and two diets including 7.5% and 15% P. palmata were pre-treated with 1N NaOH and autoclave for 30 min (CT7.5, PP7.5, PP15) and 60 min (CT7.5). Chromium oxide was included in each diet (1%) as a digestibility marker. Protein sources consisted of FM (20%), shrimp hydrolyzate (1%), and vegetal feedstuffs (>60%). Diets were blended with fish oil as a lipid source, achieving DHA, EPA, and ARA requirements. Taurine, methionine, and dicalcium phosphate were supplemented in all diets to prevent essential amino acid and phosphorus deficiencies.

Table 11 - Proximate composition of the experimental diets used in the digestibility trial.

	Diets							
	CONTROL	**CT7.5**	**CT7.5NaOH[1]**	**CT7.5NaOH[2]**	**PP7.5**	**PP7.5NaOH[3]**	**PP15**	**PP15NaOH[4]**
Feedstuff *% dry weight basis*								
Palmaria Palmata[a]	0.0	0.0	0.0	0.0	7.5	7.5	15.0	15.0
Codium tomentosum[b]	0.0	7.5	7.5	7.5	0.0	0.0	0.0	0.0
Fish meal[c]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Corn gluten[d]	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Wheat gluten[e]	5.5	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Soybean meal 48[f]	18.2	16.9	16.9	16.9	17.3	17.3	16.9	16.9
Pea Prot Concentrate meal[g]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Whole wheat meal[h]	15.0	8.1	8.1	8.1	7.7	7.7	0.0	0.0
Fish Oil	13.7	13.9	13.9	13.9	13.9	13.9	13.9	13.7
Vitamin premix[i]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[j]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Shrimp hydrolyzate[k]	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Binder[l]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	0.0	0.2	0.2	0.2	0.2	0.2	0.5	0.5
MET	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
TAU	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cr_2O_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proximate analysis (% DM)								
Dry Matter (DM, %)	92.4	88.4	94.1	92.3	94.0	92.1	92.1	93.7
Protein	47.3	47.8	47.1	48.6	48.7	48.5	48.6	49.1
Lipid	17.0	17.5	17.8	17.8	17.8	16.6	17.0	16.4
Energy ($kJ.g^{-1}$)	25.0	24.3	24.6	24.3	24.4	23.8	23.9	23.9
Ash	6.7	5.5	6.3	6.3	5.6	6.5	5.6	7.0

[a] ALGAplus Lda. Aveiro, Portugal (CP: 14.5 % CL: 1.4 %).

[b] ALGAplus Lda. Aveiro, Portugal (CP: 14.5 % CL: 1.4 %).

[c]Pesquera Centinela, Steam Dried LT, Chile (CP: 61.1 %; CL: 9.8 %). Sorgal, S.A. Ovar, Portugal.

[d]Sorgal, S.A Ovar, Portugal (CP: 75.4 % CL:2.8 %)

[e] Sorgal, S.A Ovar, Portugal (CP: 54.9 % CL: 2.2 %)

[f]Non-GMO; Cargill France SAS, St. Germain-en-Laye, France (CP: 43.5 %;CL:2.0 %)

[g]Sorgal, S.A Ovar, Portugal (CP: 49.4 % CL:X 3.3).

[h]Sorgal, S.A Ovar, Portugal (CP: 13.2 % CL: 2.4 %)

[i]Vitamins (mg/kg diet): retinol, 18,000 (IU/kg diet); calciferol, 2000 (IU/kg diet); alpha-tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[j]Minerals (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.4 (g/kg diet).

[k]Sorgal, S.A Ovar, Portugal.

[l]Aquacube. Agil, UK.

[1] NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

[2] NaOH treatment in 4:3 solid/liquid ratio + autoclave 60 min

[3] NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

[4]NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

3.2.2 Experimental design

Codium tomentosum and *Palmaria palmata* were obtained from Algaplus LDA, Aveiro, Portugal. Both algae were triturated, a part being pre-treated afterward with NaOH 1N in a 4:3 solid/liquid ratio, and posteriorly autoclaved for 30 or 60 min (depending on the diet) at 120ºC. Then, pre-treated diets were put in an oven at 60ºC, for 48h. All ingredients were mixed, dry-pelleted, and dried as indicated in the growth trial (3.1). Proximate composition of the experimental diets are presented in table 10.

The digestibility trial was performed at CIIMAR, in a thermo-regulated recirculation system equipped with a battery of 12 fiberglass tanks with 50 L water capacity and with a feces settling column connected to the outlet of each tank, designed according to the Guelph system (Cho, 1990). During the trial, a water flow of 4–5 L/min was maintained, the water temperature averaged 22.0 ± 1.0 °C, salinity 35 ± 1‰, dissolved oxygen at 8±1 mg L^{-1} and a controlled photoperiod of 12h light and 12h. Fishes were acclimated to the system conditions for 5 days, being fed with the experimental diets. After the adaption period, fish were fed by hand until apparent satiation, two times a day, seven days per week, until 3.5 grams of feces has been collected (dry weight). Feces were collected once a day, 7 days a week, before the morning meal. After each collection, feces were centrifugated at 4000 x g for 10 minutes and the supernatant was discarded. Feces from each tank were pooled and stored at –20 °C until analysis. Thirty minutes after the afternoon meal, all tanks, water pipes, and settling columns were thoroughly cleaned to remove any residue of feces or diet. The ingredients treatmens and diets used in the digestibility trial are schematized in figure 15.

Apparent digestibility coefficients (ADC) of dry matter, protein, lipid, and energy of the diets will be determined by the following formula:

$$ADC\ (\%) = 100\ - 100\left[\frac{\%\ Marker\ in\ the\ feed}{\%\ Marker\ in\ feces} \times \frac{\%\ Nutrient\ in\ feces}{\%\ Nutrient\ in\ feed}\right]$$

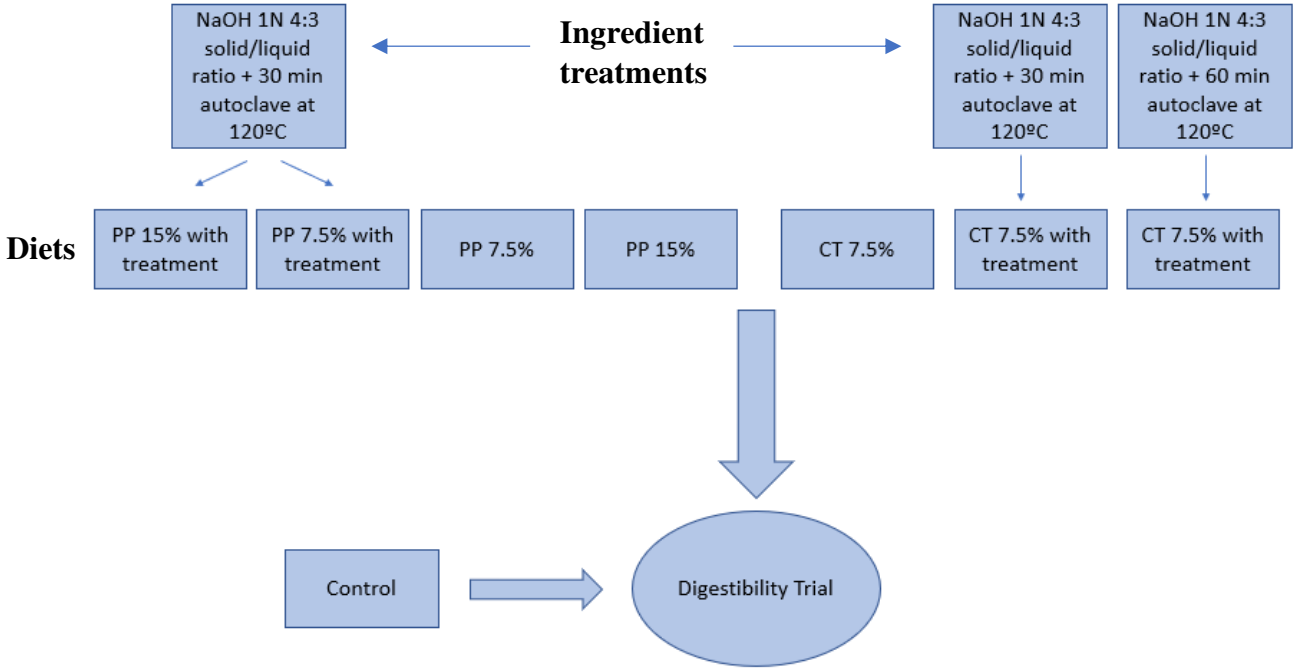


Figure 15 - Digestibility Trial Scheme

3.2.3 Statistical Analysis

Data will be presented as the mean and pooled standard error of the mean (SEM). Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. Data was normalized, if appropriate, using ln or log transformation. Statistical analysis of the apparent digestibility coefficients was done by randomized complete block design ANOVA, with feces collection periods as blocks. The null hypothesis was rejected using a probability value of 0.05. Significant differences between means were calculated by Tukey´s multiple range test. All data was analyzed using the SPSS 27.0 software (IBM® SPSS® Statistics, New York, USA). All the data analysed is included as tables or figures in this thesis document.

3.3 Proximate biochemical analysis composition

The ingredients, diets, feces, and whole-body were finely ground, homogenized, and analysed according to the AOAC (2000) procedures.

3.3.1 Diet, Carcass and Feces protein determination

Crude protein content in diets and feces were determined by the Kjeldahl method, in a Tecator digestion and distillation unit (Tecator Systems, Höganäs, Sweden; models 1015 and 1026) (Figure 16). Samples protein content is calculated by nitrogen determination, multiplying by 6.25 factor, assuming that the average nitrogen protein content is 16%. This process was divided into three stages: i) sample digestion using sulfuric acid and a Kjehdahl catalyser; ii) sample distillation by steaming on a basic medium (NaOH); and iii) Ammonia quantification by titulation, using boric and hydrochloric acid.

Total N (%) = (A-B)/P x 0.2 x 14.007 x 100

Brute Protein (%) = total N x 6.25

A- titulant volume for each samples (ml)

B- titulant volume for white (ml)

P- sample weight (mg)



Figure 16 – NUTRIMU Tecator distilillation unit 1026

3.3.2 Diet and carcass crude lipids determination

Crude Lipids were measured by sample extraction with petroleum ether in a soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046) (Figure 17). Overall, this method took 3 hours, where samples were firstly boiled for 1 hour, and then rinsed for 2 hours. Lipids were designated as the residue of the extract evaporation in the oven after ether petroleum action.



Figure 17 – NUTRIMU Soxtec system extractor

3.3.3 Diet, Carcass and Feces Gross Energy determination

Gross energy was measured by the heat produced while burning each sample with pure pressurized oxygen, using an adiabatic bomb in a PARR calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261) (Figure 18). During sample combustion, the produced heat was transmitted to a water mass inside a container, which involves a calorimeter steel bottle, where the sample is located. Thus, brute energy was determined with the correlation between the temperature differential and the energetic value of the sample. The equipment was calibrated with benzoic acid, applying the 1 cal= 4.1814 Joule conversion factor.

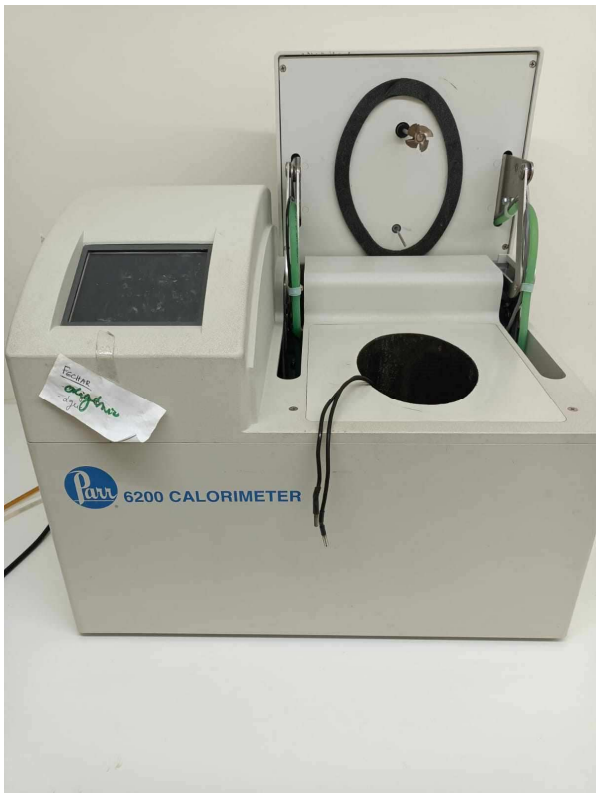


Figure 18 - NUTRIMU PARRR calorimeter model 1261

3.3.4 Diet, carcass and feces dry matter determination

Dry matter was determined by total sample weight loss after being dried in an oven at 100ºC for 24h until constant weight.

Sample Humidity (%) = ((B-A) - (C-A) x100) / (B-A)

A – Empty crucible weight

B – Crucible with fresh sample weight

C – Crucible with dried sample weight

3.3.5 Diet, carcass and feces ash determination

The ash content was the remaning organic residue that persisted after 450 ºC incineration of all organic matter in a muffle for 16 hours.

Ash (%) = (C-A)/(B-A) x 100

A – Empty crucible weight

B – Crucible with fresh sample weight

C – Crucible with incinerated sample weight

3.3.6 Feces lipid determination

Feces lipid content was quantified according to Folch et al. (1957). In this analysis, two different reagents were used (chloroform and methanol), along with water. Firstly, a chloroform:methanol reagent was used, in a 2:1 ratio, for sample homogenization, and then, a chloroform:methanol:water, in a 3:48:47 proportion, was used as a cleaning reagent.

$$\%Lipids = \frac{Centrifuge\ tube\ weight\ with\ lipids\ - Centrifuge\ tube\ weight}{Sample\ weight} \times 100$$

3.3.7 Chromium oxide determination

Chromium oxide in the diets and feces was quantified by a colorimetric method using perchloric acid ($HCLO_4$) as an oxidant agent. . It is an inert external marker, with no digestion interference. Thus, it's a capable marker to quantify nutrient digestibility (Sales & Janssens, 2003). For this quantification, chromium oxide, sodium molybdate (Na_2MoO_4), concentrated hydrochloric acid (H_2SO_4), and perchloric acid were used as reagents. The final reagent (500 ml) was composed of 10g of sodium molybdate dissolved in 150ml distilled water, of which was later added 150ml of concentrated hydrochloric acid and 200ml of perchloric acid. All procedures were performed according to Bolin et al. (1952) (Table 12).

Table 12 - Chromium oxide determination standard range

		Standard range								
Cr_2O_3 Solution (µg/ml)	Zero	5	10	20	40	60	80	100	120	140
Cr_2O_3 Solution (ml)	0	0.25	0.5	1	2	3	4	5	6	7
Distilled water (ml)	25	24.25	24.5	24	23	22	21	20	19	18
Final volume (ml)	25	25	25	25	25	25	25	25	25	25

Chromium oxide content determination:

$$\%Cr_2O_3 = \frac{\frac{Abs - b}{m} \text{x final volume (ml) x } 10^{-6}}{\text{sample weight (g)x \%DM}} \text{x } 100$$

Abs-b/m: Chromium oxide concentration (µg/ml) calculated through standard line (Abs = m x + b)

DM**:** Dry matter

m: standard line slope

b: Y intercept

3.4 Oxidative stress enzymes activity

Catalase

The quantification of catalase activity was assessed following catalase reaction on hydrogen peroxide (H_2O_2), as described by Claiborne (1985). Results were given as H_2O_2 µmol.min^{-1}.protein mg^{-1}.

Glutathione reductase

Glutathione reductase measurement was performed based on the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) while reacting with oxidized glutathione (GSSG), generating reduced glutathione (GSH) with the formation of $NADP^+$, according to Cribb et al. (1989). GR activity was expressed as $NADP^+$ $nmol.min^{-1}$ .protein mg^{-1}.

Glutathione peroxidase

Glutathione peroxidase (GPX) analysis was assessed as described in Mohandas et al. (1984). The reaction was quantified by the formation of oxidized NADPH ($NADP^+$), due to GSSH reduction to GSH. The result is expressed as $NADP^+$ $pmol.min^{-1}$ .protein mg^{-1}.

Superoxide dismutase

Superoxide dismutase (SOD) activity determination consists in the inhibition percentage of cytochrome C reduction produced by SOD. Citocrom C reduction occurs as a result of superoxide radicals, generated by the xanthine oxidase enzymatic system reacting with SOD. This analysis was performed according to McCord and Fridovich (1969), and final units were expressed as U SOD. protein mg^{-1}, in which U SOD was defined as the required enzyme quantity in order to inhibit cytochrome C reduction in 50%.

3.5 Lipid peroxidation

Malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation (LPO) level in the liver and intestine following the methodology described by Buege (1978). In the presence of thiobarbituric acid, MDA reacts, producing colored thiobarbituric acid reacting substances (TBARS) that were measured using a spectrophotometer at 535 nm. Results were calculated from an MDA calibration curve.

4. Results

4.1 Effect of dietary inclusion of *Gelidium* by-product

4.1.1 Growth trial performance

European seabass grew up up to 2.5 times their initial body weight, from an average of 38g to 95g in the diets with the best performance. There were no significant differences in weight gain (g kg ABW^{-1} day^{-1}), daily growth index (DGI), feed efficiency (FE), and protein efficiency ratio (PER) among treatments though a linear decreasing trend in these parameters were noticed with the higher dietary *Gelidium* by-product inclusion (Table 13). Nevertheless, feed intake (FI) (g kg ABW^{-1} day^{-1}) of fish-fed G15+T diet was higher than that of fish fed the G15 diets (Table 13). Survival was almost 100%, registering only one death in all 300 fish, in the G10 diet, with no apparent external signs or symptoms.

Table 13 - Growth performance and feed utilization of European seabass fed the experimental diets.

Diets	G0	G5	G10	G15	G15+T	SEM	Linear
Initial body weight (g)	38.0	38.0	38.0	38.0	38.0	0.0	Ns
Final body weight (g)	95.0	94.5	88.0	82.2	87.3	2.4	0.023
Weight gain (g kg ABW^{-1} day^{-1})	17.5	17.4	16.1	15.0	16.0	0.5	0.021
Daily growth index[1]	2.45	2.43	2.21	2.01	2.19	0.1	0.022
Feed intake (g kg ABW^{-1} day^{-1})	20.9^{ab}	21.9^{b}	21.6^{ab}	20.5^{a}	22.1^{b}	1.7	0.023
Feed efficiency[2]	0.79	0.75	0.71	0.64	0.69	0.0	0.019
PER[3]	1.83	1.74	1.67	1.61	1.65	0.0	0.028
Survival (%)	100.0	100.0	95.0	100.0	100.0	1.0	Ns

One-way ANOVA and Polynomial Contrasts: values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05), and absence of letters indicates no significant diferences. Ns: non significant.

4.1.2 Whole body composition

Whole-body composition, nitrogen, and protein retention were not affected by diet composition (Table 14). Similarly, no differences were found in the hepatosomatic and

visceral somatic index between tested diets (Table 14). However, energy retention (% intake) responded linearly to a polynomial contrasts analysis, showing a decrease in energy retention values with increasing dietary levels of Gelidium by-product.

Table 14 - Whole-body composition (% wet weight), hepatosomatic index, viscerosomatic index, and nitrogen and energy retention of European seabass juveniles fed the experimental diets.

Diets	Initial	G0	G5	G10	G15	G15+T	SEM	Linear
Dry Matter (%)	26.3	32.0	31.1	31.6	29.9	32.1	0.4	Ns
Protein	14.3	15.8	16.3	16.4	15.7	16.5	0.2	Ns
Lipids	5.6	10.8	9.3	10.3	9.4	10.4	0.3	Ns
Ash	6.4	5.0	4.9	5.1	5.0	5.0	0.0	Ns
Gross energy (kJ g^{-1})	5.2	7.5	7.4	7.5	6.9	7.4	0.1	Ns
Protein nitrogen retention (% NI[a])[b]	-	30.8	31.0	29.9	27.1	30.0	0.7	Ns
Energy nitrogen retention (% EI[c])[d]	-	35.7	33.0	31.8	29.2	31.7	1.0	0.034
HSI (%)	-	1.4	1.2	1.1	1.3	1.4	0.0	Ns
VSI (%)	-	9.8	9.3	9.5	9.2	10.7	0.2	Ns

One-way ANOVA and Polynomial Contrast: values are presented as means (n = 3) and pooled standard error of the mean (SEM). The absence of letters indicates no significant differences. Ns: non significant

[a] NI: Nitrogen intake

[c]EI: Energy intake

[b]Nitrogen retention = ((Final body weight x Final body nitrogen content – Initial body weight x Initial body nitrogen content) NI) x 100.

[d]Energy retention = ((Final body weight x Final body energy content – Initial body weight x Initial body energy content) NI) x 100

HSI - Hepatosomatic index.

VSI - Viscerosomatic index.

4.1.3 Antioxidant enzyme activity and lipid peroxidation

Catalase

Catalase activity in the liver and intestine showed no significant differences between all the dietary treatments (Figure 19). Catalase activity in liver responded linearly to the polynomial contrast analysis, in which catalase activity values were higher with the increase of Gelidium by-product inclusions in diets.

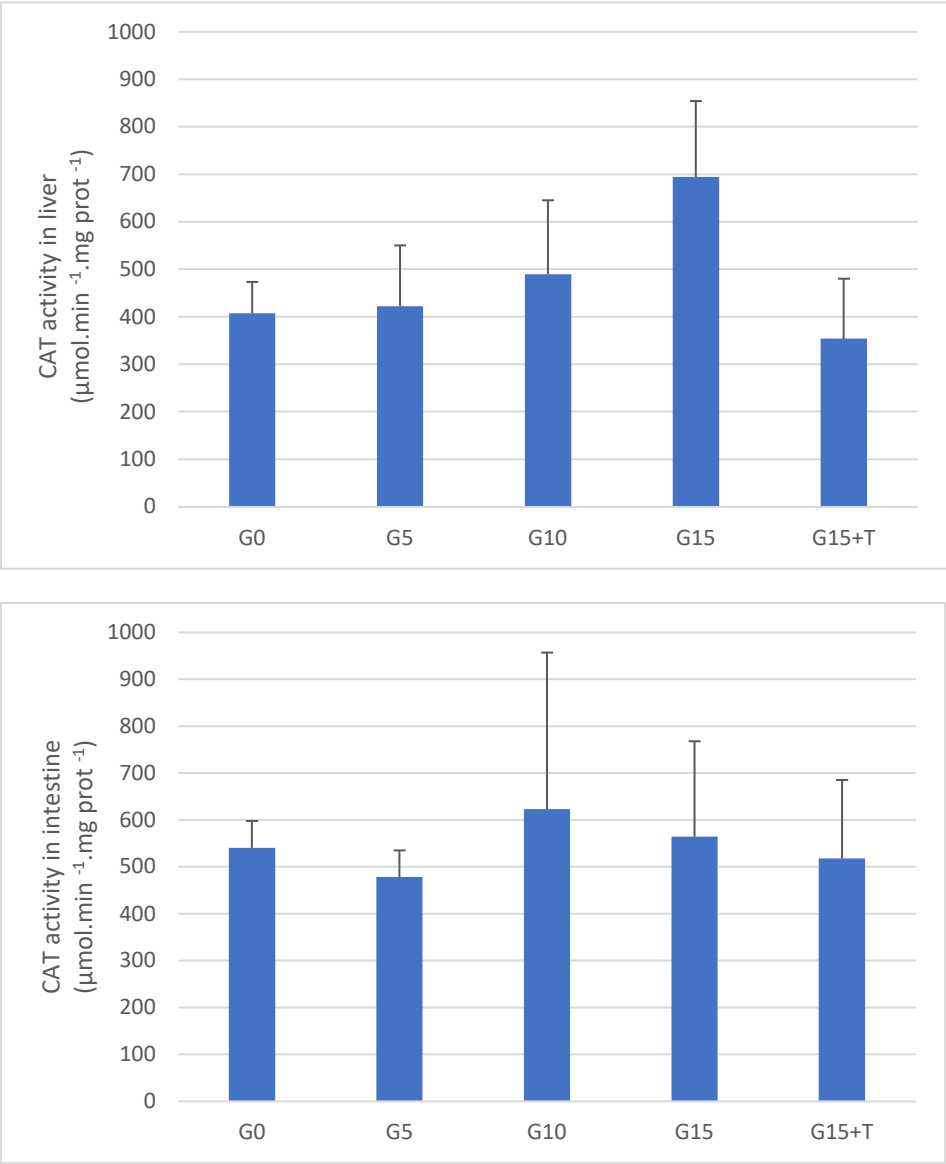


Figure 19 - Catalase (CAT) activity in the liver and intestine of *D. labrax* juveniles, fed the experimental diets. Values are presented as means (n = 9) and standard deviation (SD). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Polynomial contrast: Liver: Linear p-value = 0.03; Intestine: Linear p-value: Non-significant.

Glutathione reductase

The hepatic GR activity of fish fed the G15+T diet was higher than that of the other diets (Figure 20). However, this result was not found in the intestine GR activity, showing no significant differences between diets. In the intestine, no significant differences were observed among the diets. However, GR activity in intestine responded linearly to the polynomial contrast analysis, in which this antioxidant enzyme ativity increased with the higher inclusions levels of Gelidium by-product in diets.

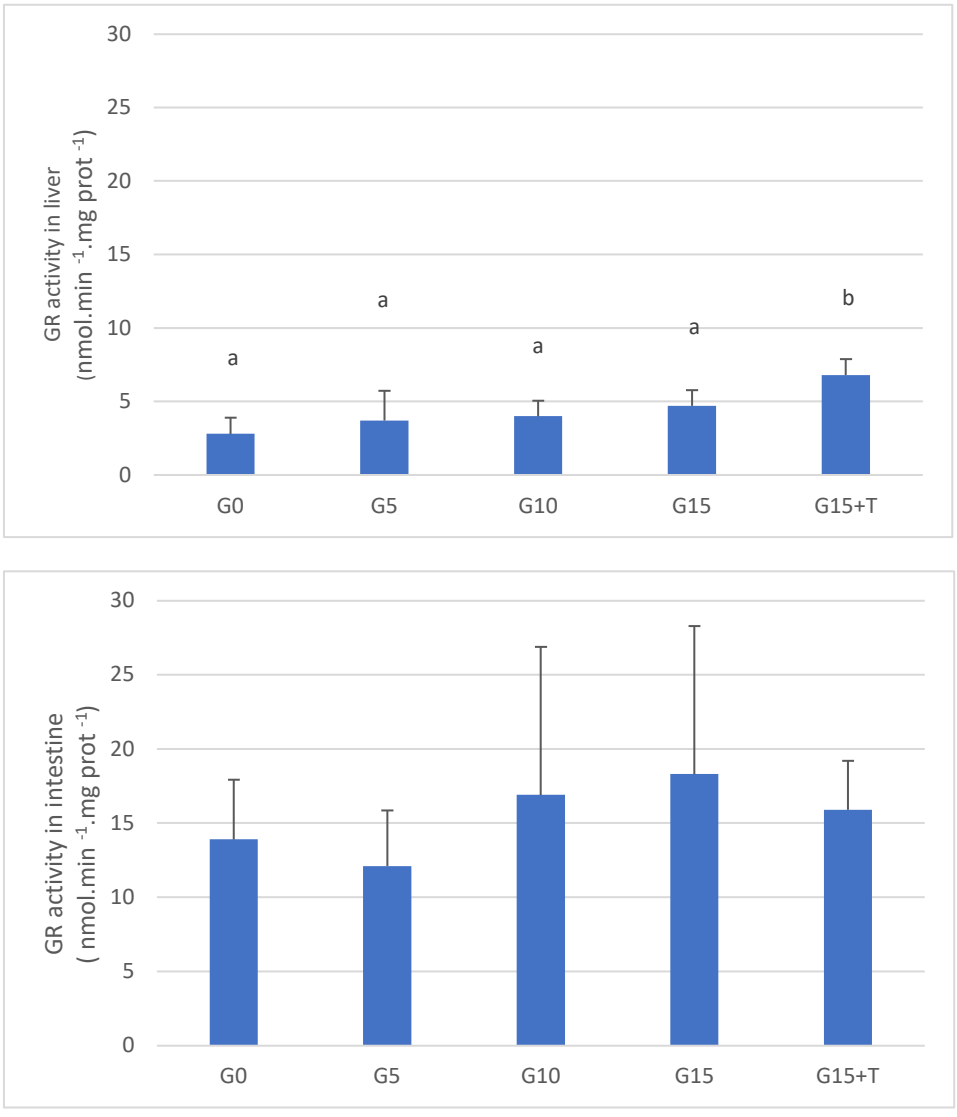


Figure 20 - Glutathione reductase (GR) activity in the liver and intestine of *D. labrax* juveniles, fed the five experimental diets. Values are presented as means (n = 9) and standard deviation (SD). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Different letters indicate significant differences (P <0.05). Polynomial contrast: Liver: Linear p-value = Non-significant ; Intestine Linear: p-value = 0.01.

Glutathione peroxidase

GPx activity in the liver of fish fed the diet G15+T was higher compared to fish fed diets control, G5, and G10 diets (Figure 21). No differences were found in GPx activity in the intestine between all dietary treatments.

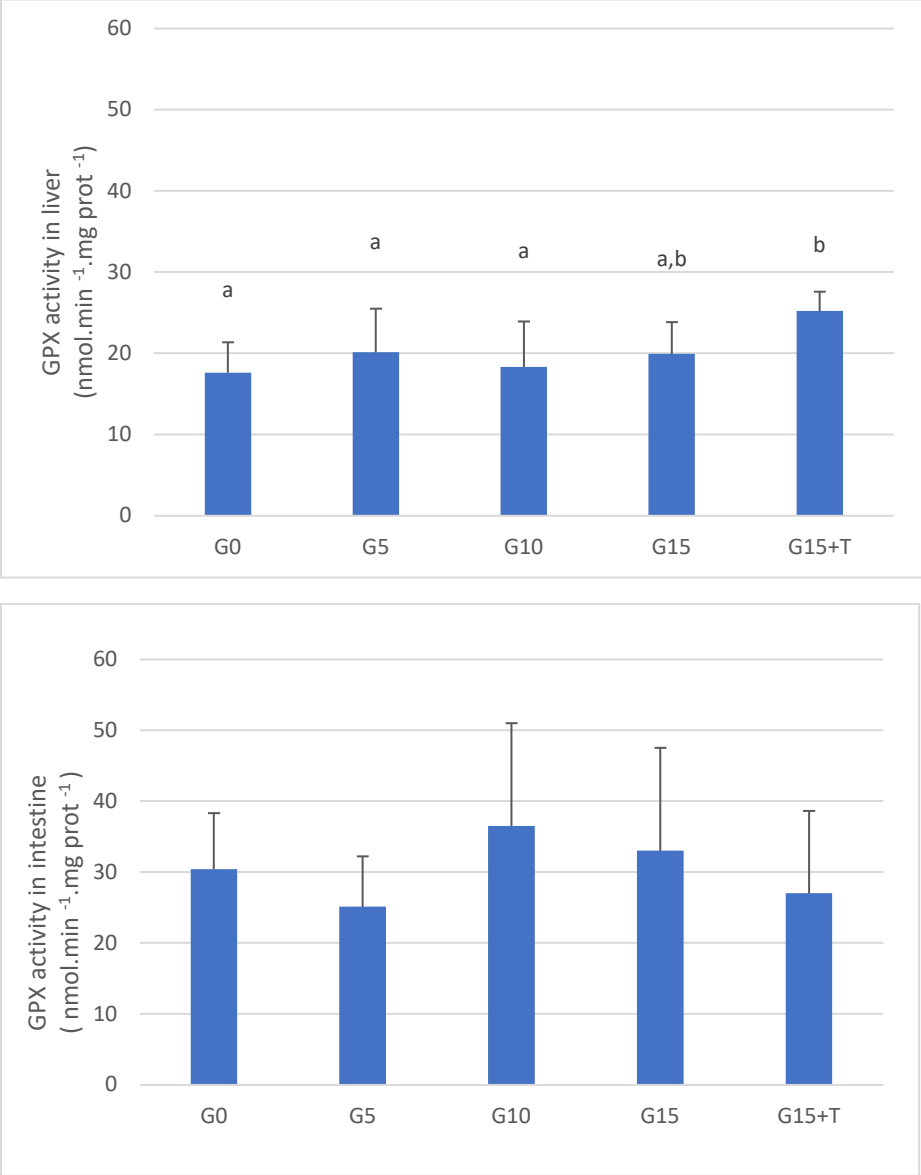


Figure 21 - Glutathione peroxidase (GPx) activity in the liver and intestine of D. labrax juveniles, fed five experimental diets. Values are presented as means (n = 9) and standard deviation (SD). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Different letters indicate significant differences (P <0.05). Polynomial contrast: Liver: Linear p-value = Non-significant ; Intestine Linear: p-value = Non-significant.

Superoxide dismutase

The hepatic and intestinal SOD activities showed no differences among experimental diets (Figure 22). However, higher SOD activity was observed in the liver compared to the SOD activity in the intestine.

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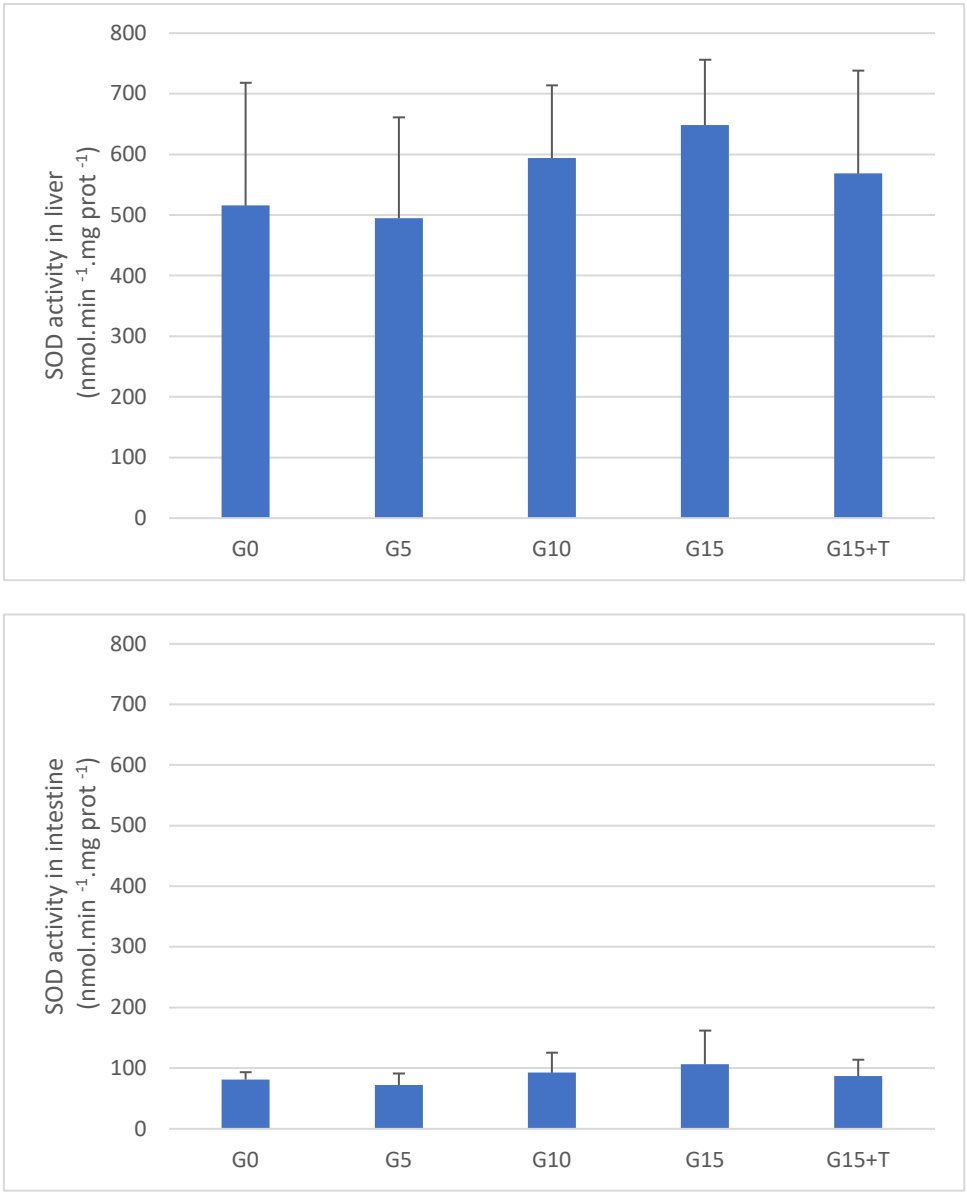


Figure 22 – Superoxide dismutase (SOD) activity in the liver and intestine of *D. labrax* juveniles, fed five experimental diets. Values are presented as means (n = 9) and standard deviation (SD). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Polynomial contrast: Liver: Linear p-value = Non-significant ; Intestine Linear: p-value = Non-significant.

Lipid peroxidation

LPO values were lower in liver than intestine of fish fed the experimental diets (Figure 23). Overall, in both tissues LPO showed no significative differences between diets, nevertheless G15+T diet showed lower LPO values in the intestine. LPO values in the intestine showed a trend for a linear increment with higher Gelidium by-product inclusion in the diets.

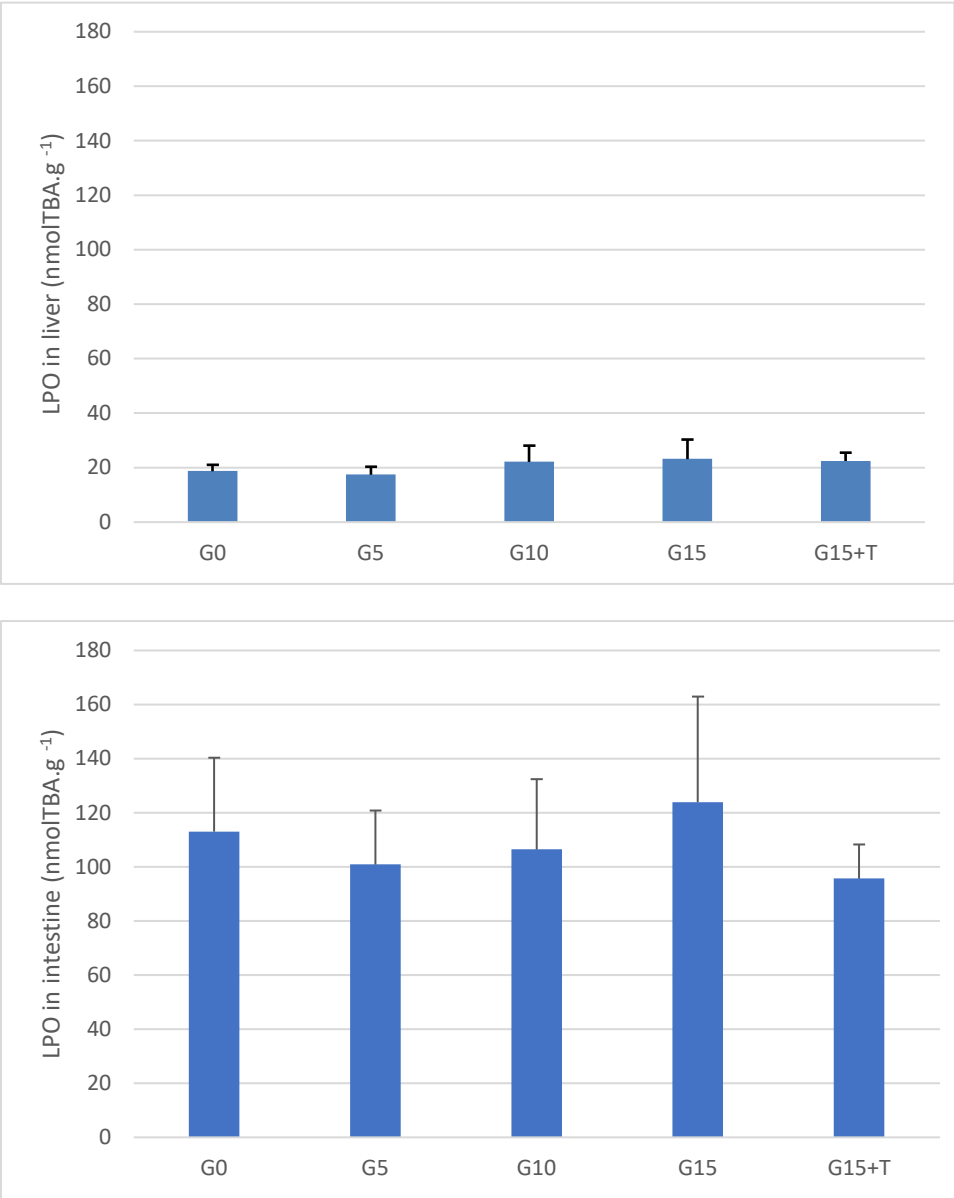


Figure 23 – One-way ANOVA and Polynomial Contrast: Lipid peroxidation (LPO) in the liver and intestine of *D. labrax* juveniles, fed five experimental diets. Values are presented as means (n = 9) and standard deviation (SD). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Polynomial contrast: Liver: Linear p-value = Non-significant ; Intestine: Linear: p-value = 0.03.

4.2 Effect of dietary inclusion of *Codium tomentosum* and *Palmaria palmata* - Digestibility trial

Apparent digestion coefficients (ADC) of crude dry matter, protein, lipids, and gross energy, are presented in Table 15. No significant differences were observed between the nutrient and energy ADC values of the experimental diets.

Table 15 - Apparent digestibility coefficients (ADC) of dry matter, protein, lipids and energy of the experimental diets.

Diets	Control	CT7.5	CT7.5 NaOH[1]	CT7.5 NaOH[2]	PP7.5	PP7.5 NaOH[3]	PP15	PP15 NaOH[4]	SEM
Dry Matter (%)	64.3	72.6	74.7	70.3	65.8	65.7	67.5	61.8	1.45
Protein	76.4	85.2	88.5	85.2	79.9	79.5	79.9	66.2	2.27
Lipids	78.8	86.1	84.0	81.7	81.7	75.8	86.3	60.4	2.79
Gross energy ($kJ.g^{-1}$)	72.7	79.3	78.1	76.6	72.5	73.4	74.1	63.6	1.60

Values are presented as means (n=3) and pooled standard error of the mean (SEM). Absence of letters indicates no significant differences.

[1] NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

[2] NaOH treatment in 4:3 solid/liquid ratio + autoclave 60 min

[3] NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

[4]NaOH treatment in 4:3 solid/liquid ratio + autoclave 30 min

5. Discussion

5.1 Growth trial

Dietary composition, attractiveness, and palatability play an important role in juvenile fish growth performance and feed utilization (Kubitza & Lovshin, 1999). Fish growth performance was very high (DGI > 2) and are within the normal range of European seabass fed high fishmeal based diets. Growth parameters DGI and WG and feed utilization FE and PER assessed had no significant differences between diets with

One-way ANOVA analysis. However, the polynominal contrasts analysis showed that a increase in Gelidium by-product inclusion led to a slight decrease in all growth and feed utilization parameters. Nonetheless, *Gelidium* by-product inclusion did not compromise fish growth, achieving similar or better results compared to studies with European seabass fed similar diets with other macroalgae inclusions such as *Ulva* spp, *Gracilaria* spp, and *Fucus* spp. up to 7.5% (Peixoto et al., 2016) or different inclusions up to 10 % of *Gracilaria cornea* and *Ulva rigida* produced from fish farm effluents in an integrated multi-trophic aquaculture (IMTA) system (Valente et al., 2006). In gilthead seabream, other important fish species for Mediterranean aquaculture, the inclusion of *Chondrus crispus* and *Ulva* spp (5%) or a mix of both macroalgae at 2.5 % negatively impacted fish growth by reducing DGI , however, feed utilization parameters such as PER, FI, and FE were not affected by this macroalgae inclusions (Guerreiro et al., 2019). In this trial, diets up to 15% *Gelidium* by-product inclusion without treatment were well accepted by the fish, denoting that tested *Gelidium* subproducts at that percentage do not negatively impact feed consumption. However, 15 % of *Gelidium* by-product inclusion reduced feed consumption but FI was restored by the Gelidium by-product treatment. Similarly, in a study of Yıldırım et al. (2009), where rainbow trout (*Oncorhynchus mykiss)* was fed graded levels (from 0% to 10%) of *Ulva lactuca* and *Enteromorpha linza*, lower feed intake was noticed in fish fed with 10% inclusions levels, compared to fish fed the control diet. Also, in a study of Al-Asgah et al. (2016), african catfish (*Clarias gariepinus*) were fed with 4 different inclusions levels of *Gracilaria arcuate* (0%, 10%, 20%, and 30%), with feed intake decreasing gradually with seaweed higher inclusions. Indeed, the presented study results showed that low inclusion *Gelidium* by-product diets (5%) allow higher feed intake than high *Gelidium* by-product inclusion levels (15%) without treatment, indicating that a *Gelidium* by-product treatment should be performed to not compromise feed intake with higher inclusions. In fact, in the presented study, a 15 % inclusion level diet with alkaline and thermal treatment showed high feed intake compared to the untreated 15 % inclusion diet denoting that the treatment was effective as a feed intake enhancer. Also, alkaline and thermal treatment in the 15% inclusion diet had no significant growth or feed utilization improvement.

5.2 Whole-body composition

Overall, results have shown similar body composition in all fish fed with 5 different diets. Protein and energy retention range from 27.1-31% N and 29.2-35.7% EI, respectively. The results are similar to those obtained in European seabass-fed graded levels (up to 10%) of *Ulva rigida* and *Gracilaria cornea* (Valente et al., 2006). However, energy retention values showed a negative linear trend with the increament of Gelidium by-product inclusions, possibly due to the lower digestible energy content of the diets. Nevertheless, this study showed that by-products derived from *Gelidium* can be included in diets up to 15%, without negative effects on fish whole body composition. HSI and VSI ranged from 1.1-1.4% and 9.2-10.7%, respectively. The relatively low hepatic somatic index (< 2) recorded in our fish suggested a good liver function without lipid and glycogen overaccumulation. These results were in agreement with studies with other macroalgae inclusions in European seabass (Peixoto et al., 2016) and in Asian seabass (*Lates calcarifer*)(Shapawi & Zamry, 2016).

5.3 Antioxidant enzyme activity and lipid peroxidation

Oxygen reduction induces reactive oxygen species (ROS) production, especially in the form of superoxide anion (O_2^-) and hydroxyl radical (OH). This ROS production is usually controlled by the organism anti-oxidant defenses however, external conditions can trigger mitochondria ROS production amplification, leading to protein denaturation and lipid peroxidation (Hoseinifar et al., 2020). Fish's anti-oxidant defense system consists of non-enzymatic anti-oxidants and enzymatic anti-oxidants, such as the glutathione cycle, SOD, GPX, GR, and CAT (Mishra et al., 2015).

Diet composition can influence fish oxidative stress, by having beneficial or detrimental depending on the ingredients and dose usage (Hoseinifar et al., 2020; Martínez-Álvarez et al., 2005; Van Doan et al., 2020). Overall, red seaweeds have bioactive compounds such as polyphenols and sulfated polysaccharides, with immunostimulant and anti-oxidant properties (Cian et al., 2015; Peixoto et al., 2019; Rudtanatip et al., 2018) However, in this case, the *Gelidium* by-product was subjected to an intense treatment for agar extraction that may have destroyed the anti-oxidant properties of this

macroalgae. As such, oxidative stress enzyme activity assessment is key to studying this ingredient's aptness to improve fish oxidative status.

SOD is the first antioxidant enzyme to act, dismutating the superoxide anion molecules into oxygen and hydrogen peroxide (H_2O_2), not allowing the formation of hydroxyl radicals (Hoseinifar et al., 2020). In this study, SOD activity showed no significant differences between diets in the liver and intestine. Similarly, in the intestine of European seabass fed diets with graded levels of *Gracilaria gracilis* (0.35%, 2.5%, and 5%)(Passos et al., 2021), and in the liver of European seabass, fed with high *Ulva ohnoi* dietary inclusion level (10%)(Martínez-Antequera et al., 2021), SOD activity was not modulated by dietary treatment. Thus, the dietary macroalgae inclusion for European seabass seems to have a low effect on hepatic or intestinal SOD activity.

After SOD action, enzymes such as CAT and GPx are responsible for eliminating non-radical ROS (H_2O_2), abundantly produced in the mitochondria matrix, converting it to harmless molecules, such as H_2O (Hoseinifar et al., 2020; Ighodaro & Akinloye, 2018). In the present study, CAT activity was similar between dietary treatments. However, the polynominal contrasts analysis showed a trend to increasing levels of CAT activity in liver with the increment of Gelidium by-product inclusion up to 15%. This results are in agreement with the results in the intestine and liver of European seabass-fed graded levels of *Gracilaria gracilis* (0.35%, 2.5%, and 5%) (Passos et al., 2021) or increasing levels of *Gracilaria* spp, *Ulva* spp and *Fucus* spp (2.5% or 7.5% of each, and a 7.5% mix of all three seaweeds) (Peixoto et al., 2016). Also, in Guerreiro et al. (2019), with *Ulva lactuca* and *Chondrus crispus* dietary inclusions (2.5% and 5% of each), despite seaweeds bioactive coumpounds content, no significative effects were observed in CAT activity in *Sparus aurata* liver.

Regarding GPx activity,differences in the European seabass intestine and liver were not found between dietary treatments with polynomial contrasts analysis. However, despite not showing also differences in intestine, One-way ANOVA analysis denoted higher GPx activity in the liver of fish-fed G15+T diet compared to fish-fed G0, 5% and 10% Gelidium diets. According to previous studies, GPx is highly dependent on selenium (Ighodaro & Akinloye, 2018; Rotruck et al., 1973; Venardos et al., 2004). Red macroalgae such as *Gracilaria* spp are usually rich in selenium (Devi et al., 2011), and thus increased GPx activity in fish-fed G15 diets may be correlated with the selenium content of these diets. In the present study, the alkaline and thermal treatments

Gelidium by-products positively affected hepatic GPx activity, which possibly indicates that treatment allowed higher selenium and other coumpounds availability. Unfortunately, selenium content was not quantified in the diets presented in this study.

GR is responsible for the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Srikanth et al., 2013). GPx oxidizes GSH to GSSH, playing an essential role in the GSSG/GSH cycle along with GR (Hoseinifar et al., 2020). As such, an increase in GPx activity will indirectly lead to an increase in GR activity, and vice versa. Not surprisingly, hepatic GR activity was also increased in fish fed the G15+T diet in agreement with GPx results. Also, polynominal contrasts analysis showed a positive linear trendin GR activity with increasing gelidium by-products dietary inclusion . In contrast, in other previously mentioned studies, the interaction between GPx and GR activitys was not found. Namely, in the study of Peixoto et al. (2016) with *D. labrax* fed with experimental diets containing different inclusion levels of *Gracilaria* spp, *Ulva* spp and *Fucus* spp (2.5% or 7.5% of each seaweed, and a 7.5% mix of all three macroalgae), GPx values in liver increased between diets, whereas GR showed no significative differences. Moreover, in the study of Guerreiro et al. (2019), with *Ulva lactuca* and *Chondrus crispus* inclusions (2.5% and 5%) in experimental diets for *S. aurata*, no significative differences were found in hepatic GR and GPx activity.

Overall, LPO in the European seabass liver and intestine was not impacted by dietary inclusions and treatments. Also, LPO was not significantly reduced in fish fed G15+T diet despite the increase of GPx and GR activity. However, a positive linear trend with increasing inclusions levels of Gelidium by-product in intestine LPO values was found. . In contrast, in European seabass-fed diets supplemented with *Gracilaria* spp and *Fucus* spp, liver LPO decreased as seaweed inclusion levels increased (Peixoto et al., 2016). One possible explanation for different results obtained with *Gelidium* by-product can be the by-product chemical composition, after severe treatments for agar extraction, and the loss of antioxidant components that can improve fish oxidative status. However, fish fed the control diet had higher absolute LPO values than thosefish fed the G5 diet. Similar results were found with a 5% dietary inclusion of *Gracilaria* spp in European seabass (Passos et al., 2021) and in meagre (*Argyrosomus regius*) (Peixoto et al., 2017). Altogether, this study showed that inclusions up to 15% of *Gelidium* by-product did not significantly increase European seabass hepatic or intestinal LPO.

Overall, this trial had no significative differences found in growth performance, but in contrast, it found differences in some oxidative stress enzymes (CAT, GR, and GPx). Therefore, *Gelidium* by-product dietary inclusion can modulate some anti-oxidant enzymes suggesting that further studys are needed to understand the underlying mechanisms associated with it.

5.4 Effect of dietary inclusion of *Codium tomentosum* and *Palmaria palmata* - Digestibility trial

Dietary inclusion of seaweed can affect nutrient digestibility depending on its inclusion level and technological treatment (Wan et al., 2019). Thus, the nutrient digestibility determination of these eco-friendly diets is important to assess their practical viability, the quantity of nutrient absorved by the fish, and the environmental impact in terms of fecal losses.

Codium tomentosum

C. tomentosum untreated diet ADC values were similar compared with *C.tomentosum* diet autoclaved for 30 min and 60 min. Similar results were achieved in a study of Güroy et al. (2013), where *Ulva rigida* was pre-treated by autoclaving at 130º for 30 min and included in diets for rainbow trout (*Oncorhynchus mykiss*) , at 5 and 10%, with no significative effects on ADC values between treated and untreated diets. Although, interestingly, fish fed the 60 min autoclave *C. tomentosum* diet showed a tendency to have lower nutrient ADC values, despite not having significative differences, in comparision to those fed with untreated *C. tomentosum* diet, which can be probably due to an excessive autoclave time exposure, partially destroying macroalgae nutritional content, as high temperatures exposure for a long time leads to protein denaturation, amino acids lower availability, and overall cellular structure damage (Papadopoulos, 1989). Despite treatments low effectiveness, *C. tomentosum* inclusions up to 7.5% did not negatively affect fish diet digestibility performance.

Palmaria palmata

P.palmata dietary inclusion did not increase or decrease nutrient ADC values compared to the control diet and similar results were found with the dietary inclusion of a different *Rhodophyta* by Soler-Vila et al. (2009) that fed diets to rainbow trout (*Oncorhynchus mykiss*) with graded inclusions of *Porphyra dioica* (5,10,15%). Nevertheless, in the presented study despite not being statistically different almost all ADC values of fish fed *Palmaria* palmata diet had a tendency to be higher than those of fish fed the control diet, except those from fish fed 15% pretreated *P. palmata* diet. Generally, untreated *P. palmata* diets had similar results to treated *P. palmata* diets, which possibly indicates that the treatment was not successful in improving the nutrient digestibility. Also, *P.palmata* ADC values were lower compared with *C.tomentosum* diets, and thus we can justify that *P. palmata* celular matrix is more resistant to European seabass digestion ability and also to the ingredient pre treatment. Therefore, other treatment methods need to be adopted in future studies, to improve these results. However, despite lower absolute values, *P. palmata* inclusions up to 15% did not negatively affect fish diet digestibility performance.

6. Conclusion

To date, there is limited information about *Gelidium* by-products in fish nutrition. Therefore, this study has shown an innovative approach, reusing industrial residues, and adopting a more eco-friendly practice, which is a positive step towards sustainable development. Dietary inclusion of up to 15% *Gelidium* by-products, specially if treated, did not negatively affect fish growth or welfare .Thus, this industrial residue is an interesting option to be included in aquafeeds as substitute of PF, namely WM and WG. However, due to the negative linear trend found in growth performance and feed utilization, an cost benefit analysis and life cycle assessment would be essential to evaluate the real impact of diets with this by-product inclusion. *Gelidium* by-products reutilization is a good example of a circular economy approach repurposing these by-products for fish nutrition, being a cheaper and more sustainable option in alliance with the European Green Deal and Farm to Fork Strategy that aims to make food systems fair, healthy, and environmentally-friendly. In the future, a more detailed chemical

composition analysis of the ingredient will provide a deeper understanding of this by-product's mineral and phenolic compound composition.

The inclusion of two different seaweeds did not affect the ADC values.The incorporations of *P. palmata* and *C. tomentosum* was successfully achieved, ensuring that fish could efficiently extract the necessary nutrients from their diets . Although, this study aimed to improve the dietary nutrients ADC values by performing two different treatments (autoclave and alkaline) and that was not successfully accomplished, hence, further studies must adopt different techniques such as ingredient sonication or exoenzymes digestion, to improve *P. palmata* and *C. tomentosum* nutrient digestibility.

7. References

Agarwal, P., Kayala, P., Chandrasekaran, N., Mukherjee, A., Shah, S., & Thomas, J. (2021). Antioxidant and antibacterial activity of Gelidium pusillum (Stackhouse) against Aeromonas caviae and its applications in aquaculture. *Aquaculture International*, *29*(2), 845-858.

Al-Asgah, N. A., Younis, E.-S. M., Abdel-Warith, A.-W. A., & Shamlol, F. S. (2016). Evaluation of red seaweed Gracilaria arcuata as dietary ingredient in African catfish, Clarias gariepinus. *Saudi journal of biological sciences*, *23*(2), 205-210.

Alasalvar, C., Taylor, K., Zubcov, E., Shahidi, F., & Alexis, M. (2002). Differentiation of cultured and wild sea bass (Dicentrarchus labrax): total lipid content, fatty acid and trace mineral composition. *Food chemistry*, *79*(2), 145-150.

Ale, M. T., Maruyama, H., Tamauchi, H., Mikkelsen, J. D., & Meyer, A. S. (2011). Fucose-containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 in vitro. *Marine drugs*, *9*(12), 2605-2621.

Ansell, A., Gibson, R., Barnes, M., & Press, U. (1998). Ecology of the green macroalga Codium fragile (Suringar) Hariot 1889: invasive and non-invasive subspecies. *Oceanography And Marine Biology: An Annual Review: Volume 36*, *36*, 1-64.

Armisen, R., & Galatas, F. (1987). Production, properties and uses of agar. *Production and utilization of products from commercial seaweeds. FAO Fish. Tech. Pap*, *288*, 1-57.

Bell, J. G., & Koppe, W. (2010). Lipids in aquafeeds. *Fish oil replacement and alternative lipid sources in aquaculture feeds*, *1*, 21-59.

Bolin, D. W., King, R. P., & Klosterman, E. W. (1952). A simplified method for the determination of chromic oxide (Cr2O3) when used as an index substance. *Science*, *116*(3023), 634-635.

Bregnballe, J. (2015). Recirculation Aquaculture. *FAO and Eurofish International Organisation: Copenhagen, Denmark*.

Buege, J. A. (1978). Microsomal lipid peroxidation. *Methods in enzymology*, *52*, 302-310.

Cavaco, M., Duarte, A., Freitas, M. V., Afonso, C., Bernardino, S., Pereira, L., Martins, M., & Mouga, T. (2021). Seasonal Nutritional Profile of Gelidium corneum (Rhodophyta, Gelidiaceae) from the Center of Portugal. *Foods*, *10*(10), 2394.

Cho, C. (1990). Fish nutrition, feeds, and feeding: with special emphasis on salmonid aquaculture. *Food Reviews International*, *6*(3), 333-357.

Cian, R. E., Drago, S. R., Sanchez de Medina, F., & Martínez-Augustin, O. (2015). Proteins and carbohydrates from red seaweeds: evidence for beneficial effects on gut function and microbiota. *Marine drugs*, *13*(8), 5358-5383.

Claiborne, A. (1985). Handbook of methods for oxygen radical research. *Florida: CRC Press, Boca Raton*, 283-284.

Cornish, M. L., & Garbary, D. J. (2010). Antioxidants from macroalgae: potential applications in human health and nutrition. *Algae*, *25*(4), 155-171.

Council, N. R. (2020). NRC.(2011). Nutrient Requirements of fish and shrimp. *Washington, DC*.

Cox's Bazar, B. (2013). Nutritional composition and amino acid profile of a sub-tropical red seaweed Gelidium pusillum collected from St. Martin's Island, Bangladesh. *International Food Research Journal*, *20*(5), 2287-2292.

Cribb, A. E., Leeder, J. S., & Spielberg, S. P. (1989). Use of a microplate reader in an assay of glutathione reductase using 5, 5'-dithiobis (2-nitrobenzoic acid). *Analytical biochemistry*, *183*(1), 195-196.

Darias, M. J., Mazurais, D., Koumoundouros, G., Glynatsi, N., Christodoulopoulou, S., Huelvan, C., Desbruyeres, E., Le Gall, M. M., Quazuguel, P., & Cahu, C. L. (2010). Dietary vitamin D3 affects digestive system ontogenesis and ossification in European sea bass (Dicentrachus labrax, Linnaeus, 1758). *Aquaculture*, *298*(3-4), 300-307.

Dawczynski, C., Schubert, R., & Jahreis, G. (2007). Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food chemistry*, *103*(3), 891-899.

de Oliveira, M. N., Freitas, A. L. P., Carvalho, A. F. U., Sampaio, T. M. T., Farias, D. F., Teixeira, D. I. A., Gouveia, S. T., & Pereira, J. G. (2009). Nutritive and non-nutritive attributes of washed-up seaweeds from the coast of Ceará, Brazil. *Food chemistry*, *115*(1), 254-259.

Devi, G. K., Manivannan, K., Thirumaran, G., Rajathi, F. A. A., & Anantharaman, P. (2011). In vitro antioxidant activities of selected seaweeds from Southeast coast of India. *Asian Pacific Journal of Tropical Medicine*, *4*(3), 205-211.

Dilshara, M. G., Jayasooriya, R. G. P. T., Kang, C.-H., Choi, Y.-H., & Kim, G.-Y. (2016). Methanol extract of Codium fragile inhibits tumor necrosis factor-α-induced matrix metalloproteinase-9 and invasiveness of MDA-MB-231 cells by suppressing nuclear factor-κB activation. *Asian Pacific Journal of Tropical Medicine*, *9*(6), 535-541.

Dumay, J., Cognie, B., Fleurence, J., Morançais, M., Turpin, V., Gavilan, M. C., Le Strat, Y., & Decottignies, P. (2022). Palmaria Species: From Ecology and Cultivation to Its Use in Food and Health Benefits. *Sustainable Global Resources Of Seaweeds Volume 1: Bioresources, cultivation, trade and multifarious applications*, 45.

Echave, J., Lourenço-Lopes, C., Carreira-Casais, A., Chamorro, F., Fraga-Corral, M., Otero, P., Garcia-Perez, P., Baamonde, S., Fernández-Saa, F., & Cao, H. (2021). Nutritional composition of the Atlantic seaweeds Ulva rigida, Codium tomentosum, Palmaria palmata and Porphyra purpurea. *Chemistry Proceedings*, *5*(1), 67.

FAO. (2022). The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. *FAO Rome*.

Ferreira, M., Salgado, J. M., Fernandes, H., Peres, H., & Belo, I. (2022). Potential of red, green and brown seaweeds as substrates for solid state fermentation to increase their nutritional value and to produce enzymes. *Foods*, *11*(23), 3864.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J biol Chem*, *226*(1), 497-509.

Foster, G., Hodgson, A., & Balarin, M. (1999). Effect of diet on growth rate and reproductive fitness of Turbo sarmaticus (Mollusca: Vetigastropoda: Turbinidae). *Marine Biology*, *134*(2), 307-315.

Galland-Irmouli, A.-V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., Bronowicki, J.-P., Villaume, C., & Guéant, J.-L. (1999). Nutritional value of proteins from edible seaweed Palmaria palmata (Dulse). *The Journal of Nutritional Biochemistry*, *10*(6), 353-359.

García-Vaquero, M., & Hayes, M. (2016). Red and green macroalgae for fish and animal feed and human functional food development. *Food Reviews International*, *32*(1), 15-45.

García-Vaquero, M., Rajauria, G., O'Doherty, J. V., & Sweeney, T. (2017). Polysaccharides from macroalgae: Recent advances, innovative technologies and challenges in extraction and purification. *Food research international*, *99*, 1011-1020.

Gee, C. S., Pfeffer, J. T., & Suidan, M. T. (1990). Nitrosomonas and Nitrobacter interactions in biological nitrification. *Journal of environmental Engineering*, *116*(1), 4-17.

Glencross, B., Fracalossi, D. M., Hua, K., Izquierdo, M., Mai, K., Øverland, M., Robb, D., Roubach, R., Schrama, J., & Small, B. (2023). Harvesting the benefits of nutritional research to address global challenges in the 21st century. *Journal of the World Aquaculture Society*, *54*(2), 343-363.

Grote, B. (2019). Recent developments in aquaculture of Palmaria palmata (Linnaeus)(Weber & Mohr 1805): cultivation and uses. *Reviews in aquaculture*, *11*(1), 25-41.

Guerreiro, I., Magalhães, R., Coutinho, F., Couto, A., Sousa, S., Delerue-Matos, C., Domingues, V. F., Oliva-Teles, A., & Peres, H. (2019). Evaluation of the seaweeds Chondrus crispus and Ulva lactuca as functional ingredients in gilthead seabream (Sparus aurata). *Journal of Applied Phycology*, *31*, 2115-2124.

Güroy, B., Ergün, S., Merrifield, D. L., & Güroy, D. (2013). Effect of autoclaved Ulva meal on growth performance, nutrient utilization and fatty acid profile of rainbow trout, Oncorhynchus mykiss. *Aquaculture International*, *21*, 605-615.

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research*, *41*(5), 770-776.

Hernández, D., Riaño, B., Coca, M., & García-González, M. (2015). Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. *Chemical Engineering Journal*, *262*, 939-945.

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, *23*(3), 543-597.

Hoseinifar, S. H., Yousefi, S., Van Doan, H., Ashouri, G., Gioacchini, G., Maradonna, F., & Carnevali, O. (2020). Oxidative stress and antioxidant defense in fish: the implications of probiotic, prebiotic, and synbiotics. *Reviews in Fisheries Science & Aquaculture*, *29*(2), 198-217.

Hough, C. (2022). *Regional review on status and trends in aquaculture development in Europe–2020*. Food & Agriculture Org.

Ighodaro, O., & Akinloye, O. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria journal of medicine*, *54*(4), 287-293.

Kang, J.-H., Lee, H.-A., Kim, H.-J., & Han, J.-S. (2017). Gelidium amansii extract ameliorates obesity by down-regulating adipogenic transcription factors in diet-induced obese mice. *Nutrition Research and Practice*, *11*(1), 17-24.

Kaushik, S. (2002). European sea bass, Dicentrarchus labrax. *Nutrient requirements and feeding of finfish for aquaculture*, 28-39.

Khalili Tilami, S., & Sampels, S. (2018). Nutritional value of fish: lipids, proteins, vitamins, and minerals. *Reviews in Fisheries Science & Aquaculture*, *26*(2), 243-253.

Kubitza, F., & Lovshin, L. L. (1999). Formulated diets, feeding strategies, and cannibalism control during intensive culture of juvenile carnivorous fishes. *Reviews in Fisheries Science*, *7*(1), 1-22.

Lall, S. P., & Kaushik, S. J. (2021). Nutrition and metabolism of minerals in fish. *Animals*, *11*(09), 2711.

Leandro, A., Pereira, L., & Gonçalves, A. M. (2019). Diverse applications of marine macroalgae. *Marine drugs*, *18*(1), 17.

Lee, S. A., Moon, S.-M., Choi, Y. H., Han, S. H., Park, B.-R., Choi, M. S., Kim, J.-S., Kim, Y. H., Kim, D. K., & Kim, C. S. (2017). Aqueous extract of Codium fragile suppressed inflammatory responses in lipopolysaccharide-stimulated RAW264. 7 cells and carrageenan-induced rats. *Biomedicine & Pharmacotherapy*, *93*, 1055-1064.

Lekang, O.-I. (2020). *Aquaculture engineering*. John Wiley & Sons.

Li, P., Mai, K., Trushenski, J., & Wu, G. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino acids*, *37*, 43-53.

Lim, S. J., Aida, W. M. W., Maskat, M. Y., Mamot, S., Ropien, J., & Mohd, D. M. (2014). Isolation and antioxidant capacity of fucoidan from selected Malaysian seaweeds. *Food Hydrocolloids*, *42*, 280-288.

Liu, H.-C., Chang, C.-J., Yang, T.-H., & Chiang, M.-T. (2017). Long-term feeding of red algae (Gelidium amansii) ameliorates glucose and lipid metabolism in a high fructose diet-impaired glucose tolerance rat model. *Journal of food and drug analysis*, *25*(3), 543-549.

Lucas, J. S., Southgate, P. C., & Tucker, C. S. (2019). *Aquaculture: Farming aquatic animals and plants*. John Wiley & Sons.

Magagula, S., Sefadi, J., Mochane, M., Mokhothu, T., Mokhena, T., & Lenetha, G. (2022). The effect of alkaline treatment on natural fibers/biopolymer composites. In *Surface Treatment Methods of Natural Fibres and their Effects on Biocomposites* (pp. 19-45). Elsevier.

Martínez-Álvarez, R. M., Morales, A. E., & Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and fisheries*, *15*, 75-88.

Martínez-Sanz, M., Gómez-Mascaraque, L. G., Ballester, A. R., Martínez-Abad, A., Brodkorb, A., & López-Rubio, A. (2019). Production of unpurified agar-based extracts from red seaweed Gelidium sesquipedale by means of simplified extraction protocols. *Algal Research*, *38*, 101420.

Mazurais, D., Glynatsi, N., Darias, M. J., Christodoulopoulou, S., Cahu, C. L., Zambonino-Infante, J.-L., & Koumoundouros, G. (2009). Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (Dicentrarchus labrax) depend on malformation type. *Aquaculture*, *294*(3-4), 262-270.

McCord, J. M., & Fridovich, I. (1969). Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). *Journal of biological Chemistry*, *244*(22), 6049-6055.

Messager, J.-L., Stéphan, G., Quentel, C., & Laurencin, F. B. (1992). Effects of dietary oxidized fish oil and antioxidant deficiency on histopathology, haematology, tissue and plasma biochemistry of sea bass Dicentrarchus labrax. *Aquatic Living Resources*, *5*(3), 205-214.

Metidji, H., Dob, T., Toumi, M., Krimat, S., Ksouri, A., & Nouasri, A. (2015). In vitro screening of secondary metabolites and evaluationof antioxidant, antimicrobial and cytotoxic properties of Gelidium sesquipedale Thuret et Bornet red seaweed from Algeria. *J. Mater. Environ. Sci*, *6*(11), 3184-3196.

Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., & Prajapati, J. (2015). Probiotics as potential antioxidants: a systematic review. *Journal of agricultural and food chemistry*, *63*(14), 3615-3626.

Mishra, V., Temelli, F., Ooraikul, B., Shacklock, P., & Craigie, J. (1993). Lipids of the red alga, Palmaria palmata.

Mohandas, J., Marshall, J. J., Duggin, G. G., Horvath, J. S., & Tiller, D. J. (1984). Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney: possible implications in analgesic nephropathy. *Biochemical pharmacology*, *33*(11), 1801-1807.

Moreira, A., Cruz, S., Marques, R., & Cartaxana, P. (2022). The underexplored potential of green macroalgae in aquaculture. *Reviews in aquaculture*, *14*(1), 5-26.

Morgan, K., Shacklock, P., & Simpson, F. (1980). Some aspects of the culture of Palmaria palmata in greenhouse tanks. *Bot. Mar.;(Germany, Federal Republic of)*, *23*(12).

Mouga, T., & Fernandes, I. B. (2022). The Red Seaweed Giant Gelidium (Gelidium corneum) for New Bio-Based Materials in a Circular Economy Framework. *Earth*, *3*(3), 788-813.

Mouritsen, O. G., Dawczynski, C., Duelund, L., Jahreis, G., Vetter, W., & Schröder, M. (2013). On the human consumption of the red seaweed dulse (Palmaria palmata (L.) Weber & Mohr). *Journal of Applied Phycology*, *25*(6), 1777-1791.

Oliva-Teles, A. (2000). Recent advances in European sea bass and gilthead sea bream nutrition. *Aquaculture International*, *8*(6), 477-492.

Oliva-Teles, A., Enes, P., & Peres, H. (2015). Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. *Feed and feeding practices in aquaculture*, 203-233.

Oliva-Teles, A., & Pimentel-Rodrigues, A. (2004). Phosphorus requirement of European sea bass (Dicentrarchus labrax L.) juveniles. *Aquaculture Research*, *35*(7), 636-642.

Olsen, R. L., & Hasan, M. R. (2012). A limited supply of fishmeal: Impact on future increases in global aquaculture production. *Trends in food science & technology*, *27*(2), 120-128.

Paiva, L., Lima, E., Neto, A. I., Marcone, M., & Baptista, J. (2017). Nutritional and functional bioactivity value of selected Azorean macroalgae: Ulva compressa, Ulva rigida, Gelidium microdon, and Pterocladiella capillacea. *Journal of food science*, *82*(7), 1757-1764.

Papadopoulos, M. C. (1989). Effect of processing on high-protein feedstuffs: a review. *Biological wastes*, *29*(2), 123-138.

Passos, R., Correia, A. P., Pires, D., Pires, P., Ferreira, I., Simões, M., do Carmo, B., Santos, P., Pombo, A., & Afonso, C. (2021). Potential use of macroalgae Gracilaria gracilis in diets for European seabass (Dicentrarchus labrax): Health benefits from a sustainable source. *Fish & shellfish immunology*, *119*, 105-113.

Peixoto, M. J., Ferraz, R., Magnoni, L. J., Pereira, R., Gonçalves, J. F., Calduch-Giner, J., Pérez-Sánchez, J., & Ozório, R. O. (2019). Protective effects of seaweed supplemented diet on antioxidant and immune responses in European seabass (Dicentrarchus labrax) subjected to bacterial infection. *Scientific reports*, *9*(1), 16134.

Peixoto, M. J., Salas-Leitón, E., Brito, F., Pereira, L. F., Svendsen, J. C., Baptista, T., Pereira, R., Abreu, H., Reis, P. A., & Gonçalves, J. F. M. (2017). Effects of dietary Gracilaria sp. and Alaria sp. supplementation on growth performance, metabolic rates and health in meagre (Argyrosomus regius) subjected to pathogen infection. *Journal of Applied Phycology*, *29*, 433-447.

Peixoto, M. J., Salas-Leitón, E., Pereira, L. F., Queiroz, A., Magalhães, F., Pereira, R., Abreu, H., Reis, P. A., Gonçalves, J. F. M., & de Almeida Ozório, R. O. (2016). Role of dietary seaweed supplementation on growth performance, digestive capacity and immune and stress responsiveness in European seabass (Dicentrarchus labrax). *Aquaculture Reports*, *3*, 189-197.

Pereira, L. (2015). Seaweed flora of the european north atlantic and mediterranean. In *Springer handbook of marine biotechnology* (pp. 65-178). Springer.

Pereira, L. (2016). *Edible seaweeds of the world*. CRC Press.

Peres, H., & Oliva-Teles, A. (1999). Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (Dicentrarchus labrax). *Aquaculture*, *179*(1-4), 325-334.

Rajauria, G. (2015). Seaweeds: a sustainable feed source for livestock and aquaculture. In *Seaweed sustainability* (pp. 389-420). Elsevier.

Randall, D. J., & Tsui, T. (2002). Ammonia toxicity in fish. *Marine pollution bulletin*, *45*(1-12), 17-23.

Rengasamy, K. R., Mahomoodally, M. F., Aumeeruddy, M. Z., Zengin, G., Xiao, J., & Kim, D. H. (2020). Bioactive compounds in seaweeds: An overview of their biological properties and safety. *Food and Chemical Toxicology*, *135*, 111013.

Rødde, R. S. H., Vårum, K. M., Larsen, B. A., & Myklestad, S. M. (2004). Seasonal and geographical variation in the chemical composition of the red alga Palmaria palmata (L.) Kuntze.

Rodrigues, D., Freitas, A. C., Pereira, L., Rocha-Santos, T. A., Vasconcelos, M. W., Roriz, M., Rodríguez-Alcalá, L. M., Gomes, A. M., & Duarte, A. C. (2015). Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. *Food chemistry*, *183*, 197-207.

Rotruck, J. T. ., Pope, A. L., Ganther, H. E., Swanson, A., Hafeman, D. G., & Hoekstra, W. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science*, *179*(4073), 588-590.

Rudtanatip, T., Lynch, S. A., Wongprasert, K., & Culloty, S. C. (2018). Assessment of the effects of sulfated polysaccharides extracted from the red seaweed Irish moss Chondrus crispus on the immune-stimulant activity in mussels Mytilus spp. *Fish & shellfish immunology*, *75*, 284-290.

Sales, J., & Janssens, G. (2003). The use of markers to determine energy metabolizability and nutrient digestibility in avian species. *World's Poultry Science Journal*, *59*(3), 314-327.

Sargent, J., Tocher, D., Bell, J., Halver, J., & Hardy, R. (2002). Fish nutrition. *The lipids*, 182-257.

Soler-Vila, A., Coughlan, S., Guiry, M. D., & Kraan, S. (2009). The red alga Porphyra dioica as a fish-feed ingredient for rainbow trout (Oncorhynchus mykiss): effects on growth, feed efficiency, and carcass composition. *Journal of Applied Phycology*, *21*, 617-624.

Srikanth, K., Pereira, E., Duarte, A., & Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish—a review. *Environmental Science and Pollution Research*, *20*, 2133-2149.

Suutari, M., Leskinen, E., Fagerstedt, K., Kuparinen, J., Kuuppo, P., & Blomster, J. (2015). Macroalgae in biofuel production. *Phycological Research*, *63*(1), 1-18.

Tacon, A. G. (2020). Trends in global aquaculture and aquafeed production: 2000–2017. *Reviews in Fisheries Science & Aquaculture*, *28*(1), 43-56.

Taşbozan, O., & Gökçe, M. A. (2017). Fatty acids in fish. *Fatty acids*, *1*, 143-159.

Tavares-Dias, M. (2022). Toxicity, physiological, histopathological, handling, growth and antiparasitic effects of the sodium chloride (salt) in the freshwater fish aquaculture. *Aquaculture Research*, *53*(3), 715-734.

Tibaldi, E., & Kaushik, S. (2005). Amino acid requirements of Mediterranean fish species. *Cahiers Options Méditerranéennes*, *63*(63), 59-65.

Tidwell, J. H. (2012). *Aquaculture production systems* (Vol. 434). Wiley Online Library.

Tolbert, B. (1979). Ascorbic acid metabolism and physiological function. *Int. J. Vitam. Nutr. Res*, *19*, 127-142.

Trigueros, E., Sanz, M. T., Alonso-Riaño, P., Beltrán, S., Ramos, C., & Melgosa, R. (2021). Recovery of the protein fraction with high antioxidant activity from red seaweed industrial solid residue after agar extraction by subcritical water treatment. *Journal of Applied Phycology*, *33*, 1181-1194.

Turchini, G. M., Torstensen, B. E., & Ng, W. K. (2009). Fish oil replacement in finfish nutrition. *Reviews in aquaculture*, *1*(1), 10-57.

Van Doan, H., Hoseinifar, S. H., Ringø, E., Angeles Esteban, M., Dadar, M., Dawood, M. A., & Faggio, C. (2020). Host-associated probiotics: a key factor in sustainable aquaculture. *Reviews in Fisheries Science & Aquaculture*, *28*(1), 16-42.

Van Ginneken, V. J., Helsper, J. P., de Visser, W., van Keulen, H., & Brandenburg, W. A. (2011). Polyunsaturated fatty acids in various macroalgal species from north Atlantic and tropical seas. *Lipids in health and disease*, *10*(1), 1-8.

Vázquez, F. J. S., & Muñoz-Cueto, J. A. (2014). *Biology of European sea bass*. CRC press.

Venardos, K., Harrison, G., Headrick, J., & Perkins, A. (2004). Effects of dietary selenium on glutathione peroxidase and thioredoxin reductase activity and recovery from cardiac ischemia–reperfusion. *Journal of Trace Elements in Medicine and Biology*, *18*(1), 81-88.

Vergara-Fernández, A., Vargas, G., Alarcón, N., & Velasco, A. (2008). Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. *Biomass and Bioenergy*, *32*(4), 338-344.

Wan, A. H., Davies, S. J., Soler-Vila, A., Fitzgerald, R., & Johnson, M. P. (2019). Macroalgae as a sustainable aquafeed ingredient. *Reviews in aquaculture*, *11*(3), 458-492.

Wan, A. H., Soler-Vila, A., O'Keeffe, D., Casburn, P., Fitzgerald, R., & Johnson, M. P. (2016). The inclusion of Palmaria palmata macroalgae in Atlantic salmon (Salmo salar) diets: effects on growth, haematology, immunity and liver function. *Journal of Applied Phycology*, *28*(5), 3091-3100.

Wang, B.-G., Zhang, W.-W., Duan, X.-J., & Li, X.-M. (2009). In vitro antioxidative activities of extract and semi-purified fractions of the marine red alga, Rhodomela confervoides (Rhodomelaceae). *Food chemistry*, *113*(4), 1101-1105.

Yalçın, S., Karakaş, Ö., Okudan, E. Ş., Başkan, K. S., Çekiç, S. D., & Apak, R. (2021). HPLC detection and antioxidant capacity determination of brown, red and green algal pigments in seaweed extracts. *Journal of Chromatographic Science*, *59*(4), 325-337.

Ye Lee, J., Li, P., Lee, J., Ryu, H. J., & Oh, K. K. (2013). Ethanol production from Saccharina japonica using an optimized extremely low acid pretreatment followed by simultaneous saccharification and fermentation. *Bioresource technology*, *127*, 119-125.

Yıldırım, Ö., Ergün, S., Yaman, S., & Türker, A. (2009). Effects of two seaweeds (Ulva lactuca and Enteromorpha linza) as a feed additive in diets on growth performance, feed utilization, and body composition of rainbow trout (Oncorhynchus mykiss).

Yoza, B. A., & Masutani, E. M. (2013). The analysis of macroalgae biomass found around Hawaii for bioethanol production. *Environmental technology*, *34*(13-14), 1859-1867.

Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005). Antioxidant activity of dulse (Palmaria palmata) extract evaluated in vitro. *Food chemistry*, *91*(3), 485-494.

Zaki, M., Nour, A., Omar, E., & Tag El-Din, A. (1994). The use of seaweed meal in feeding common carp (Cyprinus carpio L.). *Asian-Australasian Journal of Animal Sciences*, *7*(2), 183-189.

Zheng, Y., Pan, Z., & Zhang, R. (2009). Overview of biomass pretreatment for cellulosic ethanol production. *International journal of agricultural and biological engineering*, *2*(3), 51-68.