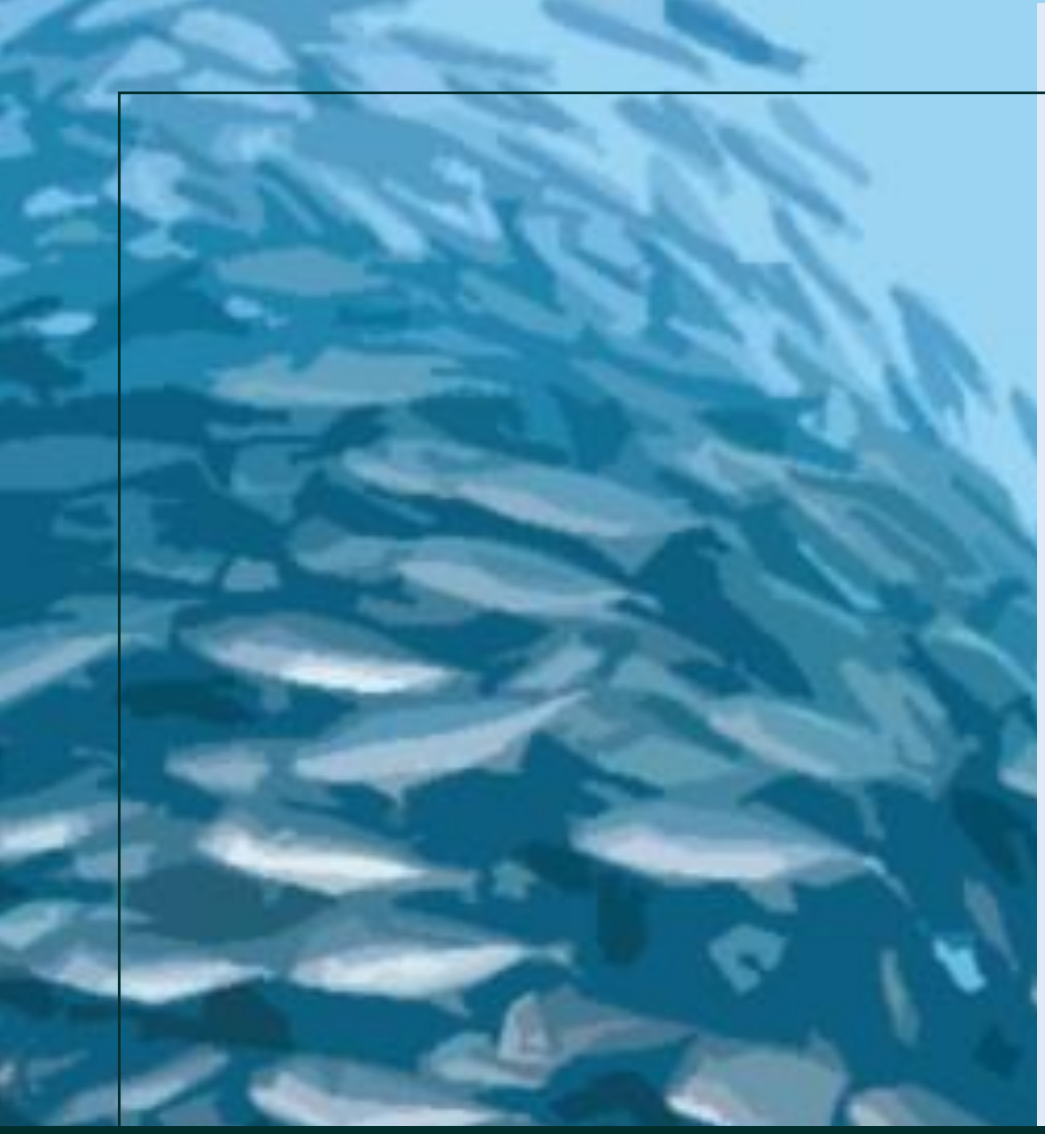

Evaluation of microalgae hydrolysates as functional ingredients for feeding gilthead seabream (*Sparus aurata*)

UNIVERSIDAD DE ALMERÍA

PhD dissertation

Alba Galafat Díaz

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Departamento de Biología y Geología

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TESIS DOCTORAL

ALBA GALAFAT DÍAZ

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Programa de Doctorado: Biotecnología y Bioprocesos Industriales aplicados a la Agroalimentación, Medioambiente y Salud

Memoria presentada por Alba Galafat Díaz para optar al Grado de Doctor por la Universidad de Almería

Fdo. Alba Galafat Díaz

UNIVERSIDAD DE ALMERÍA

Departamento de Biología y Geología

Los Dres. Francisco Javier Alarcón López, Tomás Francisco Martínez Moya y Antonio Jesús Vizcaíno Torres, profesores de la Universidad de Almería

HACEN CONSTAR:

Que la presente memoria titulada "Evaluation of microalgae hydrolysates as functional ingredients for feeding gilthead seabream (*Sparus aurata*)" que presenta Dña. Alba Galafat Díaz para optar al grado de Doctor por la Universidad de Almería, ha sido realizada en dicha universidad bajo su dirección, y autorizan su presentación y defensa.

Directores de la tesis:

Dr. D. Francisco Javier Alarcón López	Dr. D. Tomás Francisco Martínez Moya	Dr. D. Antonio Jesús Vizcaíno Torres

PROJECTS

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ABSTRACT

Currently, finding and testing alternative ingredients and novel dietary additives useful to develop sustainable and nutritious aquafeeds still is one of the main challenges in aquaculture. In this regard, the use of microalgae awakens a great interest not only as an important source of dietary protein, but also as functional ingredients in aquafeeds owing to their chemical composition and their content in bioactive compounds. However, there are technical gaps like their variability in nutrient composition and digestive bioavailability that need to be addressed before aquafeed industry can incorporate algae-based ingredients or additive into commercial formulas. Given these considerations, the present Doctoral Thesis evaluates the potential of several microalgae and cyanobacteria hydrolysates as dietary supplement in feeds for gilthead seabream.

CHAPTER 1 focuses on the *in vitro* evaluation of the protein bioaccessibility of different marine and freshwater microalgae and cyanobacteria, and their potential to be used as dietary ingredients in aquafeeds. The results revealed that all the microalgae and cyanobacteria evaluated arises as a potential protein source for feeding marine fish, particularly *Arthrospira platensis*, *Nannochloropsis gaditana* and *Chlorella vulgaris* All of them presented a high protein content as well as a balanced amino acid profile, but there were differences on their susceptibility to be hydrolysed by *Sparus aurata* digestive enzymes that must be taken into account before including them in practical diets for this species.

Given that considerations, it may be reasonable to think that any strategy aimed at improving the algae nutrient bioavailability might be of interest for including microalgae in aquafeeds. In this regard, the following chapters focus on the *in vivo* evaluation of different crude and hydrolysed microalgae biomasses in three trials carried out with gilthead seabream. The potential effects of algae biomass has been assessed studying their effects on growth, nutrient utilization, muscle proximate composition and fatty acids profile, immunity, oxidative status of fish and digestive and absorptive processes occurring in the intestinal epithelium.

CHAPTER 2 evaluates the effects of the dietary inclusion of crude or hydrolysed *Arthrospira platensis* in starter feeds for gilthead seabream fry. The

results obtained reveal that up to 10% inclusion of *A. platensis* did not cause negative effects on the parameters evaluated, although positive effects on muscle lipid peroxidation and gut functionality were found in fish fed on hydrolysed microalgae supplemented diets.

CHAPTER 3 is focus to assess the incorporation of *Arthrospira* sp. enzyme hydrolysate as dietary additive in diets for gilthead seabream juveniles. In this trial no negative effects were found on growth performance and nutrient utilization, however, the dietary supplementation with this microalgal hydrolysate improved not only the intestinal ultrastructure and functionality but also the muscle pigmentation and antioxidant capacity in fish.

Finally, **CHAPTER 4** provides information for the potential use of crude and hydrolysed *Nannochloropsis gaditana* as dietary additive in diets for gilthead seabream juveniles. In general, the lack of detrimental effects on growth and nutrient utilization and the beneficial effects observed on gut functionality, muscle pigmentation and antioxidant capacity in fish confirmed that *N. gaditana* inclusion in feeds might represent a valuable nutritional strategy for feeding *Sparus aurata* juveniles.

In general, results from *in vivo* feeding trials revealed that dietary inclusion of crude and hydrolysed *A. platensis* and *N. gaditana* biomasses did not affects the growth and nutrient utilization in gilthead seabream. Positive effects on several physiological parameters were observed when fish were fed on the diets supplemented with enzymatically hydrolysed microalgae, which seems to be associated with an increase in the nutritional and functional properties compared with the raw biomasses. Therefore, the use of hydrolysed microalgae biomasses in fish nutrition seems to be a promising strategy, not least due to the positive effects on the intestinal mucosa and oxidative status of fish observed. Further research is required with the aim of elucidating the optimum dietary inclusion level as well as the effects in longer feeding periods.

RESUMEN

La búsqueda de nuevos ingredientes y aditivos que permita el desarrollo de piensos más sostenibles y a la vez que garanticen un adecuado contenido en nutrientes es uno de los principales retos de la acuicultura actual. En este sentido, el uso de microalgas suscita gran interés no solo por su composición química caracterizada por un alto contenido en proteína y/o en lípidos ricos en ácidos grasos n-3, sino también como ingredientes funcionales, dado que además contienen compuestos bioactivos. Sin embargo, existen ciertas limitaciones que deben de tenerse en cuenta de forma previa a su incorporación en los piensos de acuicultura, ya sea como ingredientes o como aditivos, y que son la variabilidad en su composición nutricional, y la menor biodisponibilidad de sus nutrientes y/o compuestos activos derivada de la presencia de paredes celulares recalcitrantes. Teniendo en cuenta estas consideraciones, la presente Tesis Doctoral evalúa el potencial de varias especies de microalgas y cianobacterias como ingrediente funcional en piensos para dorada.

El **CAPÍTULO 1** se centra en la evaluación *in vitro* de la bioaccesibilidad de la proteína en diferentes microalgas y cianobacterias, tanto marinas como de agua dulce, y su potencial para ser utilizadas como ingredientes en piensos acuícolas. Los resultados revelan que todas las microalgas y cianobacterias evaluadas, especialmente *Arthrospira platensis*, *Nannochloropsis gaditana* y *Chlorella vulgaris*, podrían ser utilizadas como fuente de proteína para la alimentación de peces marinos. En general, las microalgas estudiadas presentan un alto contenido proteico, así como un perfil de aminoácidos equilibrado, pero existen ciertas diferencias en su susceptibilidad para ser hidrolizadas por las enzimas digestivas de la dorada (*Sparus aurata*) que debe de tenerse en consideración en la formulación de piensos para esta especie.

En base a lo anterior, parece razonable pensar que cualquier estrategia dirigida a mejorar la biodisponibilidad de nutrientes de las microalgas tiene gran interés para mejorar su valor nutricional y/o funcional. En este sentido, los siguientes capítulos se centran en la evaluación de biomasas de *A. platensis* y *N. gaditana* crudas e hidrolizadas en ensayos de alimentación con alevines y juveniles de dorada, y se ha evaluado el efecto potencial que tiene su inclusión en el pienso sobre el crecimiento, la utilización de nutrientes, la composición

química, el perfil de ácidos grasos y el estado oxidativo del músculo, la inmunidad inespecífica, y los procesos de digestión y absorción intestinal.

El **CAPÍTULO 2** evalúa los efectos de la inclusión de biomasa cruda e hidrolizada de la cianobacteria *Arthrospira platensis* en piensos de iniciación para alevines de dorada. Los resultados obtenidos revelan que hasta un 10% de inclusión de *A. platensis* no mejora el crecimiento de los peces, si bien se han encontrado efectos positivos sobre la peroxidación lipídica muscular y la funcionalidad digestiva intestinal en los peces alimentados con los piensos suplementados con microalgas, principalmente cuando éstas se utilizan hidrolizadas.

El **CAPÍTULO 3** se centra en evaluar la incorporación de un hidrolizado de *Arthrospira* sp. como aditivo en piensos para juveniles de dorada. En este ensayo tampoco se encontraron efectos negativos sobre el crecimiento y la utilización de nutrientes, sin embargo, la suplementación del pienso con este hidrolizado mejora, no solo la ultraestructura y funcionalidad intestinal, sino también la pigmentación de la piel y la capacidad antioxidante en los tejidos de los peces.

Por último, el **CAPÍTULO 4** proporciona información sobre el uso potencial de *Nannochloropsis gaditana* cruda e hidrolizada como aditivo en dietas para juveniles de dorada. En general, no se observa mejora sobre el crecimiento o la utilización de nutrientes, pero si se comprueban efectos beneficiosos sobre la funcionalidad intestinal, la pigmentación muscular y la capacidad antioxidante en los tejidos de los peces, que sugieren que el uso de *N. gaditana* como aditivo en los piensos representa una valiosa estrategia de mejora nutricional en la alimentación de los juveniles de *Sparus aurata*.

En general, los resultados de los ensayos *in vivo* revelan que la inclusión de biomasas crudas e hidrolizadas de *A. platensis* y *N. gaditana* no afectan al crecimiento y la utilización de nutrientes de los peces, y, además, se observan efectos positivos sobre varios de los parámetros fisiológicos evaluados, derivados de la suplementación con biomasa microalgal hidrolizada, lo que parece estar asociado a una mejora de las características nutricionales y funcionales de las biomasas hidrolizadas en comparación con las microalgas

crudas. Por lo tanto, el uso de biomasas de microalgas hidrolizadas en la alimentación de los peces parece prometedor, sobre todo debido al efecto estimulante sobre la mucosa intestinal y el estado oxidativo de los peces, pero se requiere una investigación más profunda que permita dilucidar el nivel óptimo de inclusión y los efectos derivados de períodos de alimentación más largos.

I. GENERAL INTRODUCTION

I.1. THE INCREASING DEMAND FOR AQUAFEEDS

According to the latest world FAO statistics, farming of aquatic animals achieved another all-time record in 2018, with 82.1 million tonnes, and it is expected to reach 109 million tonnes in 2030 (FAO, 2020). Within this production, fed aquaculture has outdistanced non-fed aquaculture, and nowadays almost 50% of all the aquaculture production is steadily dependent on commercial aquafeeds. Figure 1 shows a projection of the aquafeed production until 2025. The average growth has been of 10% per year, and the production of feeds is expected to increase up to 87 million tonnes by 2025, although this figure is quite low compared to the global feed production for terrestrial animals, which is expected to reach more than 1 billion tonnes. In spite of this fact, aquafeed production consumes 70% of fishmeal, and over 73% of fish oil marketed in the world.

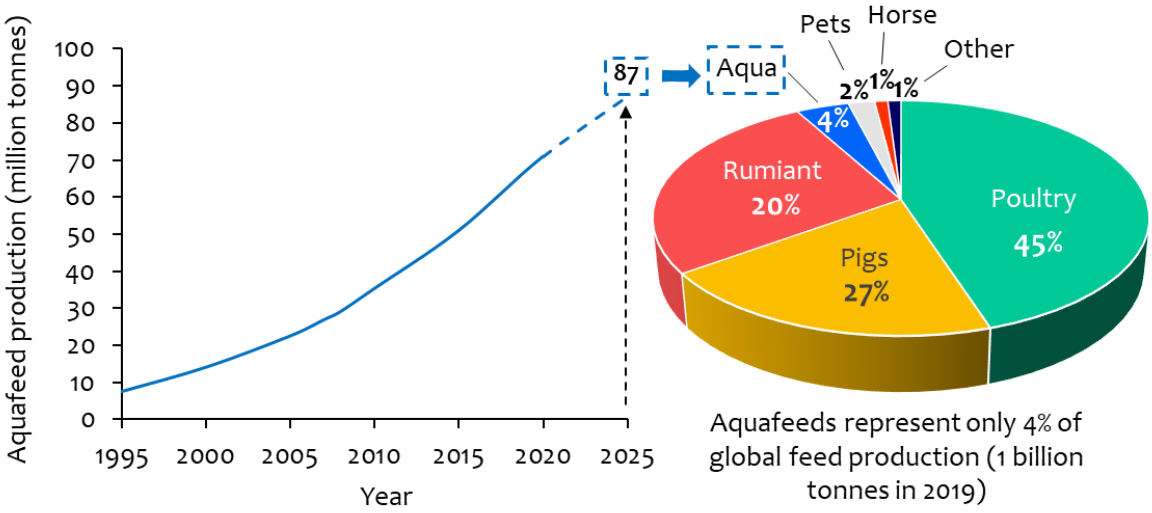

Figure 1. Estimated aquafeed production for 2025.

Indeed, aquaculture industry still heavily relies on fishmeal and fish oil as regular feed ingredients (Yadav *et al.*, 2020). Both feedstuffs are considered the most nutritious, digestible, and palatable ingredients for aquafeed production, as well as the major source of essential amino acids, phospholipids, and omega-3 fatty acids, not least docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids (Yarnold *et al.*, 2019). Both raw materials come by from wild-harvested fish populations, and so changes in the catches of the target fish species can affect their production. According to FAO (2020), the

amount of wild fish catches used for producing fishmeal and fish oil in 2018 (18 million tonnes) was significantly lower than that in 1994 (30 million tonnes). This reduction in the supply of those feedstuffs together with a raising demand driven by a fast-growing aquaculture industry have led fishmeal prices to increase by almost three-fold in the last decade (fishmeal and fish oil current costs are around 1,120-1,730 and 2,000-2,200 USD per tonne, respectively (HAMMERSMITH Marketing Ltd, September 2020). In this scenario, although the inclusion rates of fishmeal and fish oil in aquafeeds have been progressively reduced, the thriving production of all farmed species has generated a growing demand for such ingredients, mainly attributable to fish aquaculture (Naylor *et al*., 2009). Therefore, finding and testing alternative protein and lipid sources with potential for developing sustainable and nutritious aquafeeds, continue to be a challenge for today's aquaculture (Yarnold *et al*., 2019), and this is the ultimate reason why considerable research effort is being made on this topic.

In this regard, any satisfactory alternative feed ingredient must supply a nutritional value comparable to that of regular ingredients, and should also be palatable, available on a large scale, as well as economically viable (Vizcaíno *et al*., 2014). Conventional land-based crops, especially grains and pulses and their derivatives, are feasible alternatives owing to their low cost, and to the fact that they have proved successful in the elaboration of aquafeeds when used for partial replacement of fishmeal and fish oil. Thus, plant-based ingredients offer positive effects on fish growth, although there are some disadvantages that hinder their inclusion in aquafeeds, especially at high inclusion level. From a nutritional point of view, terrestrial plant feedstuffs are characterized by low digestibility and nutrient bioavailability, and low palatability (Daniel, 2018). In addition, these ingredients usually have imbalanced nutrient profiles, with deficiencies in some essential amino acids, such as lysine, methionine, threonine, and tryptophan, and they can contain anti-nutritive factors that may affect negatively both physiology and growth performance of farmed fish. Other alternatives such as plant oils (e.g. rapeseed oil, cottonseed oil, soybean oil, sunflower seed oil) are rich in n-6 fatty acids but poor in n-3 long chainm polyunsaturated fatty acids (PUFA), in such a way that their excessive use may

alter the fatty acid profile of fish fillets (Shah *et al.*, 2018). Moreover, intestinal inflammatory phenomena have been described in juvenile salmon fed on 20% soybean meal even after short periods (Booman *et al.*, 2018). In this case, even if fish growth wasn't affected, enteritis alters the integrity of the intestinal mucosa and increases the risk of disease.

The above mentioned are the reasons why numerous studies have been carried out to find other alternative ingredients without these limitations. In this point, it is very important to consider that replacing fishmeal is not just substituting the protein of fishmeal, given that fishmeal contains many other important nutrients (nucleotides, n-3 fatty acids, minerals, bioactive compounds, etc.). It should be burn in mind that fish have requirements for nutrients and not for ingredients. Likewise, fish oil is also more than a source of lipids; it is a source of n-3 fatty acids, but also of cholesterol, vitamins, carotenoids, and other factors. For those reasons, finding alternatives to fishmeal and fish oil is more than finding cheaper sources of protein or lipid, it is also replacing all the rest of essential nutrients that fish require, including essential amino acids, nucleotides, fatty acids, minerals, vitamins and pigments.

In this regard, algae are interesting alternative ingredients for aquafeeds (Figure 2). The chemical composition of some algal species has drawn the attention of researchers as an important resource, not only as dietary protein/lipid source, but also as potential additives for providing bioactive and functional compounds to aquafeeds (Shah *et al.*, 2018; Vizcaíno *et al.*, 2019a).

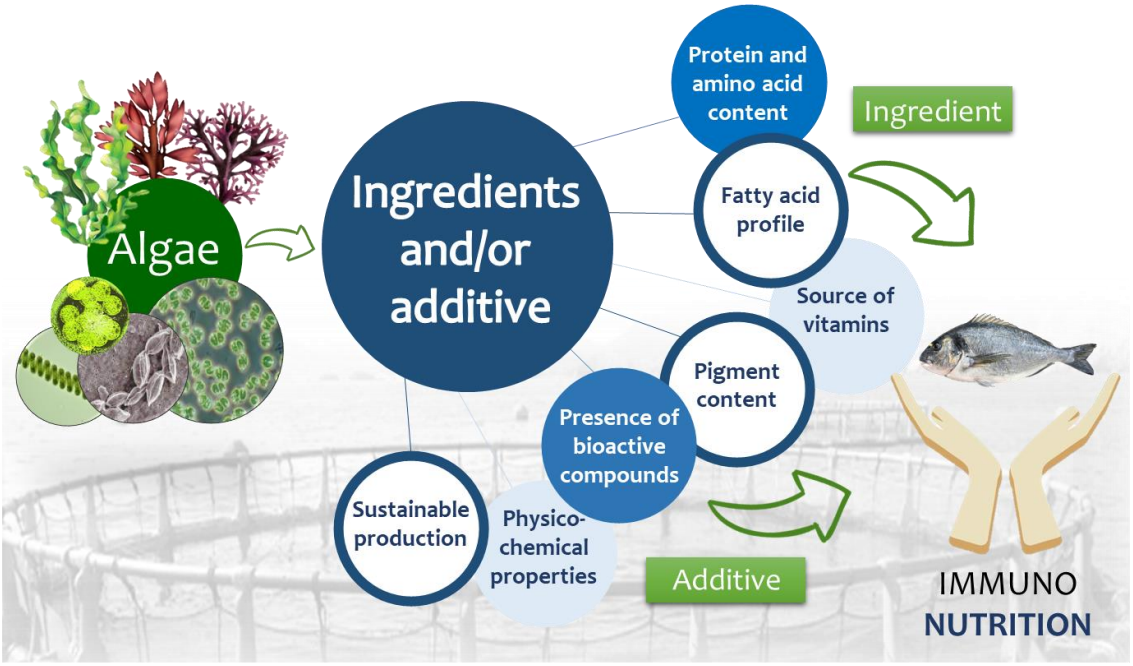

Figure 2. Interest of algae as dietary ingredients/additives for aquafeed manufacturing.

I.2. RELEVANCE OF ALGAE IN AQUAFEEDS

Since the 1980s, algae play a key role in aquaculture nutrition, either for direct or indirect consumption (Brown *et al*., 1997). However, it has been in the last 25 years when the interest in this resource as potential alternative to fishmeal and fish oil has been progressively increased owing to their nutritional value, rapid growth and its antioxidant value (Roy & Pal, 2015; Vizcaíno *et al*., 2019b).

From a nutritional point of view, microalgae can be used as a natural source of protein, lipids, vitamins, carotenoids, and energy (Shah *et al*., 2018), whereas macroalgae are more appreciated as sources of bioactive compounds, such as pigments, polysaccharides, polyphenols, and vitamins rather than as protein or lipid sources (Moutinho *et al*., 2018). However, the nutritional value of a given algae strain depends of several factors, such as cell size and shape, digestibility, presence of anti-nutritive factors, production of toxic substances, and the specific nutritional requirements of the target animal species of microalgae-supplemented diets (Brown *et al*., 1997).

The chemical composition of algae has been extensively documented in previous studies (Table 1). Overall, the protein content of microalgae ranged from 30 to 55% (on dry matter basis, DM) (López *et al*., 2010), though there are some genera, such as *Anabaena* sp., *Chlorella* sp., or *Arthrospira* sp. (Cyanobacteria) with higher values (Venkataraman & Becker, 1985). In general, microalgae protein shows a balanced amino acid profile, similar to that observed in other regular ingredients commonly used in aquafeeds (Becker, 2007). Microalgae have a lipid content ranging from 2 to 50% (DM), although some genera exceed 80%. Usual values are in the range of 20-50% (Chisti, 2007). The carbohydrate content varies from 5 to 35% and plays an important role in microalgae digestibility (Percival & Turvey, 1974). Regarding macroalgae, the protein content in brown seaweeds ranges from 3 to 15%, and from 14 to 47% (DM) in the case of green and red seaweeds (Arasaki & Arasaki, 1983). The total lipid content is relatively low (0.2-4% DM), while the total amount of carbohydrates ranges from 1.8 to 66% (DM) including simple sugars, soluble carbohydrates, pectin, alginic acid, carrageenan and agar, among others (Wan *et al*., 2019).

Table 1. Proximate composition (% dry weight, DW) of several algae species.

Marine microalgae	**Protein**	**Lipid**	**CHO**	**Source**
Anabaena sp.	60.9	14.1	-	Vizcaíno *et al*. (unpublished)
Dunaliella sp.	52.3	20.3	-	Vizcaíno *et al*. (unpublished)
Nannochloropsis gaditana	44.9	27.0	-	Vizcaíno *et al*. (unpublished)
N. gaditana	33.2	27.9	15.9	Di Lena *et al*. (2020)
Pavlova sp.	24–29	9–14	6–9	Becker (1994)
Porphyridium sp.	20.1	4.8	-	Vizcaíno *et al*. (unpublished)
Schizochytrium sp.	12.5	40.2	38.9	Shields and Lupatsch (2012)
Tetraselmis chuii	46.5	12.3	25	Tibbetts et al. (2015)
Tetraselmis sp.	27.2	14.0	45.4	Shields and Lupatsch (2012)
Tetraselmis suecica	26.0	14.7	24.1	Di Lena *et al*. (2020)
T. suecica	36.0	12.9	-	Vizcaíno *et al*. (unpublished)
Tysochrysis lutea	43.6	17.8	-	Vizcaíno *et al*. (unpublished)
Freshwater microalgae				

Aphanizomenon flos-aquae	62.0	3.0	23.0	Becker (2007)
Arthrospira maxima	60-71	6-7	13-16	Becker (2007)
Arthrospira platensis	50-65	4-9	8-14	Becker (2007)
A. platensis	46.8	1.4	3.3	Molino *et al.* (2018)
A. platensis	36.8	7.2	-	Vizcaíno *et al.* (unpublished)
Chlorella ovalis				Slocombe *et al.* (2013)
Chlorella pyrenoidosa	57.0	2.0	26.0	Becker (2007)
Chlorella sp.	43.2	6.5	-	Vizcaíno *et al.* (unpublished)
Scenedesmus almeriensis	42.8	9.6	-	Vizcaíno *et al.* (unpublished)
S. almeriensis	49.4	12.0	24.6	Sánchez *et al.* (2008)
Scenedesmus obliquus	50–56	12–14	10–52	Becker (2007)
Spirogyra sp.	25.3	9.3		Vizcaíno *et al.* (unpublished)
Macroalgae				
Gracilaria cornea	13.5	0.8	39.8	Vizcaíno *et al.* (2016a)
Gracilaria lameneiformis	19.2	0.5	61.3	Xu *et al.* (2011)
Laminaria digitata	15.9	0.5	-	Marsham *et al.* (2007)
Macrocystis pyrifera	5.3-6.1	0.7	-	Cruz-Suárez *et al.* (2009)
Ulva fasciata	8.8-12.3	3.6-5.1	-	McDermid and Stuercke (2003)
Ulva ohnoi	19.2	3.2	29.9	Vizcaíno *et al.* (2019a)
Ulva rigida	14.9	1.2	50.4	Vizcaíno *et al.* (2016a)
Reference ingredient				
Fishmeal	65.0	12.7	-	Vizcaíno *et al.* (unpublished)
Soybean meal	50.1	2.7	-	Vizcaíno *et al.* (unpublished)

CHO: carbohydrates

I.2.1. Algal protein

Protein content is the main factor that determines the value of a given alga strain for feeding purposes in aquaculture nutrition (Spolaore *et al.*, 2006). Microalgal protein from different species shows similar amino acid profiles, which are characterized by a high content in essential amino acids, as exemplified by the comprehensive study of 40 species of microalgae carried out by Brown *et al.* (1997). This study found that all species showed similar amino acid composition, comparable to that of other regular ingredients. For instance, *Arthrospira* and *Chlorella* biomasses have a protein content above

50%, whose quality is comparable to those of yeast and soybean meal (Kovač *et al*., 2013).

Overall, leucine and arginine are the predominant amino acids in microalgal protein. The content of both amino acids ranges from 5 to 9 g per 100 g protein, while histidine and methionine are typically the most limiting amino acids, with a content around 1.5 to 2 g per 100 g protein. However, microalgae strains like *Dunaliella*, *Scenedesmus* or *Arthrospira* show an amino acid content similar to that of fishmeal (Table 2). Methionine is usually the most limiting amino acid in the ingredients used for aquafeed manufacture, especially when terrestrial plant protein sources are used to replace regular ingredients like fishmeal (Mai *et al*., 2006a; b; Tibbetts *et al*., 2015). Therefore, and based on the dietary amino acid requirements of aquaculture fish (Wilson, 2003), algal protein could be able to provide most of the essential amino acids needed for an adequate growth of the animals.

Table 2. Amino acid profile (g 100 g^{-1} protein) of several algae species.

Marine microalgae	**Arg**	**His**	**Ile**	**Leu**	**Lys**	**Met**	**Phe**	**Thr**	**Val**	**S**
Dunaliella sp.	6.6	2.5	4.5	9.3	6.2	2.5	6.0	5.0	6.0	(2)
Nannochloropsis gaditana	5.2	1.6	3.9	7.3	4.3	1.2	4.7	4.6	4.9	(1)
Tetraselmis suecica	5.5	1.6	4.5	6.8	4.1	1.5	4.8	4.1	4.7	(1)
Tisochrysis lutea	6.6	1.8	4.1	6.9	3.8	1.3	5.9	4.6	5.0	(1)
Freshwater microalgae										
Arthrospira platensis	5.1	1.3	3.6	7.0	4.6	2.1	3.9	4.0	4.3	(1)
Chlorella sp.	2.3	0.4	1.8	5.0	2.7	0.6	3.3	2.4	3.0	(1)
Scenedesmus almeriensis	5.8	1.7	4.3	6.9	4.8	1.3	4.5	4.1	5.0	(1)
Scenedesmus sp.	6.4	2.6	4.4	9.2	6.6	2.4	5.6	5.6	6.2	(2)
Macroalgae										
Ulva lactuca	3.6	1.8	3.7	6.7	4.2	1.6	4.0	4.7	6.2	(4)
Ulva rigida	4.6	1.4	3.1	5.2	3.7	1.5	3.3	5.0	5.6	(4)
Reference ingredient										
Fishmeal	5.7	2.4	4.7	7.7	7.9	3.0	4.1	4.7	5.4	(5)
Soybean meal	7.3	2.7	4.5	7.7	6.4	1.4	5.0	3.9	4.8	(5)

S: source.(1) Vizcaíno *et al*. (unpublished); (2) Kent *et al*. (2015); (3) Kristaki *et al*. (2011); (4) Shuuluka *et al*. (2013); (5) Cho & Kind (2010).

Similarly, macroalgae protein can be considered relatively well balanced in terms of essential amino acids (Wan *et al*., 2019). Generally speaking, many species contain most of the essential and nonessential amino acids (Gressler *et al*., 2010). Although some commercially important species, like the red seaweed *Palmaria palmate*, lack some amino acids (e.g., cysteine), they are rich in aspartic acid and glycine, with a content of total essential amino acids comparable to soybean protein (Galland-Irmouli *et al*., 1999).

Most published values on the protein content of algae are based on estimations of crude protein, which quantifies other nitrogenous constituents of algae, such as glucosamides, amines, nucleic acids, and cell wall components, in addition to protein. This leads to overestimate of the true protein content (Becker, 2007). For instance, non-protein nitrogen can reach 11.5% in *Arthrospira*. Even with this overestimation, the nutritional value of algae is high, with average quality being similar to, and even higher than, conventional plant protein sources.

I.2.2. Algal lipid and fatty acid profile

The fatty acid content is another factor that determines the nutritional value of algae (Shah *et al*., 2018). There are numerous scientific publications reporting polyunsaturated fatty acid (PUFA) content of algae, especially microalgae, species used in aquaculture (Dunstan *et al*., 1992; Volkman *et al*., 1989).

In general, many of the microalgae and seaweed species possess a high proportion of PUFA, especially n-3 fatty acids such as docosahexaenoic acid (22:6n-3; DHA), α-linolenic acid (18:3n-3; ALA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; AA) (Brown *et al*., 1997; Wan *et al*., 2019) (Table 3). As shown in Table 1, macroalgae present lower lipid contents compared to those observed in some microalgae species, however, seaweeds may improve the fatty acid profile of feeds (Wan *et al*., 2019; Sáez *et al*., 2020).

Table 3. Polyunsaturated fatty acid content (% of total fatty acids) in several algae species.

Marine microalgae	**18:2n-6**	**18:3n-3**	**20:4n-6**	**20:5n-3**	**22:6n-3**	**Source**
Dunaliella sp.	6.3	15.6		0.2	0.2	(1)
Nannochloropsis sp.	3.5		4.6	30.1		(2)
Schizochytrium sp.	1-2	> 1	1	1-16	18-44	(3)
Tetraselmis sp.	9.7	16.2	1.0	4.7		(4)
Tetraselmis sp.	4-7	5-22	< 1-4	2-8	< 1	(3)
Tetraselmis suecica	6.9	14.9	2	6.2		(2)
Tisochrysis lutea	7.8	16.3	0.6	0.9	12.0	(2)
Freshwater microalgae						
Scenedesmus sp.	4.7	20.8				(4)
Arthrospira sp.	7.7			5.5		(5)
Chlorella sp.	17.5	20.0				(4)
Scenedesmus almeriensis	6.3	27.9				(6)
Scenedesmus sp.	1-6	> 1-3				(3)
Macroalgae						
Ulva lactuca	9.5	0.1	1.8	1.6	0.2	(7)
Ulva ohnoi	2.6	9.7		0.3		(8)
Ulva rigida	14.3	5.2	0.5	0.4	0.5	(9)

(1) Mourente *et al.* (1990); (2) Servel *et al.* (1994); (3) Tibbetts *et al.* (2018); (4) Pratoomyot *et al.* (2005); (5) Sahu *et al.* (2013); (6) Vizcaíno *et al.* (2019b); (7) Cardoso *et al.* (2017); (8) Sáez *et al.* (2020); (9) Ivanova *et al.* (2013).

In general, long-chain n-3 PUFAs are mostly present in marine microalgae strains. Fatty acids like ARA, EPA and DHA can be directly produced by several microalgae species like *Porphyridium*, *Nannochloropsis* and *Schizochytrium* sp. Indeed, the last strain can be used as source of PUFA owing to the high DHA content (up to 49% total lipids) (Ren *et al.*, 2010). On the other hand, green microalgae (Chlorophyta) are deficient in long chain PUFA, but contain other fatty acids like linoleic and linolenic acids that are essential for many freshwater fish species. Therefore, the fatty acid content makes algae (especially microalgae) a valuable novel ingredient for replacing fish oil, given that they can mimic the average fatty acid profile found in forage fish.

However, the use of a single strain does not allow to create a "*fish-free*" fish oil alternative. At least two marine microalgae strains should be blended for achieving that purpose.

I.2.3. Algal carbohydrates

The polysaccharide composition of microalgae varies notably among species (Brown, 2002). In general, glucose is the predominant sugar in some microalgae commonly evaluated for aquaculture purposes, like *Tisochrysis* sp. and *Chlorella* sp. (28-86% total carbohydrates). Also, mannose, fucose, galactose and xylose, among others, are present in different proportions. Most of these sugars are components of the microalgae cell wall, and play a key role as a protective barrier that, in turn, reduces the bioavailability of intracellular nutrients. Extracellular polysaccharides may interfere with nutrient absorption, or conversely, be useful binding agents when it comes to forming feed pellets.

On the other hand, carbohydrates make up one of the largest fractions of seaweeds composition reaching values from 2% to 66%. Polysaccharides, such as cellulose, hemicellulose and lignin, are present in seaweeds, which can have different influence in both feed overall quality (as gelling and stabilising agents), and in animals, as a source of non-starch polysaccharides. While certain carbohydrate fractions as ulvan or β-glucan can have functional effects on fish, other complex carbohydrates as non-starch polysaccharides can induce negative effects on nutrient absorption, growth performance and gut morphology. The latter can be of great interest in the case of farmed carnivorous fish, where carbohydrates are poorly digested and metabolised as energy source (Wan *et al*., 2019). The efficiency of fish to digest the cell wall depends on, the carbohydrate composition, and how carbohydrate fractions are linked to each other, and also on the existence of the appropriate digestive enzymes in the different fish species. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases than carnivorous fish.

I.2.4. Algal pigments

Broadly speaking, algae have an adequate content in high-value carotenoids, such as β-carotene and astaxanthin (Figure 3), which are commonly used in aquaculture mainly for their colouring and antioxidant properties, improving the quality and commercial value of farmed fish (Yarnold *et al.*, 2019). β-carotene is one of the most demanded pigments with a wide variety of market applications: i) pro-vitamin A (retinol) in food and animal feed, ii) as food colouring agent, additive to cosmetics and multivitamin preparations, and iii) as a food additive under the antioxidant category. This pigment can be naturally produced by the microalgae genus *Dunaliella* that may synthesize and accumulate up to 16% of its dry weight in the form of cellular β-carotene (Lers *et al.*, 1990). Astaxanthin is another pigment that can be synthetized by *Haematococcus* microalgae, *Chlorella zofingiensis* and *Chlorococcum* sp. (Del Campo *et al.*, 2004). This compound presents such a antioxidant activity that is has been proposed as "super-vitamin E" (Nakagawa *et al.*, 2011). As a natural pigment, astaxanthin is commonly used as a pigmentation source in the aquaculture industry (Canales-Gómez *et al.*, 2010). Out of total carotenoid (astaxanthin, cantaxanthin), the astaxanthin was determined as the most important carotenoid in salmon and rainbow trout (Shah *et al.*, 2016; Tolasa *et al.*, 2005). Astaxanthin cannot be synthesized *the novo* by salmonids, and therefore carotenoid pigments must be supplied in aquafeeds.

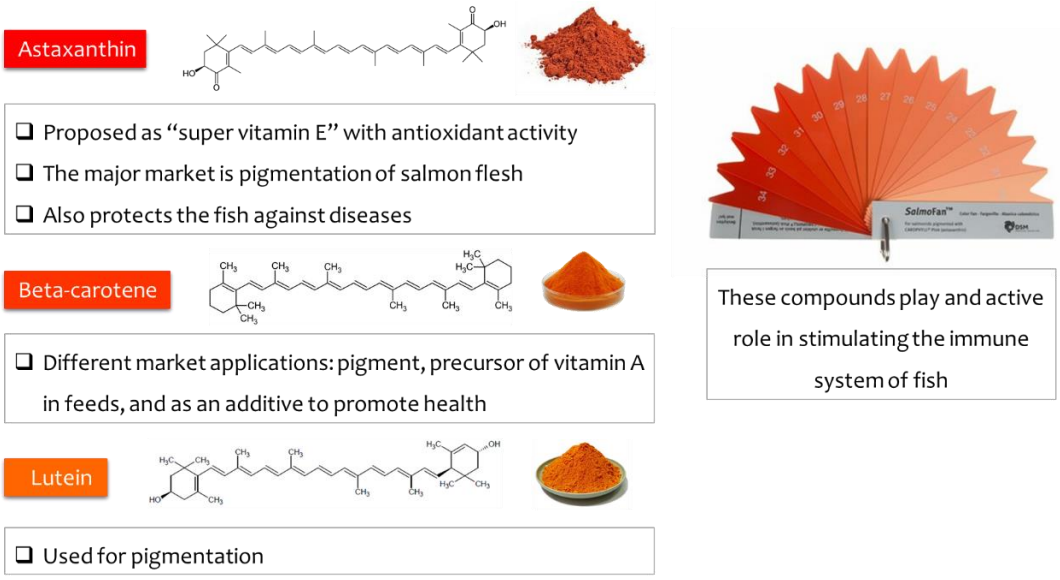

Figure 3. The interest of algae as source of pigments for aquafeeds.

Recently, Sales *et al*. (2020) analyzed the composition of the carotenoid fraction obtained from the microalgae *Nannochloropsis gaditana* (Table 4), and they found that violaxanthin, β-carotene, and neoxanthin were the most abundant carotenoids in extracts, and vaucheroxanthin ester and canthaxanthin, the less abundant. Those authors affirmed that lipid extract from the microalgae *N. gaditana* can be used as dietary additive for feeding juvenile gilthead seabream. The use of extracted compounds instead of the whole biomass can be effective for enhancing the bioavailability of these compounds. In fact, extracted pigments from *Arthrospira platensis*, *Haematococcus pluvialis* and *Chlorella* sp. have been successfully incorporated as supplements for feeding *Lates calcarifer* larvae (Gora *et al*., 2019).

Table 4. Carotenoid content (mg kg^{-1}) of the non-saponifiable lipid extract obtained from *Nannochloropsis gaditana* (Data from Sales *et al*., 2020).

Carotenoid	Non-saponifiable lipid extract
Neoxanthin	754.97 ± 149.19
Violaxanthin	2137.29 ± 254.97
Antheroxanthin	417.51 ± 70.36
Vaucheroxanthin	78.81 ± 10.60
Zeaxanthin	58.32 ± 8.14
Vaucheroxanthin ester	13.47 ± 2.38
Canthaxanthin	14.09 ± 2.24
β-carotene	925.51 ± 145.40
Total	4399.97 ± 643.27

Values are mean ± SD of triplicate determination.

I.3. THE USE OF ALGAE AS INGREDIENT IN AQUAFEEDS

The nutritional profile and the variety of nutraceutical compounds in algae justify their potential use as major ingredients, and, also as additives in aquafeeds. Indeed, there is abundant scientific literature related to the use of algae-supplemented diets for feeding fish. Thus, studies focussed on the use of microalgae as potential sources of protein, lipid, and functional additives for

aquafeeds have been increasing exponentially in the last decade (Tables 5 and 6).

Table 5. Recent studies on applications of microalgae as dietary ingredient or additive in aquafeeds.

Microalgae	Fish species	Use	Effect on fish	S
Arthrospira sp.	*Pelteobagrus fulvidraco*	4% AI	(↔) growth performance, (↑) antioxidant and immune response	(1)
Arthrospira sp.	*Oncorhynchus mykiss*	7.5% FMR	(↔) fish growth, (↑) carotenoid concentrations in skin and fillet	(2)
Arthrospira sp.	*Sparus aurata*	4% AI	(↑) activity digestive enzymes, (↑) intestinal mucosa structure, and (↓) oxidation of muscle lipids	(3)
Chlorella vulgaris	*Paralichthys olivaceus*	15% FMR	(↑) growth performance, (↑) antioxidant enzyme activity and lipid metabolism	(4)
Desmodesmus sp.	*Salmo salar*	10% FMR	(↔) growth rate, condition factor, protein efficiency ratio and body proximate composition	(5)
Isochrysis sp.	*Dicentrarchus labrax*	36% FOR	(↔) feed intake, growth performance (↑) greenish pigmentation of the skin	(6)
Nannochloropsis and *Isochrysis* sp.	*Gadus morhua*	15% FMR	(↑) feed intake and growth	(7)
Nannochloropsis and *Schizochytrium* sp.	*P. olivaceus*	100% FOR	(↔) growth performance and nutrient utilization	(8)
Nannochloropsis sp.	*D. labrax*	15% FMR	(↔) growth performance, proximate composition and intestinal integrity	(9)
Pavlova viridis and *Nannochloropsis* sp.	*D. labrax*	100% FOR	(↔) growth performance and nutrient utilization	(10)
Phaeodactylum tricornutum	*S. salar*	6% FMR	(↔) growth, nutrient digestibility and feed utilization	(11)
Scenedesmus almeriensis	*S. aurata*	38% FMR	(↔) growth and nutrient utilization. (↑) intestinal enzyme activities and absorptive surface	(12)

Scenedesmus sp.	*S. salar*	10% FMR	(↔) feed intake, growth, chemical composition. (↑) n-3 PUFA content	(13)
Schizochytrium sp.	*S. salar*	5% FMR	(↔) health and fillet nutritional quality	(14)
Schizochytrium sp.	*Oreochromis niloticus*	100% FOR	(↑) growth performance, nutrient utilization, and n-3 FUFA in fillet	(15)
Spirulina maxima	*O. niloticus*	30% FMR	(↔) growth	(16)
Spirulina sp.	*Puntius gelius*	20% FMR	(↑) growth and feed conversion ratio	(17)
T. galbana, S. almeriensis and *N. gaditana*	*Solea senegalensis*	15% FMR	(↔) growth performance and nutrient utilization. (↑)intestinal absorptive capacity	(18)
Tetraselmis suecica	*D. labrax*	20% FMR	(↔) growth performance	(19)

Use: FMR: fishmeal replacement: **FOR**: fish oil replacement; **AI**: algae inclusion.
Effect: (↓) reduced; **(↑)** increased/improved; **(↔)** no effect.
S: (1) Liu *et al*. (2020); (2) Teimuri *et al*. (2013); (3) Galafat *et al*. (2020); (4) Rahimnejad *et al*. (2017); (5) Kiron *et al*. (2016); (6) Tibaldi *et al*. (2015); (7) Walker & Berlinsk (2011); (8) Qiao *et al*. (2014); (9) Valente *et al*. (2019); (10) Haas *et al*. (2016); (11) Sørensen *et al*. (2016); (12) Vizcaíno *et al*. (2014); (13) Gong *et al*. (2019); (14) Kousoulaki *et al*. (2015); (15) Sarker *et al*. (2016); (16) Rincón *et al*. (2012); (17) Hajiahmadian *et al*. (2012); (18) Vizcaíno *et al*. (2018); (19) Tulli *et al*. (2012).

Table 6. Recent studies on applications of seaweeds as dietary ingredient/additive in aquafeeds.

Seaweed	Fish	Use	Effects on fish	S
Palmaria palmata	*Salmo salar*	15% AI	(↔) growth performance and feed utilization	(1)
Porphyra dioica and *Ulva* sp.	*Oreochromis niloticus*	10% AI	(↔) growth performance or body composition	(2)
Sargassum horneri	*Scophthalmus maximus*	10% AI	(↔) growth performance. (↑) non-specific immune response and resistance to pathogenic bacteria	(3)
Ulva lactuca	*Solea senegalensis*	10% AI	(↔) growth performance and feed utilization	(4)
U. ohnoi	*S. senegalensis*	5% AI	(↓) growth and pancreatic protease activity. (↑) absorptive surface of the intestinal mucosa	(5)

U. rigida and *Gracilaria cornea*	*Sparus aurata*	15% AI	**(↔)** growth and fish chemical composition. **(↔)** intestinal structure	(6)

Use: AI: algae inclusion. **Effect: (↓)** reduced; **(↑)** increased/improved; **(↔)** no effect. **S:** (1) Wan *et al*. (2016); (2) Silva *et al*. (2015); (3) Wang *et al*. (2019); (4) Moutinho *et al* (2018); (5) Vizcaíno *et al* (2019a); (6) Vizcaíno *et al*. (2016a).

According to previous studies, low level of fishmeal replacement (around 0.5 - 10%) has positive effects of fish. For instance, the inclusion up to 7.5% *Arthrospira* sp. in diets for rainbow trout (*Oncorhynchus mykiss*) did not negatively affect growth and nutrient utilization. Weight gain and carotenoid concentration in skin and fillets both increased in fish fed on 7.5% microalgae-supplemented diet (Teimouri *et al*., 2013). Similarly, *Scenedesmus* sp. and *Desmodesmus* sp. at 5 or 10% replacement of fishmeal in practical diets for Atlantic salmon (*Salmo salar*) have been successfully evaluated without causing negative effects on growth, nutrient utilization, and body proximate composition of fish (Kiron *et al*., 2016), and even an increase in total n-3 and PUFA content in fish was reported (Gong *et al*., 2019). The inclusion up to 15% of *Nannochloropsis gaditana*, *Tisochrysis lutea* and *Scenedesmus almeriensis* were successfully used in diets for Senegalese sole (*Solea senegalensis*) (Vizcaíno *et al*., 2019b). Vizcaíno *et al.* (2014) also pointed out that the dietary inclusion up to 38% *S. almeriensis* in diets for gilthead seabream (*S. aurata*) juveniles caused positive effect on gut functionality.

On the other hand, microalgae could be a key ingredient for designing a fish oil replacement that contains essential fatty acids, such as EPA and ARA. Encouraging results were recently obtained in marine fish species by Tibaldi *et al*. (2015). These authors used dried *T. lutea* biomass to replace up to 36% fish lipid in a diet with low level of fish oil, and they did not find adverse effects on growth performance in European seabass (*Dicentrarchus labrax*). Similarly, the heterotrophic microalgae *Schizochytrium* sp. has been successfully used on different fish species. The high DHA content of this microalga has allowed the total replacement of fish oil in diets for Nile tilapia (*Oreochromis niloticus*) (Sarker *et al*., 2016). The results obtained revealed positive effects on growth

and nutrient utilization, as well as an increase on n-3 long chain PUFA accretion in fillets.

Seaweeds have also been evaluated in recent years as a novel and sustainable resource for aquafeed manufacturing (Vizcaíno *et al*., 2016a, 2019a). In spite of the fact that their protein content is low compared to other ingredients used in aquafeeds, seaweeds are also rich in biologically active compounds, such as polysaccharides, pigments, polyphenols, and vitamins, which might exert certain beneficial effects on farmed fish (Wan *et al*., 2019). Overall, there are numerous studies pointing to promising results in terms of growth, survival, and nutrient utilization in different farmed fish fed on seaweeds up to 10 - 15% inclusion level. Some species of the genus *Ulva* have been successfully evaluated as a dietary ingredient for gilthead seabream (Vizcaíno *et al*., 2016a), Senegalese sole (Moutinho *et al*., 2018) or Nile tilapia (Silva *et al*., 2015) without negative effects on growth and nutrient utilization. Similarly, the inclusion up to 10% of *Sargassum horneri* had no adverse effects on growth performance of juvenile turbot (*Scophthalmus maximus*). The inclusion of *S. horneri* also enhanced the activity of non-specific immune enzymes and the resistance against pathogenic bacteria (Wang *et al*., 2019).

In summary, several beneficial effects of algae have been reported in numerous studies, such as: i) improved growth, feed utilisation, and survival rate, ii) increased lipid metabolism, iii) antioxidant properties, iv) enhanced body composition and flesh quality, v) enriched skin and flesh pigmentation, vi) improved integrity of intestinal mucosa, vii) enhanced activity of digestive enzymes, viii) reinforced immune system, and, ix) invigorated resistance to stress, and against pathogens.

Given the nutritional composition and the reported effect on fish, it seems that algae are interesting alternative ingredients/additives for aquafeeds. Herbivorous and omnivorous species tolerate well high inclusion levels of algae compared to carnivorous fish species. However, the results reported suggest that the optimum dietary algae inclusion level should vary depending on the algae and on the farmed fish species considered. In this regard, the effect of algae seems to be dose-dependent, and species-specific, and consequently, it is difficult to establish a general rule about the use of algae in

aquafeeds. Therefore, specific research should be carried out on each specific case, and more research is needed to evaluate the potential of algae as protein sources, and pinpointing factors affecting their effectiveness should be undertaken.

I.4. CURRENT CHALLENGES IN THE USE OF MICROALGAE IN AQUAFEEDS

I.4.1. Safety and regulatory aspects of algae in aquafeeds

The use of algae in foods and feeds is increasingly relevant as the components of microalgae have the potential to be competitive with the same components of other origins. For instance, microalgae used commonly for the production of food supplements, such us species of the genus *Isochrysis*, *Chaetoceros*, *Tetraselmis*, *Pavlova*, *Skeletonema*, *Dunalliela*, *Nannochloropsis*, *Phaedactylum*, *Chlorella*, do not produce toxins. But, it should be considered that, even within a given species, differences exist between toxic and non-toxic strains. For this reason, it is very important to know their safety aspects at species level. The competitiveness of algae-based products is based not only on technical and economical aspects, but also on the regulations ruling their use (Enzing *et al*., 2014). The safety aspects of algae used in aquafeeds have been analysed by the European Food Safety Authority (EFSA). The placing in the European Union market of algae or its components for food and feed purposes is regulated by three legal dispositions: i) EC regulation 178/2002, on food safety; ii) EC regulation 258/97, on novel foods and novel food ingredients; and iii) EC regulation 1924/2006, on nutrition and health claims for foods. Given that several compounds derived from algae are used as feed additives, the Regulation (EC) 1333/2008 also applies. In the past, prior to placing in the market, food and feed business operators were obliged to apply for authorization for the use of proteins derived from algae in feed under Directive 82/471/EEC. Assessment of safety and nutritional value should be done according to the guidelines in Directive 83/228/EEC using the "*Guidance for the assessment of biomasses for use in animal nutrition*" published by the European Food Safety Authority (EFSA, 2011).

Algae belong to the group "*algae and prokaryotes organism*" in the Annex I of Regulation EC 752/2014. Algae or their products used as feed should fulfil all the legal maximum levels mentioned in Directive 2002/32/EC. The regulation EU 68/2013 established the "*Catalogue of Feed Materials*" and includes algae within the group 7 of the list of feed materials, specifically in "*Other plant, algae, and products derived thereof*" (Table 7). Within the European Union, the EFSA requires the safety assessment of any new compound intended for use in food and feed before it is authorized to be placed on the market.

Table 7. Information in the group "*Other plants, algae and products derived thereof*" (source: Commision Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials).

Number	Name [1]	Description	Compulsory declarations
7.1.1	**Algae**	Algae, live or processed, including fresh, chilled or frozen algae. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.2	**Dried algae**	Product obtained by drying algae. This product may have been washed to reduce the iodine content. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.3	**Algae meal**	Product of algae oil manufacture, obtained by extraction of algae. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.4	**Algal oil**	Product of the oil manufacture from algae obtained by extraction. May contain up to 0.1% of antifoaming agents	Crude fat Moisture if > 1%
7.1.5	**Algae extract**	Watery or alcoholic extract of algae that principally contains carbohydrates. May contain up to 0.1% of antifoaming agents	

7.2.6	**Seaweed meal**	Product obtained by drying and crushing macro-algae, in particular brown seaweed. This product may have been washed to reduce the iodine content. May contain up to 0.1% of antifoaming agents	Crude ash

(1) The name shall be supplemented by the species.

I.4.2. Price

The use of algae proteins instead of fishmeal in aquafeeds would allow for a decoupling of aquaculture production from wild fisheries. Until now, fishmeal and fish oil are substantially cheaper than microalgae, which prevents microalgae to enter the aquafeed market. The lack of alternatives to microalgae for feeding fish larvae and juveniles assures a market for microalgae in fish hatcheries. The use of algae as feed ingredient in aquafeeds looks promising, as microalgae have a favourable protein and lipid composition. They can be used in feeds at inclusion levels of about 10%, and have additionally positive effects on the health of animals. The replacement of soybean meal in compound feed production represents a potentially large market for algae (Pereira *et al*., 2020). The outlook for microalgae to be used as feed additives is promising, due to the many positive effects on animal health described. Furthermore, the aim in certain countries to reduce the use of antibiotics is a driver to the use of other health-improving feed additives, being algal and their derivatives potential candidates for this purpose.

A critical factor that will determine the commercial viability of algae is their competitiveness compared to other raw sources currently on the market. For example, a major application for the production of microalgae is lipid extraction for obtaining biofuel. However, although algal biomass is a greener, environmentally friendly alternative, biofuel it is not yet competitive compared to fossil fuels (Cuellar-Bermudez *et al*., 2015). The same situation applies in the case of algae for commercial aquafeeds. The competitiveness of microalgae could be further increased by taking a holistic view, maximising the extraction of all available high-value components by cascading biorefinery. Similarly,

seaweeds are ideally suited for cascading biorefinery, because they contain many high-value components, together with bulky low-value components that are considered raw materials for the bio-based industry, as with an economic value (Cian *et al.*, 2012). Currently, the relatively high cost of microalgae compared to regular ingredients (Figure 4) limits their use in high-value fish production (Yarnold *et al.*, 2019). It has been estimated that algal meal prices of 0.66 and 2.65 € per kg would be needed to replace fishmeal and soybean meal, respectively, in diets for tilapia (a relatively low-value fish). The study of Vizcaíno *et al.* (2014) pointed out that culture technologies should reduce substantially the cost of microalgae production, and even considering a cost of 5.5 € per kg (Norsker *et al.*, 2011), the large scale utilization of microalgae in aquafeeds remains a constrain for the aquaculture industry. Given that algae are expensive to produce, especially microalgae, their use as bulk ingredients for aquafeed formulae is likely to require improved production efficiency and further cost reduction by using biorefinery approaches. However, their main advantage from an economical standpoint their richness in fatty acids, pigments, vitamins, minerals, and bioactive compounds, which make them excellent high-value additives and supplements to blend into a wide range of aquafeeds, even when using at low dietary inclusion level.

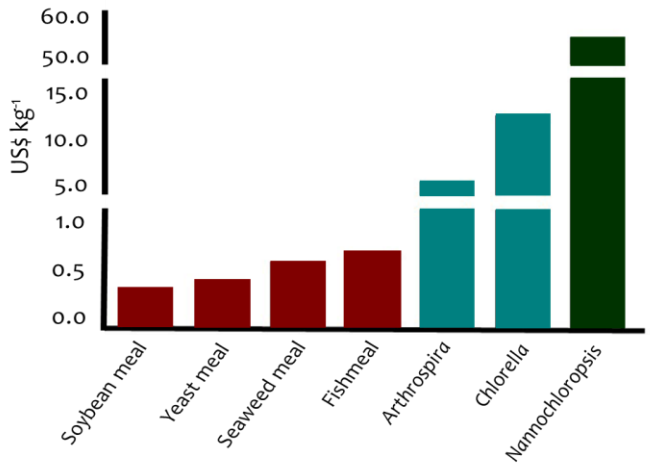

Figure 4. Price comparison of fishmeal alternatives as formulated-feed ingredients. Image modified from Yarnold *et al.* (2019).

An example of cascading biorefinery is the SABANA project (grant # 727874 from the European Union's Horizon 2020 Research and Innovation program), which aims at developing a large-scale integrated microalgae-based

biorefinery for the production of valuable products for the aquaculture (Figure 5). In this project, natural seawater and sunlight are used to growth microalgae. Instead of chemical fertilizers, wastewaters are used as nutrient source for producing large amounts of biomass that are being processed for obtaining valuable products for aquafeeds. The project is divided in two major tasks; i) one is related to the improvement of technology for large scale biomass production (including biological, engineering and sustainability aspects), and ii) the second one focuses on the development of methods for integral utilization of the biomass (including harvesting, processing, and evaluation of pre-commercial products). The objective of this project is to achieve a zero-waste process in a demonstration facility of 5 hectares located in the University of Almería, with capacity to produce 300 tonnes of algal biomass per year with an economic cost of around 1 € per kg dry weight.

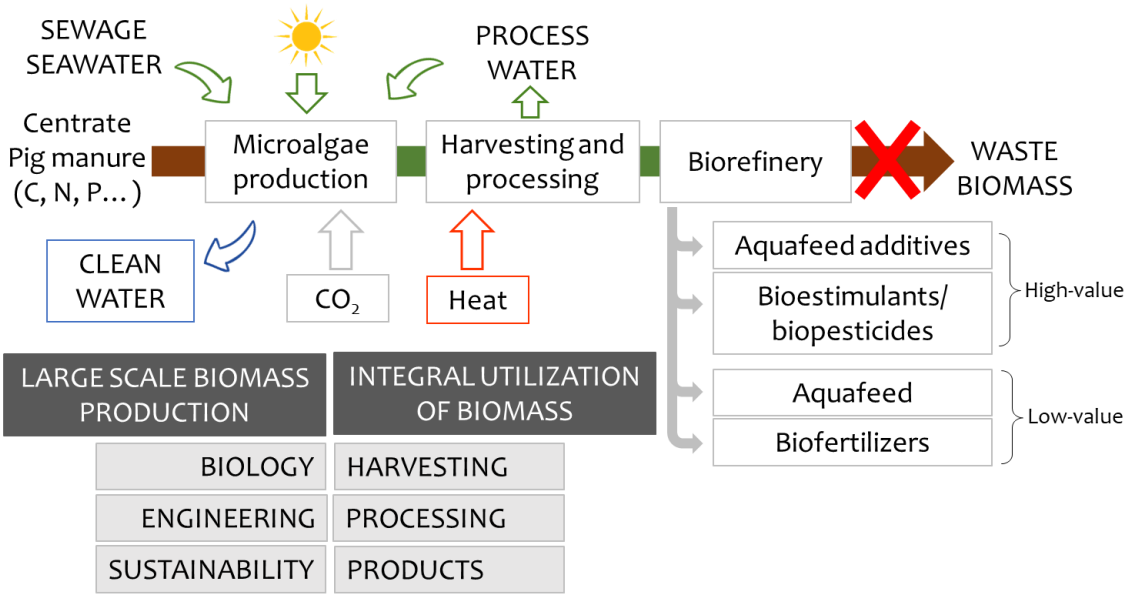

Figure 5. Detail of the block diagram for producing and processing the microalgal biomass in the Project SABANA.

I.4.3. Variability in nutrient composition

An additional challenge, particularly relevant in algae production, is the variability in their chemical composition, which is highly dependent on the species strain, the growth medium, the harvesting period, and the production method, among other factors. For instance, the protein content can vary by season, temperature, and location in which the algae are cultured and/or

harvested (Joubert & Fleurence, 2008). The relative composition of specific proteins can also differ, changing the concentrations of amino acids consequently. For example, annual monitoring of *Palmaria palmata* harvested on the Atlantic coast showed that protein levels were highest in winter and spring months, varying from 9 to 25%, and peaking in May (Galland-Irmouli *et al*., 1999). In microalgae, Adams *et al*. (2013) described that short-term effect of nitrogen limitation generally includes an increase in lipid and carbohydrate contents, and a decrease in the growth rate and the content of crude protein, although the extent of this response varies markedly between species. In the light of the data, the development of protocols for optimizing the biochemical composition of algae should expect to play an important role in the future production processes.

I.4.4. Presence of anti-nutritional factors

The presence of anti-nutritive factors is one of the most important issues derived from using novel dietary ingredients in aquafeeds. These compounds can exert negative effects on the absorption of nutrients and micronutrients, hampering the normal functioning of certain organs, so they are one of the main drawbacks limiting their practical use in formulated feeds (Vizcaíno *et al*., 2020).

Anti-nutritive factors comprise a wide variety of compounds, such as protease inhibitors, phytohemagglutinin, lectins, phytic acid, saponins, phytoestrogens or antivitamins (Prabhu *et al*., 2017). In general, these substances have been related to plant-derived feedstuffs, although recent studies have also documented their presence in some algae species (Oliveira *et al*., 2009; Mæhre, 2015; Vizcaíno *et al*., 2020). Overall, the ability of microalgae to inhibit fish digestive proteases seems to be low, with inhibition values lower than 20% against control assays (Vizcaíno *et al*., unpublished data). However, other studies pointed to the existence of protease inhibitors in some macroalgae species such as *Ulva rigida*, *Ulva ohnoi*, *Gracilaria cornea* and *Sargassum sp*., which may exert not only reduced proteolysis within digestive tract, but also increased pancreatic secretion as an attempt to

overcome their anti-nutritional effects (Sáez *et al*., 2013; Diken *et al*., 2016; Vizcaíno *et al*., 2019a; 2020).

Sáez *et al.* (2013) evaluated the effect of the inclusion of *Gracilaria cornea* and *Ulva rigida* as dietary ingredients on the intestinal proteolytic activity of juvenile gilthead seabream. The results obtained evidenced that digestive proteases were affected by algae-supplemented diets, as fish displayed different alkaline protease enzyme activity levels after a 70-day feeding trial. In particular, the proteolytic activities in fish fed *Ulva* supplemented-diets were significantly lower than those of fish fed on control diet. The presence of protease inhibitors in macroalgae may contribute to the progressive decrease in the proteolytic activity in fish fed diet with increasing levels of *Ulva*. However, the decline in the level of alkaline protease activity was not accompanied by a decrease in fish growth and feed utilization, since all fish grew similarly. The existence of a compensation mechanism against dietary protease inhibitors in juvenile gilthead seabream has been proved by Santigosa *et al*. (2010), who found similar results when fish were fed on diets with soybean trypsin inhibitor.

Vizcaíno *et al*. (2020) also assessed the existence in *Ulva ohnoi* of substances able to inhibit the digestive proteases of gilthead seabream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*) and seabass (*Dicentrarchus labrax*) (Figure 6). In that study, inhibition plots and zymograms were obtained in order to illustrate the response of fish proteases after incubation with crude or thermal treated *U. ohnoi.* Digestive proteases of all marine fish tested showed susceptibility to *Ulva* protease inhibitors, although considerably high concentration of *Ulva* was needed to cause high inhibition values. According to the estimation of Vizcaíno *et al*. (2020) the amount of *Ulva* required to reach 50% inhibition of digestive proteases would represent a dietary inclusion of approximately 40% to 53%, being these values quite far from those used in the formulation of commercial aquafeeds. Moreover, the effect of thermal treatmentS on the capacity of *Ulva* to inhibit trypsin activity evidenced that such inhibitors are susceptible to temperature. A treatment of 80°C for 15 min reduced the inhibitory capacity by 50%, and above 75% as prolonged times were applied. Deactivation of anti-nutritive factors is also an important issue to be

considered in raw material processing during aquafeed manufacture, and in the case of *U. onhoi* the heat treatment seems to be enough for inactivating these compounds.

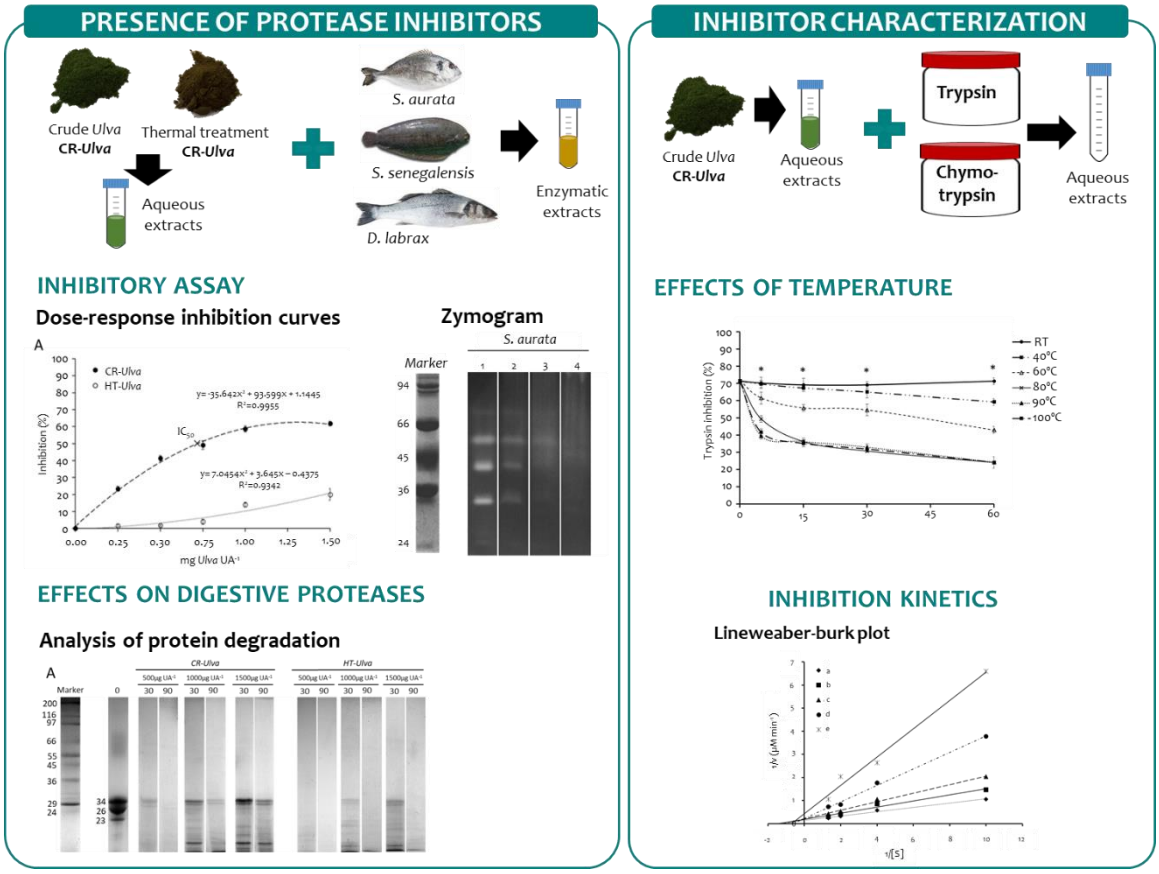

Figure 6. A summary of the results achieved in the study by Vizcaíno *et al.* (2020).

I.4.5. Algae digestibility

Not only the nutrient composition of algae determines their interest as ingredients in aquafeeds, but also factors such as feed attractiveness (e.g., smell, taste), accessibility (e.g., cell/pellet size, buoyancy), and nutrient availability should be considered. The high cell-wall recalcitrance of most algae is detrimental to digestibility and assimilation of intracellular nutrients, especially for carnivorous fish with a short digestion phase (e.g., seabass, turbot). The efficiency of marine animals to digest the cell walls depends on carbohydrate composition, on how they are linked to each other, as well as on the existence of adequate fish digestive carbohydrases. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases, but carnivorous fish do not, and this fact should be taken into consideration when formulating aquafeeds (Shi *et al.*, 2017). Consequently, it may be reasonable to

think that any strategy aimed at improving the bioavailability of the inner compounds might be of great interest for using algae in aquafeeds. Several procedures have been evaluated with the aim of releasing inner components of algae (Tibbetts *et al*., 2017; Teuling *et al*., 2019) but when they come to large-scale cell lysis, the enzymatic hydrolysis is likely one of the most promising strategies, not least owing to its economic viability. By following this procedure, even low inclusion level of enzyme-hydrolysed algae might well improve the physiological aspects in fish in a manner similar to including higher amounts of raw algae in aquafeeds (Tchorbanov & Bozhkova, 1988; Galafat *et al*., 2020). Alternatively, fermentation can also increase protein digestibility due to the degradation of insoluble polysaccharides, such as xylan. This last has been reported after fermentation of *Palmaria palmata* biomass using the fungal mould *Trichoderma pseudokoningii*, which was found to decrease the xylan content (Marrion *et al*., 2003).

Little research has been carried out to assess the digestibility of algal protein by fish digestive enzymes (Tibbetts *et al*., 2015; 2016; Vizcaíno *et al*., 2019b). Previous studies provided useful species-specific information about the manner that digestive enzymes of farmed fish hydrolyse algal proteins. The *in vitro* study of Vizcaíno *et al.* (2019b) reported that microalgae show *in vitro* protein degradation values around 50%, which are similar to those described in other raw materials commonly used in aquafeeds, such as soybean protein concentrate or fishmeal (Figure 7). Some microalgae species like *Tysocrysis* sp. or *Dunaliella* sp. may reach even high proteolysis by *S. aurata* digestive proteases (> 75%). This high *in vitro* protein hydrolysis is related to the fact that these microalgae have no distinct cell wall, and consequently, it is expected that cells could be easily hydrolysed by fish digestive enzymes (Vizcaíno *et al*., 2019b). On the contrary, in other species like *Nannochloropsis* sp., *Chlorella* sp. or *Scenedesmus* sp., protein is less susceptible to the action of fish proteases, and consequently, proteolysis values are lower than 50% (Vizcaíno *et al*., unpublished data). These microalgae possess a thick cell wall containing cellulose, hemicellulose, pectin and glycoprotein, which determines a low bioavailability of intracellular components (Bernaerts *et al*., 2018). In spite of the presence of intestinal amylase activity in some marine fish, like gilthead

seabream, the lack of digestive cellulases prevent the breakdown of the algal cell wall. The effective breakdown of algae cellulosic cell wall is a key factor for improving nutrient bioavailability in monogastric animals, not least in fish. Consequently, it would be advisable to include a previous step for cell wall disruption and/or hydrolysis before using algal biomass in aquafeed manufacturing.

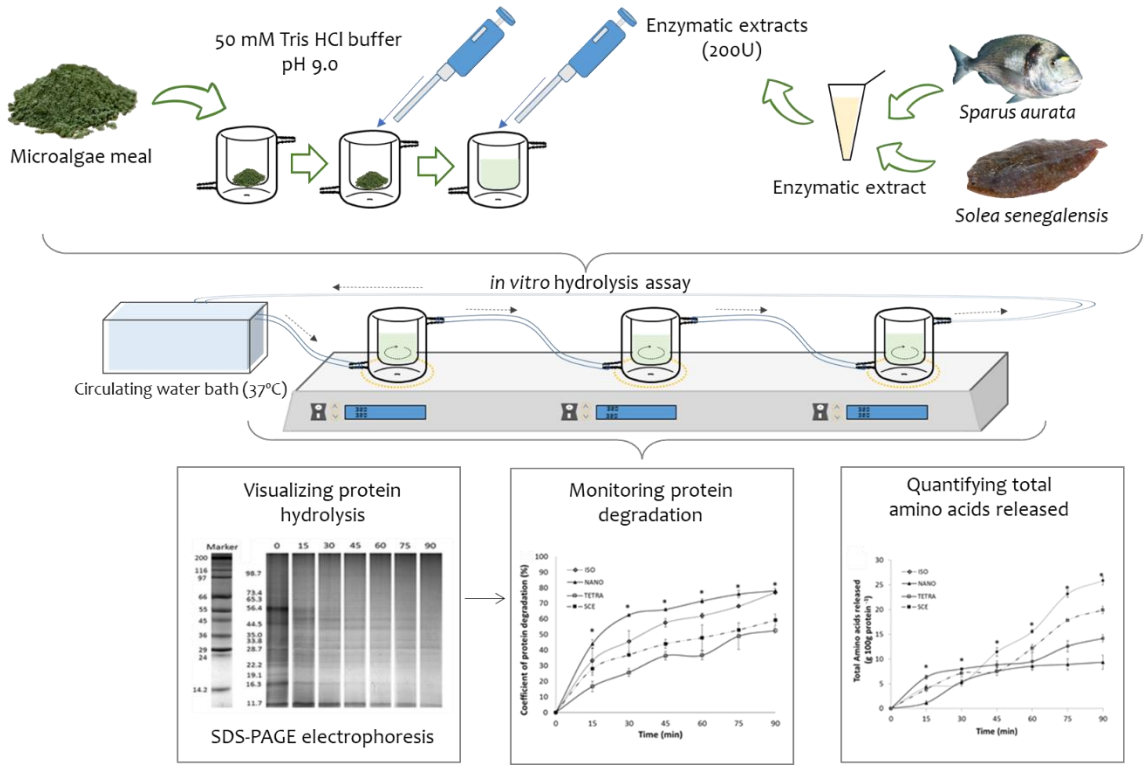

Figure 7. A summary of the *in vitro* study carried out of assessing the hydrolysis of algal protein by digestive proteases of juvenile gilthead seabream (Vizcaíno *et al.*, 2019).

I.5. EVALUATION OF ALGAE IN AQUAFEEDS

I.5.1. Effect on growth and nutrient utilization

Several studies have reported that the dietary inclusion of microalgae for feeding farmed fish doesn't impact negatively on growth performance and nutrient utilization (Shah *et al.*, 2018; Roohani *et al.*, 2019). Nevertheless, adverse effects on fish growth have also been reported (Walker & Berlinsky, 2011; Gong *et al.*, 2019). Differences in response seem to be influenced by several factors, such as fish and algae species, inclusion level, and nutritional composition of algae (Shah *et al.*, 2018). Several microalgae species, such as

Tisochrysis lutea, *Tetraselmis suecica*, *Nannochloropsis gaditana*, *Arthrospira platensis* and *Scenedesmus almeriensis*, have been successfully tested as dietary ingredients for different farmed fish species (Figure 8). The dietary inclusion of *T. suecica* (5%) improved growth performance of gilthead seabream fry (Vizcaíno *et al*., 2016b). Similarly, Vizcaíno *et al*. (2018) confirmed that microalgae inclusion up to 15% did not cause negative effects on growth performance of Senegalese sole juveniles. The study of Perera *et al.* (2020) evaluated two commercial compounds extracted from microalgae, i) LB-GREENboost® (LBGb), and ii) LB-GUThealth® (LBGh) developed by LifeBioencapsulation S.L. (Almería, Spain) included at 0.5% and at 1% in feeds. In this case, all fish groups grew allometrically from 12 - 13 g to 37 - 39 g with an overall weigh gain (WG) of ~200 % and specific growth rates (SGR) of 1.26 - 1.30%. In the above-mentioned studies, the inclusion of microalgae did not affect feed intake, although altered feeding behaviour and decreased feed consumption have been reported owing to high inclusion level (Dallaire *et al*., 2007). On the other hand, it is important to keep in mind that despite microalgae do not Increase growth performance of fish, they have a significant effect on nutrient utilization, reducing feed conversion ratio (FCR) (Perera *et al*., 2020).

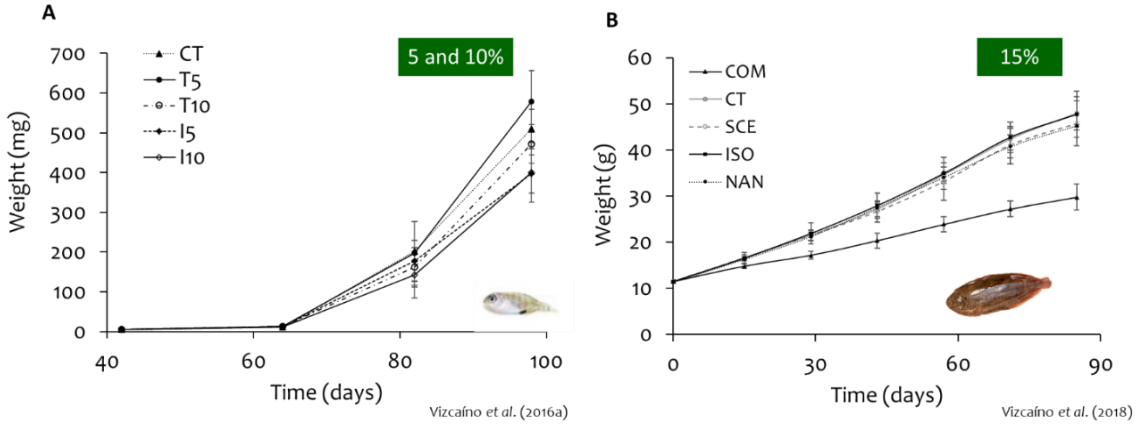

Figure 8. Examples of the effects of microalgae-supplemented diets on fish growth. A) Gilthead seabream fry fed control (CT), and *Tetraselmis suecica* (T5 and T10) and *Isochrysis galbana* (I5 and I10) at 5 and 10% dietary inclusion. B) Senegalese sole juveniles fed commercial (COM), control (CT), and *Scenedesmus almeriensis* (SCE), *Isochyrsis galbana* (ISO) and *Nannochloropsis gaditana* (NAN) at 15%.

Similarly, the effects of macroalgae on fish seem to be species-specific, and they depend on the proportion of biomass used. It has been reported that low dietary level of macroalgae leads to positive effects on growth performance and nutrient utilization of farmed fish (Vizcaíno *et al*., 2016a; 2019a). On the contrary, Valente *et al.* (2006) reported that the incorporation of 10% *Gracilaria cornea* affected negatively growth performance in *Dicentrarchus labrax*. These detrimental effects have been attributed to the existence of anti-nutritive factors, as described Vizcaíno *et al*. (2020), which might affect the bioavailability and/or digestibility of nutrients. In this sense, Vizcaíno *et al*. (2019a) found that FCR was increased as a result of including 5% *Ulva onhoi* in experimental diets for Senegalese sole juveniles. This fact might be a consequence of the high content in soluble and insoluble polysaccharides that can provoke a rapid transit of feed through the fish digestive tract, this increasing FCR and even impairing the specific growth rate (Vizcaíno *et al*., 2016a). Nevertheless, Moutinho *et al*. (2018) reported that the dietary inclusion of 10% *U. lactuca* for 5 months (from 23 g up to 60 g body weight) did not cause any detectable impact on growth performance and feed utilization in juvenile Senegalese sole. The disparate response of fish after *Ulva* dietary administration described in the literature might be related to differences in factors such as fish physiological maturity, the strain of *Ulva*, the length of the feeding trial, and even the dietary inclusion level of the seaweed.

I.5.2. Effect on muscle proximate composition

Overall, the use of microalgae in aquafeeds has yielded dissimilar effects on the chemical composition of fish. Thus, whereas Vizcaíno *et al*. (2014) reported that the inclusion of *Scenedesmus almeriensis* of up to 39% in diets for gilthead seabream juveniles did not affect fish body composition, other studies showed that microalgae modify the protein and lipid content in liver and muscle (Vizcaíno *et al*., 2016b; Galafat *et al*., 2020). Specifically, the last study found that dietary microalgae decrease muscle lipid content in gilthead seabream fry and juveniles. Similar findings were reported by Roohani *et al.* (2019), who described that *Spirulina platensis* increased protein and decreased lipid content in *Salmo trutta* juveniles. These authors pointed out that several algal

compounds, especially vitamins, minerals, essential amino acids and fatty acids, may activate fish metabolism, and particularly the use of lipid as energy source, which leads to reduced tissue storage. In the case of macroalgae, Sáez *et al*. (2020) also described a reduction in muscle lipid content in Senegalese sole fed with diets supplemented with 5% *Ulva onhoi,* and Ergün *et al*. (2009) described that fish fed *Ulva*-enriched diets showed increased muscle protein content. Previous studies attributed the effects on lipid metabolism and muscle fat deposition to the high content in vitamin C of *Ulva* (Ortiz *et al*., 2006). From the above-mentioned studies, it is clear that algae supplementation is an interesting strategy aimed at modulating the composition of fish muscle, which might be highly desirable in terms of quality of aquaculture products.

Another advantage of algae for feeding fish is that the animals generally reflect in muscle the dietary fatty acid profiles. This way, inclusion of algal biomass with the appropriate fatty acid profile can lead to significant increase in n-3 PUFAs content in the fillet. Regarding the modulation of fatty acid content, García-Márquez *et al*. (2020) described in Nile tilapia that a short feeding pulse with *Scenedesmus almeriensis*-enriched diets reduced the levels of saturated fatty acid in muscle, whilst increased levels of ARA, EPA and DHA in muscle. In this case, the partial replacement of fish derivatives by 25% with *S. almeriensis,* and its administration over a short period of time (30 days) represents an opportunity for producers to further improve the nutritional value of tilapia fillets, this leading to higher market value of fish products. In Senegalese sole, also 5% dietary supplementation with *Ulva* reduced muscle total lipid content and favoured muscle selective retention of n-3 PUFA, not least eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Sáez *et al*., 2020). A similar effect was observed by Vizcaíno *et al*. (2016b) in gilthead seabream fry fed with low dietary level of microalgae, whereas the inclusion of *Tetraselmis suecica* significantly increased the proportion of 18:3n-3 and the addition of *Tisochrysis galbana* increased DHA content in fish tissues. Moreover, the use of algae oils containing either EPA and DHA was effective in the total replacement of fish oil in practical (15% fishmeal) diets for marine fish, and ensured high nutritional quality of the fish fillet, increasing DHA content

(Carvalho *et al*., 2020). The results of the previous studies clearly indicate that algae and their derivatives are useful for providing n-3 PUFA in aquafeeds, and can contribute to reduce the use of fish oil worldwide.

I.5.3. Effects on gut functionality

One of the most important aspects related to the dietary inclusion of algae is its effect on the functionality and integrity of fish gut, given that growth is directly linked to nutrient digestion and absorption processes (Vizcaíno *et al*., 2019a). One of the key roles of the intestinal epithelium is to complete the final stages of the digestion, as well as to absorb dietary nutrients. A healthy gut helps the better absorption and bioavailability of nutrients from feed while acts as a physical barrier for avoiding the diffusion of pathogens and toxins from the lumen to the mucosal tissues. A single layer of epithelial cells separates the intestinal lumen from the underlying sterile tissue, and any alteration in the barrier integrity strongly activates immune cells and cause chronic inflammation of the intestinal tissues. This is why the integrity of the intestinal mucosa is a key factor in fish nutrition.

In this regard, various studies performed by Vizcaíno *et al*. (2014; 2016a; 2016b; 2018; 2019a) demonstrated that the dietary inclusion of algae induces noticeable changes in the activity of several enzymes involved in the digestive and absorptive processes. Pancreatic and intestinal brush border enzymes are correlated with the nutritional status of fish (Alarcón *et al*., 1998). Hence, their activities are used as indicators of the digestive and absorptive capacity of fish. With the latter in mind, the digestive enzyme activities quantified in those studies were differentiated in two groups; i) on the one hand, total alkaline protease activity, trypsin and chymotrypsin activities are used as indicators of the digestive capacity of fish to hydrolyse dietary protein, and ii) on the other, leucine aminopeptidase and alkaline phosphatase activities are used as indicators of the intestinal absorptive capacity. Results obtained in gilthead seabream and Senegalese sole showed a lack of negative effects on the activity of enzymes from the pancreatic secretion or on brush border enzymes; on the contrary, increased activity of the enzymes associated to the brush border membrane were observed owing to the dietary inclusion of some

microalgae (*Scenedesmus almeriensis*, *Tisochrysis lutea* or *Tetraselmis suecica*). These enzymes play a key role in the final digestion stages of dietary protein, allowing amino acid absorption and transport by enterocytes. Particularly, alkaline phosphatase is a dominant enzyme in the intestinal mucosa, and it is commonly used both as an indicator of the intestinal integrity, and as a general marker of nutrient absorption. For this reason, the increase in these activities can be associated with an amelioration in the overall efficiency of digestive and absorptive processes. Regarding the use of aquafeeds supplemented with seaweeds, Vizcaíno *et al.* (2016a; 2019a) described contrasting results; whereas *Ulva lactuca* caused a decrease in the intestinal proteolytic activity in gilthead seabream and Senegalese sole juveniles, the use of *Ulva onhoi* increased alkaline phosphatase activity in Senegalese sole. An explanation of this differential effect could be attributable to the different dietary level used in these feeding trials. In this way, the higher dietary level, the lower digestive proteolytic activity. In the case of *Ulva* sp., Vizcaíno *et al*. (2019b) suggested its use as an additive for improving the intestinal epithelium of Senegalese sole, but only for a short period of time, in order to avoid undesirable effects on digestive proteolytic enzymes. A healthy gastrointestinal tract is also crucial for optimal growth performance. According to Sweetman *et al*. (2008), the study of the intestinal mucosa can be used as a valuable tool to know how diet or other factors, such as infectious diseases or anti-nutritional compounds, can influence its structure and morphology. One of the major limitations for using proteins from plant origin in aquafeeds is their impact on the digestive system, including the reduced height of *villi* and enterocytes, low brush border integrity and supranuclear vacuolization in enterocytes, presence of leucocytes in *lamina propria* and submucosa, as well as the presence of different inflammatory symptoms, among other events (Cerezuela *et al*., 2012). Light and electron microscopy are useful tools for assessing how algae-supplemented diets might affect the integrity of the intestinal mucosa of fish. Both light microscopy and electron microscopy, transmission and scanning (TEM and SEM), images obtained from intestinal sections of fish fed algae-supplemented diets have been used for assessing the integrity of the apical brush border. This way, while TEM images offer information of the length, diameter and absorption surface of *microvilli*, SEM images enable to measure

the enterocyte apical area. The studies carried out on marine fish species (gilthead seabream and Senegalese sole) at different developmental stages (fry and juvenile) revealed that the inclusion of algae provoked positive changes in *microvilli* length, enterocyte apical area, and increased absorption surface in enterocytes (Vizcaíno *et al*., 2014; 2016b; 2019a). It is a general rule in these studies that those changes revealed an overall increased absorptive capacity in the intestine, as well as a reinforced intestinal mucosa as physical barrier in fish fed with microalgae-supplemented diets.

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II. HYPOTHESIS AND OBJECTIVES

In view of the antecedents previously exposed, the following hypotheses are raised:

Hypothesis 1. Evaluation of microalgae protein hydrolysis by action of digestive enzymes of gilthead seabream, through *in vitro* techniques allows estimating the potential of these raw materials for their inclusion, as a partial substitute for fish meal, in aquafeeds.

Hypothesis 2. Raw and hydrolysed *Arthrospira platensis* biomass can be used as a functional ingredient in feeds for gilthead seabream fry, without causing negative effects on growth, nutritional utilization and digestive functionality in the animals.

Hypothesis 3. *A. platensis* hydrolysed biomass can be included as functional ingredient, at low dose, in feeds without exerting negative effects on growth and general condition gilthead seabream juveniles.

Hypothesis 4. Inclusion, at a low dose, of lyophilized and hydrolysed *Nannochloropsis gaditana* biomass in feeds for seabream juveniles does not exert negative effects on growth, proximal muscle composition and digestive functionality.

Based on the above hypothesis, the following objectives are set:

Objective 1. Nutritional characterization of the biomass of different species of microalgae and evaluation of the protein bioavailability through *in vitro* digestive simulation assays using enzymatic extracts of gilthead seabream (*Sparus aurata*).

Objective 2. Evaluation of the effects derived from the use of *Arthrospira platensis* raw and hydrolysed biomass in starter feeds on growth, nutritional utilization, structure and digestive functionality in gilthead seabream fry.

Objective 3. Analysis of the effects caused by the inclusion of *A. platensis* hydrolysed biomass in feeds for gilthead seabream juveniles on growth and general condition.

Objective 4. Study of the effects derived from the use of raw and hydrolysed biomass of microalgae *Nannochloropsis gaditana* on growth, feed utilization, proximal composition and lipid oxidation in muscle, as well as on the digestive functionality in gilthead seabream juveniles.

III. EXPERIMENTAL WORK

III.1. EVALUATION OF THE *in vitro* PROTEIN BIOACCESSIBLITY OF SEVERAL MICROALGAE AND CYANOBACTERIA AS POTENTIAL DIETARY INGREDIENTS FOR GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES

III.1.0. ABSTRACT

This work addresses the evaluation of different marine (*Dunaliella salina* REC-0214B and *Nannochloropsis gaditana* REC-0215) and freshwater (*Anabaena* sp. BEA-0300, *Arthrospira platensis* BEA-0007B, *Chlorella vulgaris* BEA-0753, *Spirogyra* sp. BEA-0666) microalgae and cyanobacteria in terms of their protein bioaccessibility as a measurement of their potential suitability as dietary ingredients in aquafeeds. For this purpose, the microalgae were evaluated in terms of total protein content, amino acid composition, and the eventual presence of protease inhibitors. In addition, protein bioaccessibility was estimated by means of a species-specific *in vitro* assay using *Sparus aurata* digestive enzyme extracts. Overall, all the microalgae showed high protein content ranging from 25% to 61%, and a balanced essential to non-essential amino acid ratio (ranging from 0.81 to 0.95). The inhibition assay confirmed the absence of protease inhibitors whatever the microalgae considered. Finally, the *in vitro* assessment of protein hydrolysis showed differences in the degree of protein hydrolysis with values for the coefficient of protein degradation ranging from 49.4% for *Spirogyra* sp. BEA-0666 to 85.5% for *D. salina* REC-0241B. Similarly, the total amount of free amino acids released *in vitro* from the microalgal biomass (ranging from 12.8 to 20.8 g equivalents L-leucine 100 g protein^{-1}), as well as their qualitative aminoacidic profile varied among the different microalgae species, although the profile can be considered as well balanced in all cases. In conclusion, the results obtained revealed that, even if significant differences were observed among species in terms of their susceptibility to be hydrolysed by *S. aurata* digestive enzymes, however, all the microalgae and cyanobacteria evaluated presented an adequate protein content and a balanced amino acid profile.

III.1.1. INTRODUCTION

Aquaculture industry is continuously trying to reduce the inclusion rates of fishmeal and fish oil in aquafeeds for the different aquacultured species. However, production of farmed species still depends on these ingredients as usual feedstuffs (Yadab *et al*., 2020), owing to the fact that they set basis for balancing the formulation of commercial aquafeeds, especially for feeding crustaceans and carnivorous fish (Tacon & Metian, 2008; Younis *et al.,* 2018; Hua *et al*., 2019).

The increased demand, together with the stagnation of wild fisheries, the over-exploitation of pelagic fishes, and the current environmental concerns associated with extractive fishing, have driven fishmeal prices up by almost three-fold the past decade. Therefore, finding and testing alternative protein sources, as well as designing sustainable and nutritious aquafeeds including those ingredients, remains a challenge for current industrial aquaculture (Yarnold *et al*., 2019). In this regard, it is important to point out that any satisfactory alternative feed ingredient must be able to provide a nutritional value comparable to that of regular ingredients but must also be readily available at en affordable cost (Vizcaíno *et al*. 2014; Oliva-Teles *et al*., 2015; Guedes *et al*., 2015).

Over the last decades, proteins of plant origin have been introduced in aquafeeds in order to reduce the dependence on fishmeal, as they provide, roughly, nutrients for adequate fish growth. Indeed, soybean meal is one of the most widespread plant ingredients for aquafeed manufacturing, owing to its high-quality as protein source, with reliable supply at competitive cost. However, terrestrial plant proteins have some nutritional disadvantages, such as imbalance in essential amino acids like lysine, methionine, threonine, and tryptophan, and also contain anti-nutritional factors (Zheng *et al*., 2019), which altogether reduce the nutritional quality of aquafeeds (Shah *et al*., 2018), and jeopardize the bioavailability and digestibility of nutrients (Daniel, 2018). Furthermore, recently sustainability concerns are also considered owing to extensive soybean farming areas in developing countries contribute to

worldwide deforestation and loss of biodiversity (Pereira *et al*., 2020). Those above-mentioned make a priority the emergence of other novel protein resources for feeding fish.

Microalgae, including some species of Cyanobacteria, have the potential for reducing the dependence on conventional ingredients for aquafeeds, as they are a more reliable and less volatile source of protein (Hemaiswarya *et al*., 2011; Guedes *et al*., 2015; Hua *et al*., 2019). They have the potential to provide protein, lipids, vitamins, carotenoids, among other compounds (Shah *et al*., 2018). Overall, protein content of, microalgae is in the region of 30 - 55% (López *et al*., 2010), although in some genera such as *Arthrospira* sp., *Scenedesmus* sp. and *Chlorella* sp., can be even higher (Molino *et al*., 2018; Shah *et al*., 2018). In addition, microalgae protein display well-balanced amino acid profiles, comparable to those of other regular ingredients (Becker, 2007; Guedes *et al*., 2015). Microalgae show high content of aspartate and glutamate (2.9 - 7.1%), whereas cysteine, methionine, tryptophan, and histidine contents are in the range of 0.4 to 3.2%, with other amino acids ranging from 3.2 to 13.5% (Wilson, 2003).

Despite this, there are some important drawbacks and challenges to the use of microalgae in aquafeeds owing to the fact that some species have recalcitrant cell walls that act as a protective barrier that reduces the accessibility to intracellular nutrients (Teuling *et al*., 2019; Vizcaíno *et al*., 2019). The efficiency of fish enzymes for hydrolysing microalgae cell walls heavily relies on carbohydrate composition (Vizcaíno *et al*., 2019). In this regard, scarce research has been carried out addressing the extent of the hydrolysis of microalgal protein by fish digestive (Tibbetts *et al*., 2017; Vizcaíno *et al*., 2019).

The aim of this work was carry out a comprehensive characterization of the protein nutritional profile and the *in vitro* protein bioaccessibility of marine (*Dunaliella salina* REC-0214B, *Nannochloropsis gaditana* REC-0215) and freshwater (*Anabaena* BEA-0300, *Arthrospira platensis* BEA-0007B, *Chlorella vulgaris* BEA-0753, *Spirogyra* sp. BEA-0666) microalgae and cyanobacteria, in

order to assess their potential as dietary ingredients for gilthead seabream (*Sparus aurata*) juveniles.

III.1.2. MATERIAL AND METHODS

III.1.2.1. Microalgae

Freeze-dried biomass of six species of cyanobacteria or microalgae (*A. platensis* BEA-0007B, *D. salina* REC-0214B, *N. gaditana* REC-0215, *Spirogyra* sp. BEA-0666, *C. vulgaris* BEA-0753 and *Anabaena* sp. BEA-0300) was provided by the Spanish Bank of Algae of the University of Las Palmas (Canary Islands, Spain). Strains were cultivated under laboratory conditions according to the standardised methodology: Erlenmeyer flasks with f/2 nutrient medium for seawater strains and BG11 medium for freshwater strains, mean light intensity at 240 µmol photons m^{-2} s^{-1}, photoperiod 12:12 (L:D), temperature 25 ± 2 °C and 1.5% CO_2 enriched air continuously supplied during the light period. Artificial light was provided with six white light lamps (Phillips PL-32W/840/4p) simulating the solar cycle. The biomass was harvested by centrifugation (RINA centrifuge, Riera Nadeu SA, Spain), frozen at −18 °C, lyophilized, and finally milled (RM200 mill, Retsch, Spain) during 20 min to obtain a fine powder (<100µm) that was stored in the dark at −20 °C until further analysis.

III.1.2.2. Protein analysis

Chemical analyses of microalgae biomasses were carried out as follows: crude protein (N × 6.25) was determined by elemental analysis (C:H:N) using a Fisons EA 1108 analyzer (Fisons Instruments, Beverly, MA, USA). For amino acid analysis, microalgae biomass was hydrolysed (20 mg in 1 mL HCl 6M) at 110 °C during 24 h under an inert atmosphere (N_2). Then, 50 µL of the hydrolysate were mixed with 50 µL 6 M NaOH. Then 100 µL of internal standard (2.5 mM norleucine) and 800 µL sodium citrate loading buffer (pH 2.2) were added and mixed in a vortex for 5 s, and then filtered (0.2 µm). A sample (20 µL) of this mixture was analysed with an amino acid analyser (Biochrom 30+ amino acid

analyser, Biochrom LTD Cambridge, UK) according to the manufacturer's protocol. Norleucine acid was added as internal standard.

III.1.2.3. Testing the presence of protease inhibitors

III.1.2.3.1. Preparation of fish digestive enzyme extracts

Twenty gilthead seabream specimens (25 g average body weight) were obtained from a commercial nursery (Predomar SA, Almería, Spain), and were fed with a commercial diet (Skretting, crude protein: 47% DM) twice per day (9:00 and 13:00) at a rate of 3% biomass daily during a week. After that, the fish were sacrificed 5 h after feeding by severing their spine according to the requirements of the European Union (Directive 2010/63/UE) and Spanish (Real Decreto 53/2013) legislation, under the protocol number 06/02/2020/011. The abdomen was opened, and the whole gut was obtained. Intestines from each five fish were pooled, and manually homogenized (1:2, w/v) in distilled water at 4 °C. Supernatants were obtained after centrifugation (12,000 rpm, 12 min, 4 °C) and stored in aliquots at –20 °C until use. Total alkaline protease activity of the enzymatic extracts was measured spectrophotometrically using 5 g L^{-1} casein in 50 mM Tris HCl (pH 9.0) as substrate (Alarcón *et al*., 1998). One unit of total protease activity was defined as the amount of enzyme that released 1 µg of tyrosine per min in the reaction mixture, considering an extinction coefficient for tyrosine of 0.008 μg^{-1} mL^{-1} cm^{-1}, measured at 280 nm. Samples were analysed in triplicate.

III.1.2.3.2. Inhibition assay

Inhibitory extracts (0.1 g mL^{-1}) were prepared from microalgae by homogenizing 100 mg biomass in 1 mL of distilled water, then shaking for 30 min at room temperature, and then for 24 h at 4°C. The mixtures were centrifuged (20 min at 13,000 g and 4°C) and supernatants were stored at 4°C until using in inhibitory assays. The inhibition of *S. aurata* digestive proteases by microalgae extracts was evaluated according to Alarcón *et al*. (2001). Briefly,

increasing volumes of the microalgae aqueous extracts were added to the reaction mixture containing the enzyme extracts, in order to provide different ratios of µg biomass per unit of proteolytic activity (UA) ranging from 0 to 400 µg microalgae UA^{-1}. Enzyme inhibition was expressed as the percentage of inhibition after comparing with a control assay carried out in presence of digestive proteases but without any inhibitory extract (0% inhibition).

III.1.2.4. *in vitro* species-specific digestive simulation

III.1.2.4.1. in vitro protein hydrolysis assay

The *in vitro* protein hydrolysis of microalgae was simulated in 10 mL-jacketed reaction vessels connected to a circulating water bath at 37 °C, under continuous agitation by a magnetic stirrer. Although that temperature is not physiological for sparids, it was selected in order to increase the activity of the enzymes and reducing the time requested for thes assay (Hamdan *et al*., 2009).

An amount of each microalga biomass, providing 80 mg of crude protein, was suspended in 50 mM Tris HCl buffer pH 9.0. After 15 min stirring, the hydrolysis was started by the addition of volumes of the *S. aurata* intestinal extracts providing 200 UA of total alkaline proteolytic activity (Vizcaíno *et al*., 2019). Blank assays with microalgae biomass, but without digestive extract, were carried out. The hydrolysis was maintained for 90 min, and samples of the reaction mixture at different sampling points (0, 15, 30, 60 and 90 min) were withdrawn. Each assay was repeated in quadruplicate.

The hydrolysis of microalgae protein was assessed by sequential characterization of the hydrolysis products released, which were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Prior to the electrophoretic separation, samples were diluted (1:1) in sample buffer (0.125 M Tris HCl, pH 6.8; 4 % (w/v) SDS; 10% (v/v) β-mercaptoethanol; 20 % (v/v) glycerol; 0.04 % (w/v) bromophenol blue) and boiled for five minutes to stop the reaction and denature protein fractions. Electrophoresis was performed at a constant voltage of 100 volts per gel for

60 min at 4 °C. Gels (12% polyacrylamide and 8 × 10 × 0.075 cm) were stained overnight with 0.1% Coomassie brillant blue (BBC R-250) in a methanol-acetic acid solution (50:20:50). For destaining, a methanol-acetic acid-water solution (35:10:55) was used. A protein standard consisting of twelve proteins ranging from 6.5 kDa (aprotinin, bovine lung) to 200 kDa (myosin, porcine heart) was used (wide range molecular weight marker, S-84445, Sigma, St. Louis, USA). The relative molecular mass (Mr, in kDa) of separated proteins fractions was estimated using a linear plot of log Mr of protein standards vs relative mobility (Rf). Changes resulting from protein hydrolysis were measured by densitometric analysis of SDS-PAGE gels (My Image Analysis Software, Thermo Scientific) for determination of the coefficient of protein degradation (CPD) according to Alarcón *et al*. (2001).

III.1.2.5. Quantification of free amino acids released

Total released amino acids from microalgae protein were determined by the o-phthaldialdehyde method (Church *et al*., 1983) using L-leucine as standard. Prior to the assays, undigested protein was discarded by precipitation with 200 g L^{-1} trichloroacetic acid (1:1) followed by centrifugation at 12,000 g for 15 min. Blank assays were run to estimate free amino acids from enzyme extracts and microalgae suspensions, which enabled to determine the net release of amino acids attributable to the enzymatic hydrolysis. Results were expressed as accumulated values of free amino acids released during the digestive simulation (g of L-leucine equivalents 100 g $protein^{-1}$). Assays were performed in triplicate.

III.1.2.6. Statistical analysis

Results are expressed as mean ± standard deviation (SD). In order to test data normality and variance homogeneity, the Kolmogorov-Smirnov's test and Levene's F-test were used, respectively. Data with parametric distribution were analysed using a one-way analysis of variance (ANOVA) and the significant differences between treatments ($p < 0.05$) were determined using

Tukey's multiple comparison test. Data with nonparametric distribution were analysed using Kruskal-Wallis test, and significant differences were determined using Box and Whisker Plots graphs. All statistical analyses were performed using the Stagraphics Plus 4.0 (Rockville, MD, USA) software. A hierarchical cluster analysis (nearest neighbour method, squared Euclidean) was used to determine a global similarity among the different microalgae evaluated. Clustering is a multivariate technique of grouping together strains that share similar values. This procedure can be used to classify data into groups that are relatively homogeneous within themselves and heterogeneous between each other, on the basis of a defined set of variables. Finally, in order to assess the feasibility of the protein characterization and *in vitro* assay to discriminate the protein bioaccessibility of the different microalgae, a Principal Components Analysis (PCA) was carried out. All statistical analyses were performed using the Stagraphics Plus 4.0 (USA) software.

III.1.3. RESULTS

III.1.3.1. Protein characterization of microalgae

The protein content of microalgae and cyanobacteria is detailed in Table 1. Crude protein ranged from 25 to 61% on dry matter (DM) basis. The highest protein content was found in *Anabaena* sp. BEA-0300 and *D. salina* REC-0214B with 60.9% and 52.3%, respectively, whereas *Spirogyra* sp. BEA-0666 showed the lowest protein content (25.3%).

The amino acid profile is summarised in Table 2. Overall, the microalgae and cyanobacteria showed similar amino acid profiles, and although *Anabaena* sp. BEA-0300 presented the highest absolute values, however, in relative terms, proportion of amino acids was similar to that found in the rest of species. Similarly, *Spirogyra* sp. BEA-0666 presented the lowest content in amino acids, but also showing a profile similar to the rest of microalgae and cyanobacteria. The *EAA/NEAA* ratios ranged from 0.91 to 0.95, which were higher than that of soybean meal (0.7), and within the range of fish meal (0.92). *D. salina* REC-

0214B and *Anabaena* sp. BEA-0300 showed the highest (0.95 ± 0.02 and 0.95 ± 0.01, respectively) and *Spirogyra* sp. BEA-0666 showed the lowest values (0.81 ± 0.01). Figure 1 graphs the essential amino acid content in the microalgae and cyanobacteria studied. As described, comparable profiles were observed in all of them, with a slightly higher proportion of some amino acids being observed in *Anabaena* sp. BEA-0300 (i.e. Arg) and *D. salina* REC-0214B (i.e. Leu and Val).

Table 1. Crude protein content (% dry matter, DM) in the different microalgae and cyanobacteria.

	Crude protein
A. platensis BEA-0007B	36.8 ± 0.1^{c}
D. salina REC-0214B	52.3 ± 0.6^{e}
N. gaditana REC-0215	31.4 ± 0.1^{b}
Spirogyra sp. BEA-0666	25.3 ± 0.2^{a}
C. vulgaris BEA-0753	43.6 ± 0.1^{d}
Anabaena sp. BEA-0300	60.9 ± 0.2^{f}
p-value	*<0.0001*

Values are mean ± SD of triplicate determination. Values with different lowercase letters indicate significant differences ($p < 0.05$).

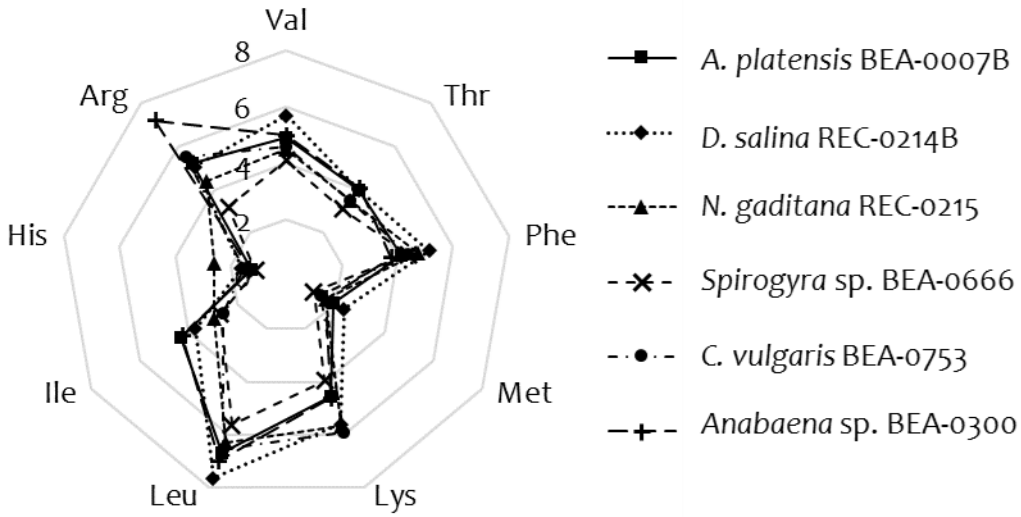

Figure 1. Essential amino acid content (g 100 g protein^{-1}) in the microalgae and cyanobacteria studied.

Table 2. Amino acid content (g 100 g biomass^{-1}) of the selected microalgae and cyanobacteria. Soybean and fish meal amino acid profiles were also included (values are the mean of triplicate determination ± SD).

	A. platensis BEA-0007B	*D. salina* REC-0214B	*N. gaditana* REC-0215	*Spirogyra* sp. BEA-0666	*C. vulgaris* BEA-0753	*Anabaena* sp. BEA-0300	Soybean meal	Fishmeal	*p-value*
NEAA									
Ala	2.17 ± 0.08^{b}	2.94 ± 0.05^{c}	1.57 ± 0.06^{a}	1.38 ± 0.01^{a}	2.73 ± 0.13^{c}	4.26 ± 0.20^{d}	1.99	4.57	*<0.0001*
Asp	2.87 ± 0.10^{c}	4.18 ± 0.14^{d}	2.19 ± 0.09^{b}	1.64 ± 0.08^{a}	3.20 ± 0.20^{c}	5.65 ± 0.23^{e}	6.55	6.81	*<0.0001*
Cys	0.30 ± 0.01^{ab}	0.47 ± 0.01^{c}	0.25 ± 0.01^{ab}	0.21 ± 0.02^{a}	0.32 ± 0.01^{b}	0.51 ± 0.06^{c}	0.63	0.67	*0.0002*
Glu	4.08 ± 0.14^{c}	5.20 ± 0.07^{d}	3.10 ± 0.014^{b}	2.06 ± 0.02^{a}	4.02 ± 0.20^{c}	5.70 ± 0.20^{e}	9.73	9.16	*<0.0001*
Gly	1.53 ± 0.05^{c}	2.81 ± 0.04^{f}	1.28 ± 0.06^{b}	1.03 ± 0.01^{a}	1.96 ± 0.10^{d}	3.21 ± 0.09^{e}	2.40	3.97	*<0.0001*
Pro	0.95 ± 0.01^{a}	2.21 ± 0.56^{c}	1.57 ± 0.01^{b}	0.81 ± 0.01^{a}	1.28 ± 0.05^{ab}	1.59 ± 0.02^{b}	4.50	3.26	*0.0081*
Ser	1.46 ± 0.05^{b}	2.02 ± 0.02^{c}	1.05 ± 0.04^{a}	0.92 ± 0.02^{a}	1.58 ± 0.09^{b}	2.55 ± 0.12^{d}	2.89	3.05	*<0.0001*
Tyr	1.61 ± 0.06^{b}	1.81 ± 0.01^{e}	1.07 ± 0.08^{b}	0.79 ± 0.02^{a}	2.20 ± 0.09^{c}	2.44 ± 0.15^{d}	2.09	2.59	*<0.0001*
EAA									
Arg	1.91 ± 0.06^{c}	2.65 ± 0.04^{e}	1.38 ± 0.07^{b}	0.79 ± 0.02^{a}	2.40 ± 0.11^{d}	4.39 ± 0.16^{f}	4.03	4.63	*<0.0001*
His	0.46 ± 0.02	0.84 ± 0.01	0.81 ± 0.50	0.28 ± 0.01	0.66 ± 0.04	0.81 ± 0.03	1.43	1.78	*0.1820*
Ile	1.59 ± 0.06^{d}	1.95 ± 0.12^{e}	0.93 ± 0.04^{b}	0.69 ± 0.01^{a}	1.13 ± 0.05^{c}	2.60 ± 0.12^{f}	2.36	3.08	*<0.0001*
Leu	2.45 ± 0.08^{c}	3.99 ± 0.36^{e}	1.98 ± 0.08^{b}	1.42 ± 0.02^{a}	2.82 ± 0.14^{d}	4.25 ± 0.18^{e}	4.10	5.44	*<0.0001*
Lys	1.68 ± 0.06^{b}	2.93 ± 0.04^{d}	1.78 ± 0.02^{b}	1.00 ± 0.01^{a}	2.56 ±0.13^{c}	2.81 ± 0.10^{d}	4.62	5.76	*<0.0001*
Met	0.70 ± 0.02^{b}	1.23 ± 0.02^{e}	0.51 ± 0.02^{b}	0.28 ± 0.01^{a}	0.60 ± 0.02^{c}	0.99 ± 0.05^{d}	0.66	1.80	*<0.0001*
Phe	1.52 ± 0.06^{b}	2.69 ± 0.01^{e}	1.49 ± 0.15^{b}	1.11 ± 0.08^{a}	1.89 ± 0.08^{c}	2.29 ± 0.10^{d}	2.80	3.21	*0.0001*
Thr	1.47 ± 0.05^{c}	1.10 ± 0.03^{d}	1.10 ± 0.04^{b}	0.79 ± 0.02^{a}	1.50 ± 0.09^{c}	2.47 ± 0.08^{e}	2.38	3.27	*<0.0001*

Val	1.81 ± 0.31^{c}	1.41 ± 0.03^{d}	1.41 ± 0.05^{b}	1.03 ± 0.01^{a}	1.99 ± 0.08^{c}	3.04 ± 0.14^{d}	2.42	5.01	*0.0001*
EAA/NEAA	0.91 ± 0.02^{b}	0.95 ± 0.02^{b}	0.91 ± 0.05^{b}	0.81 ± 0.01^{a}	0.91 ± 0.01^{b}	0.95 ± 0.01^{b}	0.70	0.92	*0.0098*

EAA: essential amino acids; *NEAA*: non-essential amino acids. Values in the same row with different superscript letters denote significant differences among microalgae and cyanobacteria biomasses.

III.1.3.2. Presence of protease inhibitors

The inhibitory effect of microalgae and cyanobacteria aqueous extracts on the intestinal proteases of gilthead seabream is shown in Figure 2. A dose-response inhibition curve was obtained by measuring the reduction in the proteolytic activity of a standardized seabream intestinal extract when incubated with different proportions of microalgae extracts. *C. vulgaris* BEA-0753 showed the highest protease inhibition capacity (23% inhibition), whereas inhibition with other microalgae and cyanobacteria never reached values higher than 10%. Each inhibition curve was fitted to a quadratic equation that can be used to predict the expected inhibition for a given amount of each microalga.

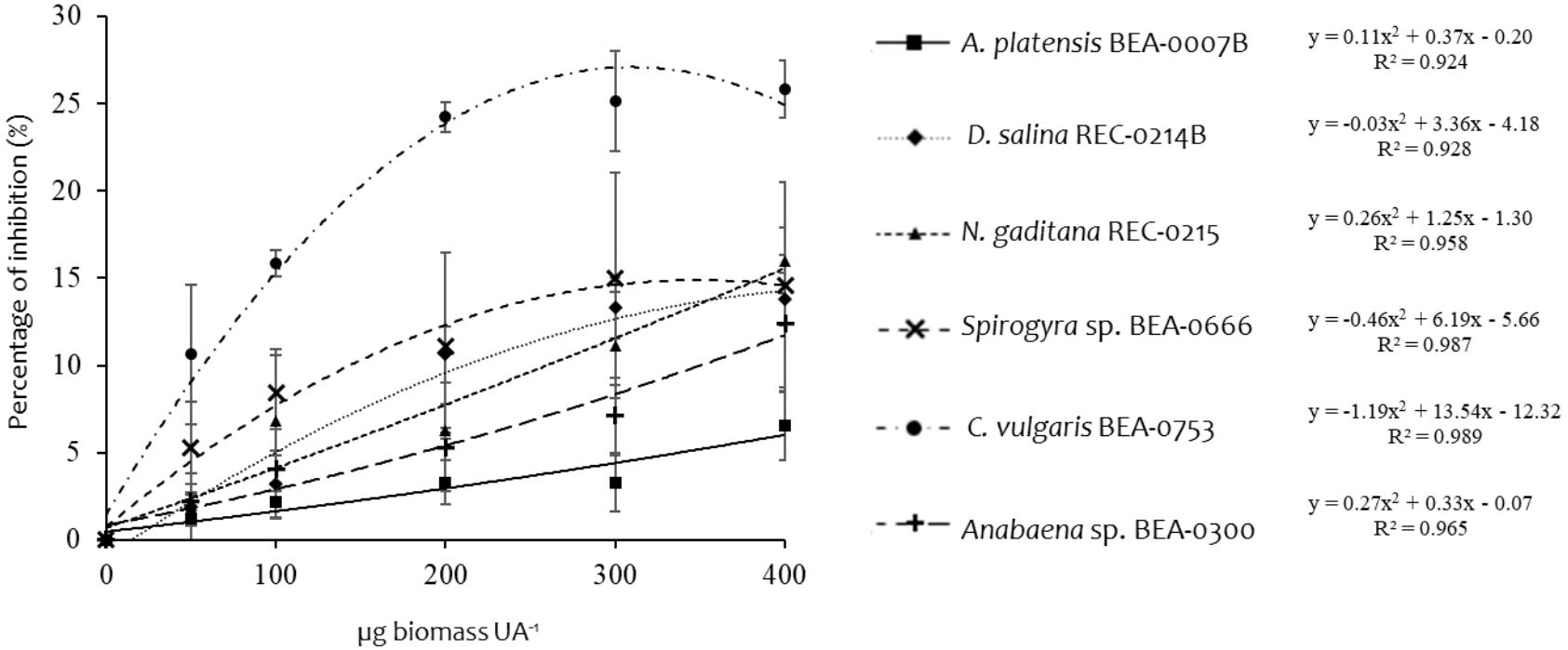

Figure 2. Dose-response curves of *S. aurata* intestinal proteases by increasing the concentration of microalgae and cyanobacteria in the inhibitory assay. Each point represents the mean of three replicates ± SD.

III.1.3.3. *in vitro* digestive simulation assay using gilthead seabream enzymes

The time-course of protein hydrolysis by the digestive proteases of *S. aurata* is shown in Figure 3**.** Electrophoretic analysis evidenced several protein fractions with different relative molecular mass for each microalga and cyanobacteria biomass. No protein auto-hydrolysis was observed when the *in vitro* digestive simulation was carried out in absence of fish enzymes (lanes control at 0 and 90 min). Changes in optical density in the electrophoresis gels were assessed by selecting five protein fractions ranging from 19 to 85 kDa, and from 24.0 to 126.0 kDa in *N. gaditana* REC-0215 and *D. salina* REC-0214B, respectively. Four fractions ranging from 11.0 to 59.0 kDa, and from 23.0 to 63.0 kDa in *Anabaena* sp. BEA-0300 and *C. vulgaris* BEA-0753 sp., respectively. Six fractions ranging from 10.0 to 63.0 kDa in *A. platensis* BEA-0007B and three fractions ranging from 24.0 to 62.0 kDa in *Spirogyra* sp. BEA-0666. In all the microalgae and cyanobacteria, a gradual hydrolysis of most of the protein fractions above mentioned was observed through the 90 min digestive simulation. In the case of *D.* salina REC-0214B, a noticeable hydrolysis of all the proteins was found, especially after 30 min (Figure 4). Some of the microalgae and cyanobacteria presented two protein fractions around 60 - 65 kDa and 20 - 25 kDa whose hydrolysis by fish enzymes was less marked than that observed for the rest of the proteins. This finding was also evidenced in *C. vulgaris* BEA-0753, where both proteins remained almost undigested after 60 min.

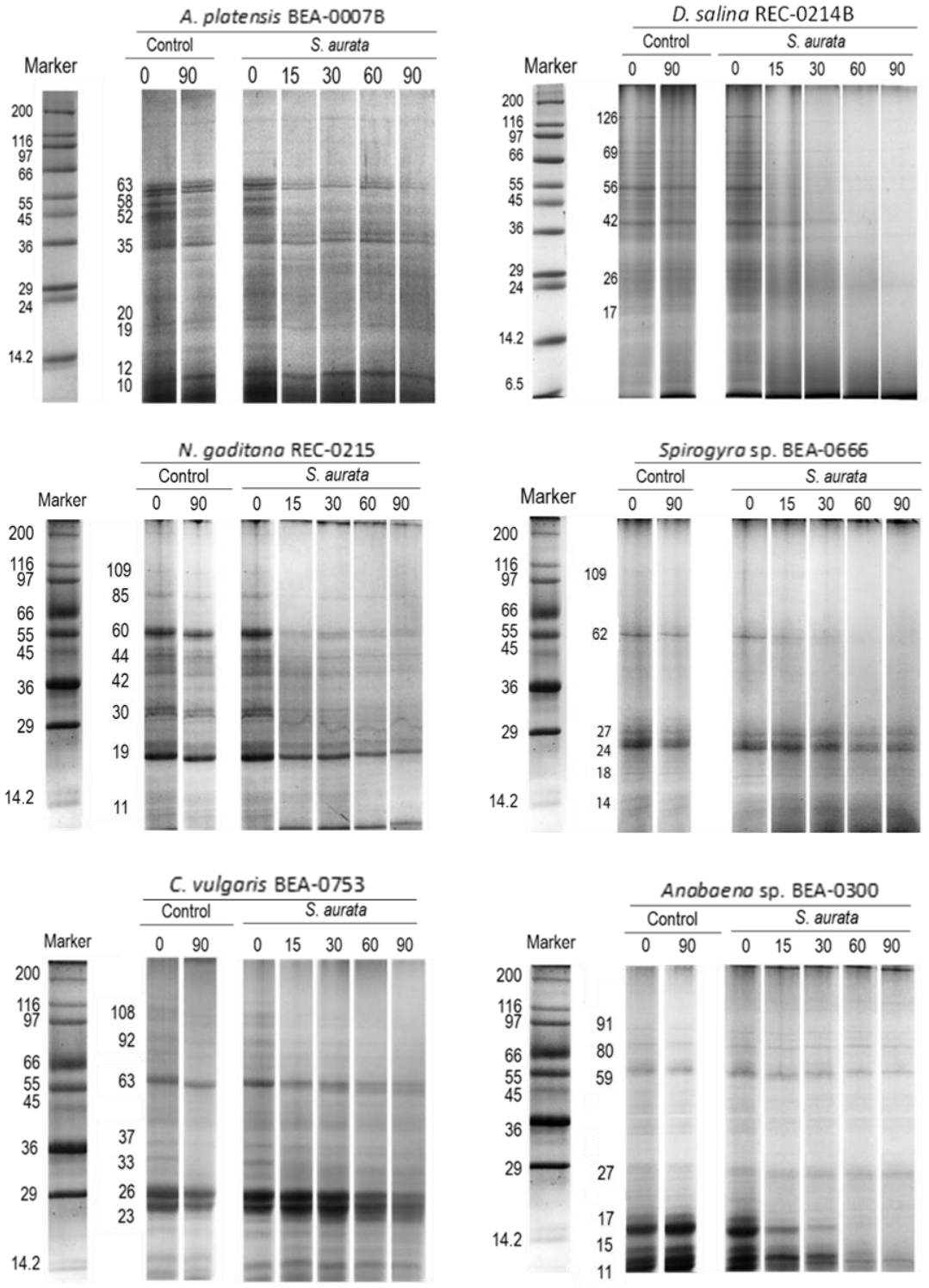

Figure 3. Time-course of *in vitro* protein hydrolysis by *S. aurata* intestinal proteases. Images show SDS-PAGE hydrolysis patterns obtained at different sampling times (0, 15, 30, 45, 60, 75 and 90 min). Numbers at the left of the proteinograms show the molecular weight (kDa) of the main proteins studied. Control represents the protein pattern of each microalgae or cyanobacteria in an assay without fish enzymes at the beginning (0 min) and the end (90 min) of the *in vitro* procedure.

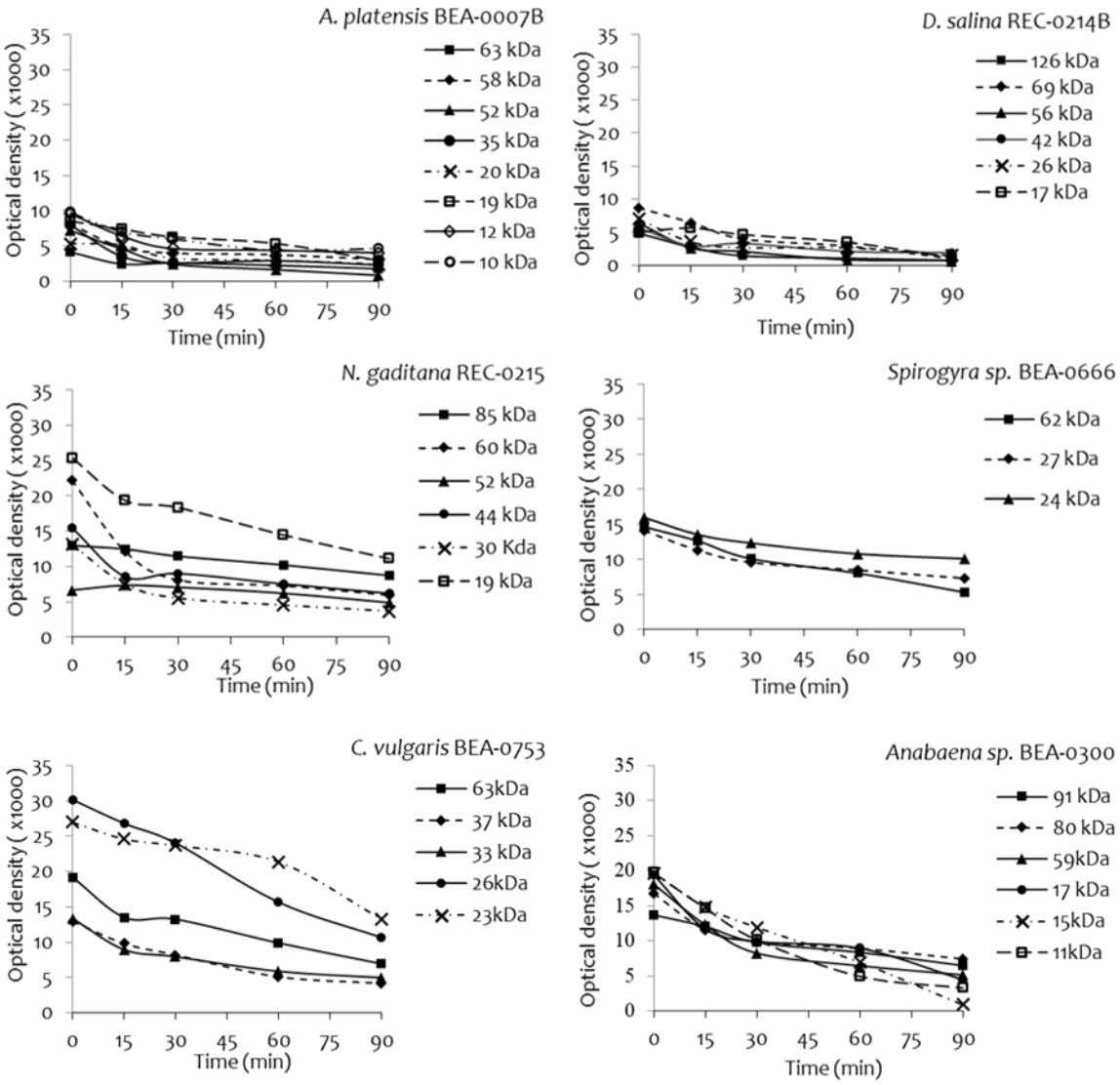

Figure 4. Changes in the optical density (OD, measured as pixels per cm^{-2}) of the main protein fractions throughout the *in vitro* hydrolysis with *Sparus aurata* digestive extracts.

Changes in the average CPD values during the *in vitro* digestive simulation are shown in Figure 5. *D. salina* REC-0214B showed CPD values higher than 80% revealing high bioaccessibility of their proteins to gilthead seabream digestive proteases. The progression revealed an initial quick proteolysis followed by a less marked but sustained hydrolysis until the end of the *in vitro* assay. Protein hydrolysis in *Anabaena* sp. BEA-0300, *A. platensis* BEA-0007B and *C. vulgaris* BEA-0753 yielded similar patterns, reaching CPD values over 60%. The lowest CPD value was obtained for *Spirogyra* sp. BEA-0666.

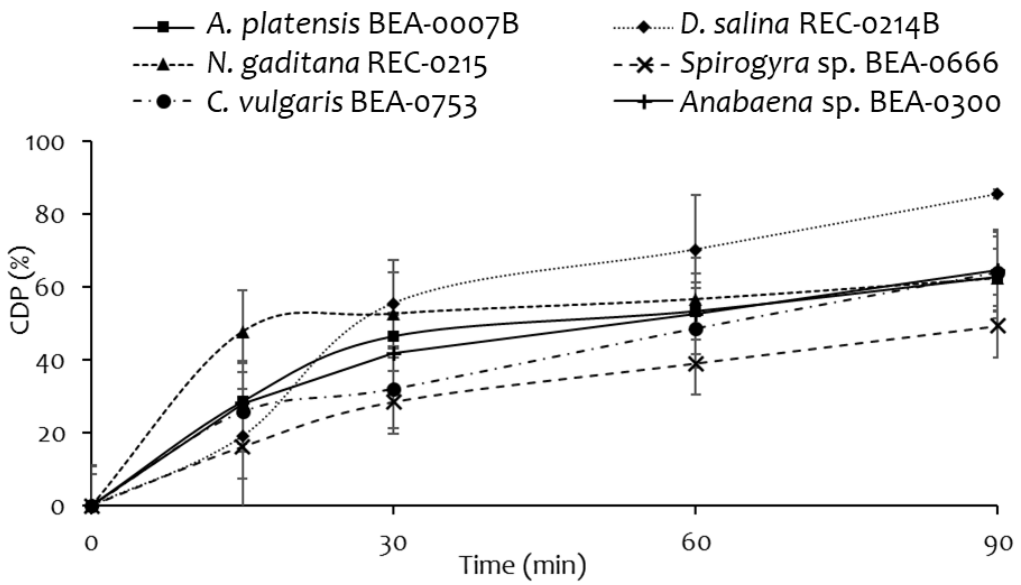

Figure 5. Changes in the coefficient of protein degradation (CPD) during the *in vitro* digestive simulation with *S. aurata* intestinal proteases.

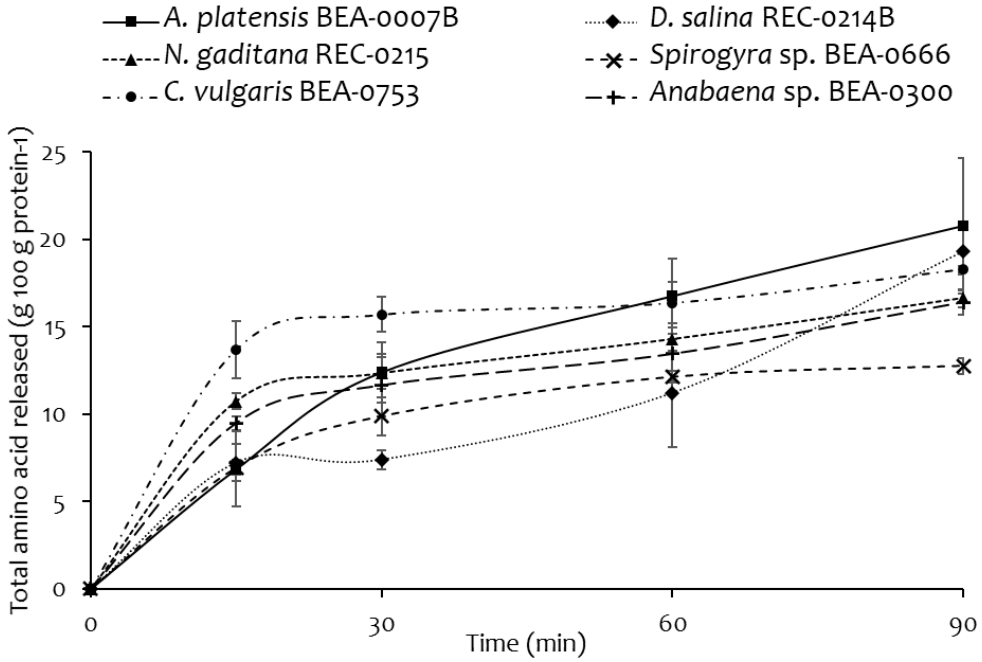

Figure 6. Concentration of free amino acids released (*AAR*, g 100 g protein^{-1}) during the *in vitro* proteolysis of microalgae and cyanobacteria by *S. aurata* intestinal proteases.

The amount of free amino acids released (*AAR*) during the enzymatic hydrolysis of protein is shown in Figure 6. Overall, the accumulation of amino acids in the reaction vessel was progressive in all the microalgae and cyanobacteria evaluated. At the end of the *in vitro* assay, total free amino acids released ranged from 12.8 to 20.8 g equivalents of L-leucine 100 g protein^{-1}. The

highest cumulative values of free amino acids were observed in *D. salina* REC-0214B ($p < 0.05$), whereas the lowest value was obtained for *Spirogyra* sp. BEA-0666.

The specific amino acids released after 90 min of *in vitro* proteolysis are shown in Figure 7. In general, both essential and non-essential amino acids were released by owing to the activity of fish digestive enzymes. Specifically, *Anabaena* sp. BEA-0300 and *D. salina* REC-0214B showed the highest values of arginine released, whist the lowest values for this amino acid were obtained in *Spirogyra* sp. BEA-0666. *A. platensis* BEA-0007B and *C. vulgaris* BEA-0753 yielded relatively high levels of glutamic acid after the hydrolytic process, reaching, in the case of *A. platensis* BEA-0007B, values up to four times higher than those observed for the rest of microalgae and cyanobacteria. Furthermore, *Anabaena* sp. BEA-0300 had the highest tyrosine values, while *D. salina* REC-0214B showed high values of essential amino acids such as valine, leucine or lysine.

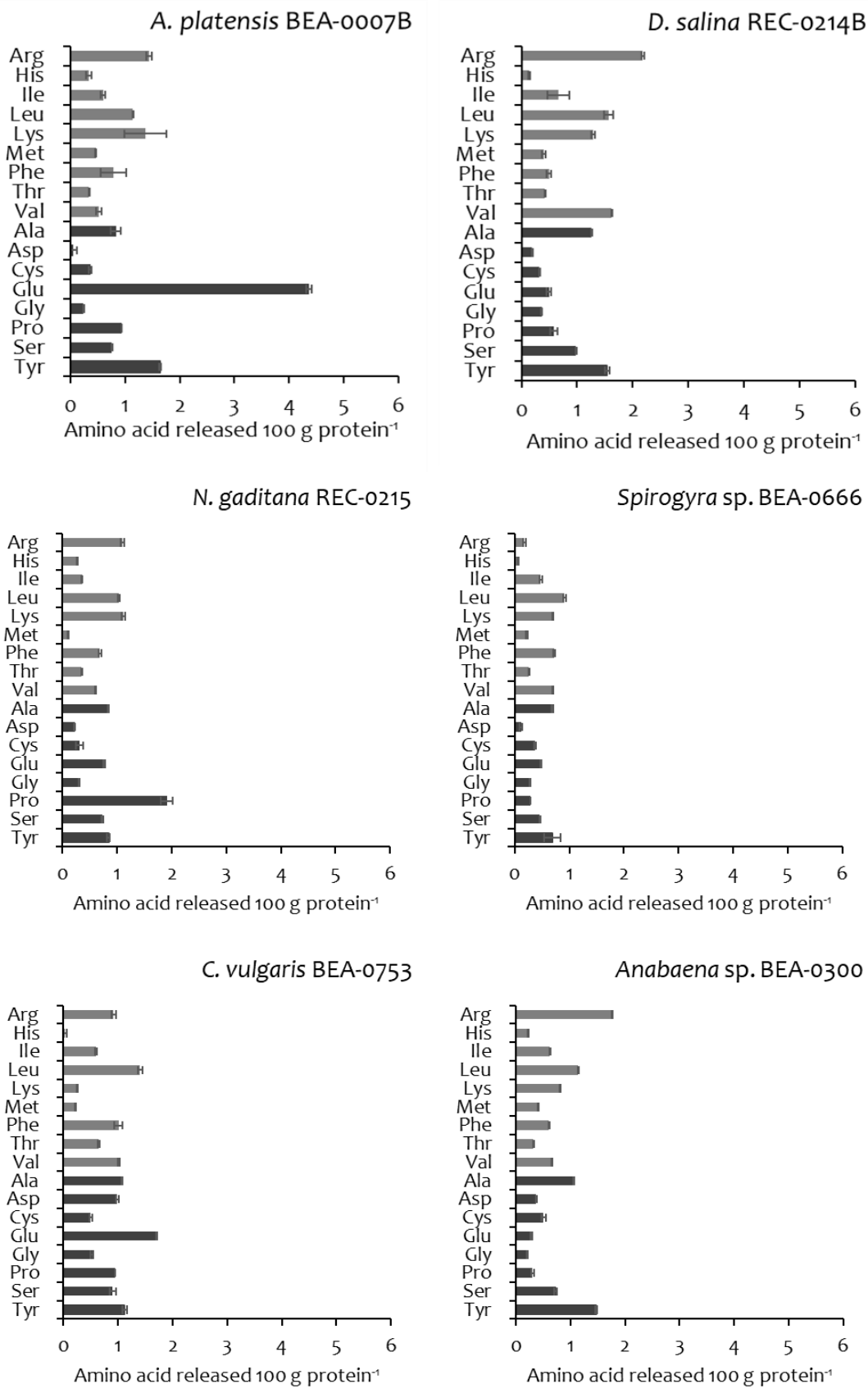

Figure 7. Profile of amino acid released (g 100 g protein^{-1}) from the selected microalgae and cyanobacteria at the end of the *in vitro* assay.

When the essential amino acids / non-essential amino acids (*EAAR*/*NEAAR*) ratio was calculated (Figure 8), it was observed that *N. gaditana* REC-0215 showed the most balanced ratio, with a value close to 1, which represents an equal proportion of essential and non-essential amino acids. *D. salina* REC-0214B, *Spirogyra* sp. BEA-0666 and *Anabaena* sp. BEA-0300 showed values higher than 1 (1.52, 1.25 and 1.32, respectively), which means that essential amino acids released prevailed in these species, whereas while *A. platensis* BEA-0007B and *C. vulgaris* BEA-0753 showed values lower than 1, which represents a higher release of non-essential amino acids.

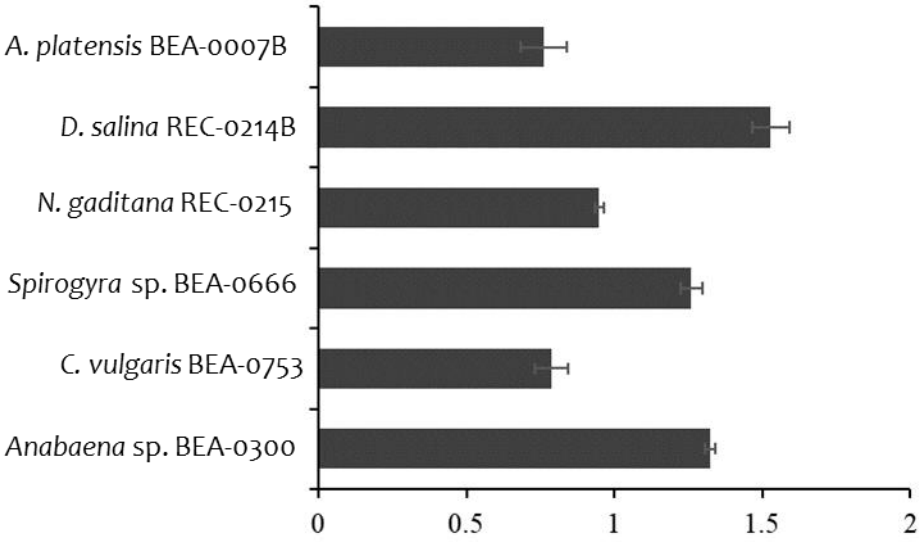

Figure 8. *Essential/non-essential* amino acid ratio after 90 min of *in vitro* hydrolysis.

Figure 9 shows the results of a cluster analysis that provides a global view of the results obtained in this work. This analysis grouped the different microalgae and cyanobacteria species according to the similarity of their characteristics. The dendrogram showed three different groups. *A. platensis* BEA-0007B, *C. vulgaris* BEA-0753 and *N. gaditana* REC-0215 appear close with less than 5% distance. The second group was shaped by *Anabaena* sp. BEA-0300 and *D. salina* REC-0214B (13% distance), whereas *Spirogyra* sp. BEA-0666 appears clearly separated (28% distance).

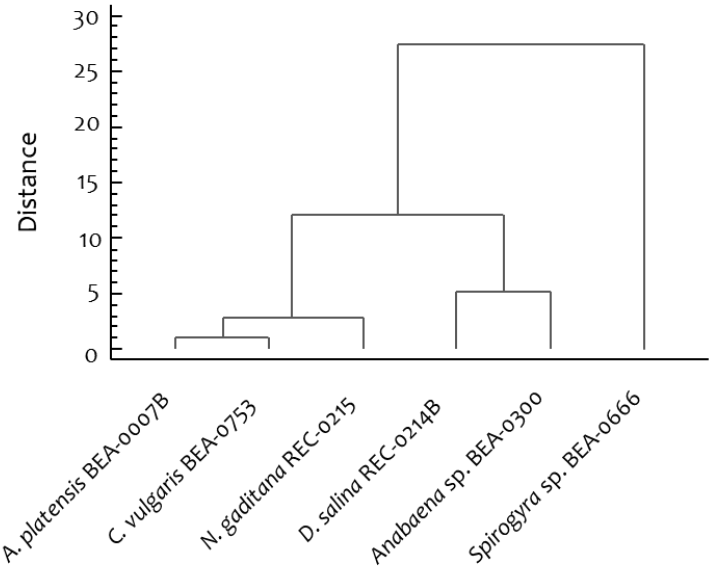

Figure 9. Dendrogram of the Euclidean distances between different microalgae and cyanobacteria. Cluster analysis was carried out using the complete data obtained in the *in vitro* assay.

Finally, the data on protein characterization and *in vitro* bioaccessibility were analysed using a Principal Component Analysis (PCA). Principal component (PC) scores were then used in the discrimination analysis to assign each sample to a particular group. A PCA was used here as a simple method to project data to a two-dimensional plane. The PCA revealed that the most influential factors to group the data were the amount of amino acids released (*AAR*) and their qualitative profile (*EAAR*/*NEAAR* ratio). The PCA results are shown in Figure 9, which captured 88% of the variance observed in the experiment in the first two PCs. Overall, the groups identified were same as those from the clustering analysis.

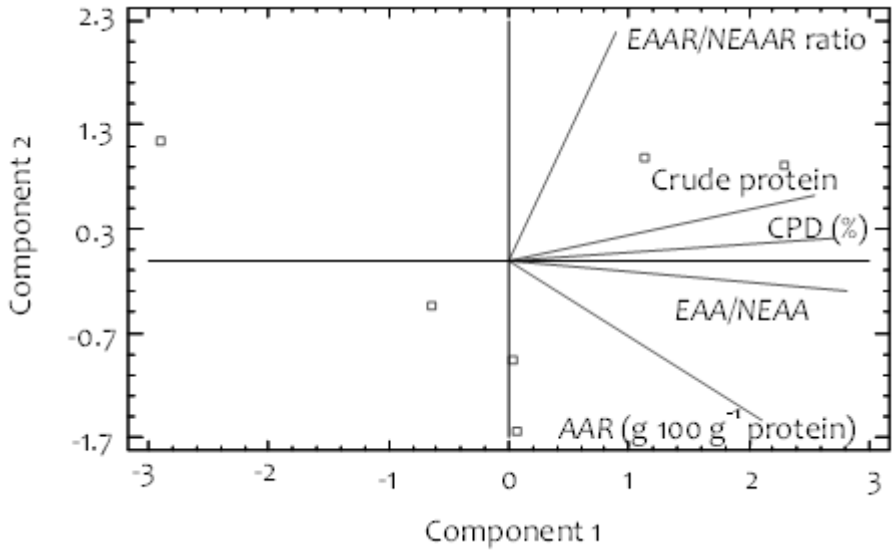

Figure 10. Principal Components Analysis (PCA) performed on data from microalgae and cyanobacteria protein characterization and *in vitro* bioaccessibility. *EAAR* essential amino acids released; *NEAAR:* non-essential amino acids released; *EAA:* essential amino acids; *NEAA:* non- essential amino acids; *AAR:* amino acids released; *CPD:* coefficient of protein degradation.

III.1.4. DISCUSSION

In the last decade, there has been an exponential increase in the number of studies aimed at evaluating microalgae as potential ingredients for aquafeeds (Shah *et al*., 2018). However, the characterization of algal biomass is still incomplete, especially in terms of protein and amino acid availability, as well as in the lack of knowledge on protein digestibility by marine fish.

The high protein content is one of the major advantages for using microalgae biomass as dietary ingredient for aquafeeds (Shah *et al*., 2018). In this piece of work, total protein content of the different microalgae and cyanobacteria species evaluated ranged from 25% to 61%. *Anabaena* sp. BEA-0300 and *D.* salina REC-0214B displayed the highest protein content (> 50%, DM), which agree with the values reported previously (Becker, 2007; Cheng *et al*., 2015). However, results obtained for *A. platensis* BEA-0007B or *N. gaditana* REC-0215 disagree with those found in the literature. Thus, some authors reported a higher protein content in these microalgae species reaching values within the range of 40 - 60% DW (Tibbetts *et al*., 2015; Batista *et al*., 2013; Teuling *et al*., 2019). On the other hand, *Spirogyra* sp. was the microalga with the lowest protein content (25.3%, DM), among the species tested, although this value was considerably higher than that reported by Harish *et al*. (2004). These dissimilar results might well be attributed to different culture conditions (Brown *et al*., 1997; Kumaran *et al*., 2021). Indeed, it is well-known phenomenon that the protein content increased when microalgae are grown in culture media rich in nitrogen (Batista *et al*., 2013).

Beyond protein content, however, the amino acid composition, specifically the content in essential amino acids, is a major quality criterion for determining the nutritional value of any alternative protein sources for aquafeeds (Webb & Chu, 1983). Roughly, all microalgae and cyanobacteria evaluated in this work showed very similar amino acid profiles, which agrees with Brown *et al*. (1997) who analysed 40 species of microalgae from six algal classes and pointed out that all species showed similar amino acid composition. As an intrinsic characteristic of microalgae, glutamic acid (2.06 – 5.70 g 100 g biomass^{-1}) and aspartic acid (1.64 – 5.65 g 100 g biomass^{-1}) were the predominant *NEAA* (Tibbets *et al*., 2015). Regarding *EAA*, it is widely acknowledged that lysine and

methionine are the most limiting amino acids in ingredients for aquafeeds, not least in land-based crops like grains, pulses and their derivatives (Mai *et al*., 2006; Tibbetts *et al*., 2015). In this work, the specific contents measured for these two amino acids were in the range of 1.0 - 2.9 g 100 g biomass^{-1} for lysine, and of 0.3 to 1.0 g 100 g biomass^{-1} for methionine, which are values similar to those reported previously by NRC (2011), ranging around 1.2 - 2.2 g 100 biomass^{-1} for lysine and 0.6 - 1.5 g 100 biomass^{-1} for methionine. Therefore, and based on the amino acid requirements of farmed fish (Wilson, 2003), all the microalgae and cyanobacteria studied could provide most of the required essential amino acids for ensuring adequate fish growth.

Besides protein content and amino acids profile, some alternative protein sources used in aquafeeds contain antinutritive factors such as protease inhibitors, which can exert negative effects on the digestion and absorption of nutrients, a fact that can reduce the nutritional value of aquafeeds (Alarcón *et al.,* 1999; Gatlin *et al.,* 2007; Vizcaíno *et al*., 2020). To date, scarce research has been done assessing the presence of protease inhibitors in microalgae, and their effect on the activity of fish digestive proteases (Diken *et al.,* 2016). The results obtained in this study revealed that considerably high inclusion level of microalgae would be needed to reach noticeable inhibition values. Thus, according to Martínez-Antequera *et al*. (2020), a juvenile fish of approximately 20 g body weight, total protease activity released after the intake would be around 1,000 and 1,300 activity units (UA). If fish were fed at 2% rate with an hypothetical feed supplemented with 15% microalgae, the microalgae to activity ratio would be around 60 µg of microalga UA^{-1}, which would account for less than 3% inhibition in the case of *N. gaditana* REC-0215, *Anabaena* sp. BEA-0300, *D. salina* REC-0214B or *A. platensis* BEA-0007B and less than 10% inhibition when using *C. vulgaris* BEA-0753 or *Spirogyra* sp. BEA-0666. In the worst-case scenery, it should also be taken into account that fish have mechanisms to overcome the effects of dietary antinutrients (Haard *et al.,* 1996; Santigosa *et al*., 2010). Consequently, it is likely that negligible effects would be expected on fish growth if microalgae were included at low level in practical feeding formulas.

This study also evaluated the digestive capacity of gilthead seabream proteases to hydrolyse microalgae proteins by using a species-specific *in vitro* protein digestibility assay. This *in vitro* model has been used previously for estimating other potential feedstuffs for aquafeeds, and it has proven useful not only in assessing the suitability of novel dietary ingredients for feeding fish (Alarcón *et al*., 2002; Vizcaíno *et al*., 2019), but also in greatly reducing the need to use of experimental animals in the preliminary evaluation of such ingredients.

Protein hydrolysis was monitored by electrophoretic separation, and the results confirmed, overall, that most of microalgae and cyanobacteria proteins were easily hydrolysed by digestive proteases of *S. aurata*. Thus, progressive and almost complete *in vitro* hydrolysis of the main protein fractions was observed in proteinograms, with the exception of two protein fractions (60-65 kDa and 20-25 kDa) that remained undegraded at the end of the *in vitro* assay. Coefficient of protein degradation (CPD) values ranged from 49.4 to 85.5%, which are similar to those described for other microalgae (Vizcaíno *et al*., 2019), as well as for other conventional raw materials, such as soybean protein concentrate or fishmeal (Sultana *et al*., 2010; Hernández *et al*., 2015). Likewise, the quantification of total free amino acids revealed significant differences among the biomasses evaluated. Thus, fish digestive proteases were able to release only 12.8% of total amino acids in *Spirogyra* sp. BEA-0666, but up to 20.8% in *D. salina* REC-0214B after the 90-min *in vitro* hydrolysis.

Broadly speaking, the results indicate high bioavailability of microalgae protein, although the significant differences found in protein hydrolysis among samples suggest the existence of several factors that might have played a role in such variability. On the one hand, the presence of cell walls in microalgae (characterized by great variability in the structure and composition among species), together with the limitations of the digestive physiology of fish, certainly influence protein digestibility (Kamalam *et al*., 2017; Bernaerts *et al*., 2018). Indeed, microalgae cell wall determines to which extent the intracellular nutrients are accessible to the digestive enzymes. On the other hand, the structure and spatial conformation of proteins itself can also determine their susceptibility to be hydrolysed, as is also the difference in amino acid

composition (Vizcaíno *et al*., 2019). The activity of digestive enzymes against proteins with different conformational structures, but similar amino acid composition, might result in different amino acid bioavailability (De la Higuera & Cardenete, 1993). Therefore, the analysis of amino acids released during protein hydrolysis by fish enzymes provides useful information about how balanced the biomass of a given microalga is, and consequently, the suitability for its inclusion in aquafeeds. The results obtained in this study revealed different profiles of essential and non-essential amino acid released (*EAAR* and *NEAAR*, respectively) in each biomass studied, in spite of the fact all of them had similar amino acid profiles in relative terms (Figure 7). Regarding essential amino acids, it is worth mentioning the high amount of lysine (Lys) released from *A. platensis* BEA-0007B, *D. salina* REC-0214B, and *N. gaditana* REC-0215, which is one of the most limiting amino acids in regular ingredients used in aquafeeds (Li *et al*., 2009). The same was found for arginine and branched-chain amino acids (leucine and valine), which play a key role in protein synthesis, immune function, and fish health (Hosseini *et al*., 2020; Ahmad *et al*., 2020).

Although most attention is usually paid to *EAAR*, however, the amount of *NEAAR* acids released, as well as the *EAAR* to *NEAAR* ratio (*EAAR/NEAAR*), are also important factors when it comes to achieving the highest efficiency in the use of dietary protein, thereby minimizing nitrogen excretion into the environment (Peres & Oliva-Teles, 2006). Indeed, if only essential amino acids were considered when formulating inert diets, the requirements of *NEAA*, or of non-specific N source used to synthesize non-essential amino acids, might not be completely fulfilled (Oliva-Teles *et al*., 2020).

In this context, *N. gaditana* REC-0215, *C. vulgaris* BEA-0753 and *A. platensis* BEA-0007B showed a well-balanced profile of released amino acids, yielding values for *EAAR/NEAAR* ratios close to 1 (0.95, 0.79 and 0.76, respectively), which is the optimal relation for fish nutrition (Oliva-Teles *et al*., 2020), while *D. salina* REC-0214B, *Anabaena* sp. BEA-0300 and *Spirogyra* sp. BEA-0666 showed ratios even higher than 1. According to Gómez-Requeni *et al*. (2003), the best growth performance in gilthead seabream is reached when feeding fish on diets that resemble the *EAA* profile and *EAA/NEAA* ratio found in muscle tissue.

Finally, the overall view of the whole results obtained in this study revealed that microalgae and cyanobacteria tested might be grouped in three different categories, based on all the data obtained from the different experiments, but mostly influenced by both the amount of amino acids released, and their qualitative profile (Figure 9). *N. gaditana* REC-0215, *C. vulgaris* BEA-0753 and *A. platensis* BEA-0007B were grouped together. In general, these species showed an optimal balance between their crude protein content and the bioaccessibility to the digestive enzymes of gilthead seabream juveniles, this fact suggesting that they could provide an adequate profile of free amino acids for further enterocyte absorption. Close to this group, *D. salina* REC-0214B and *Anabaena* sp. BEA-0300 were grouped, whereas *Spirogyra* sp. BEA-0666 appeared clearly distanced from both groups, likely owing to the poor results obtained in the in vitro digestive simulation.

In conclusion, the present study provides useful species-specific information of microalgae and cyanobacteria as potential protein ingredients for aquafeeds. The results obtained revealed that all the microalgae and cyanobacteria evaluated showed an adequate protein content, as well as a balanced amino acid profile, although significant differences were observed in their susceptibility to *S. aurata* digestive enzymes, and therefore in the bioavailability of their protein fraction for this fish species.

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III.2. ASSESSMENT OF DIETARY INCLUSION OF CRUDE OR HYDROLYSED *Arthrospira platensis* BIOMASS IN STARTER DIETS FOR GILTHEAD SEABREAM (*Sparus aurata*)

III.2.0. ABSTRACT

This work evaluates the effects of the dietary inclusion of crude or hydrolysed *Arthrospira platensis* (cyanobacteria) biomass on growth, muscle composition, digestive functionality and immune activities in gilthead seabream (*Sparus aurata*) fry (20.32 mg mean body weight). A 40-day feeding trial was conducted, aimed at assessing four experimental diets that included 5 or 10% (w/w) *A. platensis*, either crude or hydrolysed, plus a microalgae-free diet as control batch. Overall, none of the dietary treatments caused negative impacts on fish growth, body composition, muscle fatty acid profile, or innate immune response. Thus, the dietary inclusion of both crude and hydrolysed *A. platensis* reduced significantly the oxidation of muscle lipids, especially when using hydrolysed biomass, regardless of the dietary inclusion level. In relation to digestive enzymes, significantly higher levels of trypsin, chymotrypsin and leucine aminopeptidase activities were measured in fish fed on *A. platensis*-supplemented diets compared to control fish. In addition, within each inclusion level (5 or 10% w/w), those animals fed with diets that included the hydrolysed biomass yielded consistently higher digestive enzyme activities than those receiving the crude biomass. Microalgae dietary inclusion also induced favourable changes in fish gut morphology, according to the increase in *microvilli* length and diameter observed. This fact might well have contributed to reinforce the role of the intestinal mucosa as a protective barrier against microorganisms, as well as to enhance the absorptive capacity of the intestinal mucosa. Finally, 10% inclusion of microalgae hydrolysate enhanced lysozyme activity in liver, this fact suggesting improved protection against infectious diseases. In conclusion, the positive effects observed in fish fed with the diets including *A. platensis* up to 10% (not least the hydrolysed biomass) with regard to the different parameters assessed (digestive enzyme activities, intestinal epithelium ultrastructure, muscle lipid oxidation, and lysozyme activity) suggest the benefits of including this product in starter feeds for gilthead seabream fry.

III.2.1. INTRODUCTION

Early stages in the life cycle of marine fish are critical for the subsequent developmental changes taking place, in which fish undergo drastic morphological and physiological changes that determine further viability (Torres *et al*., 2020). Besides organ differentiation and morphogenesis, the larval stage is characterized by the highest growth rate throughout the productive cycle of commercial fish (Savoie *et al*., 2011). Consequently, considerable research effort has been made to develop inert microdiets that must fulfill certain premises for adequate larval development. Besides being tasty and economical, the most important of such requirements is to provide the necessary protein, amino acids, and fatty acids (Conceição *et al*., 2007; Vizcaíno *et al*., 2016; Khoa *et al*., 2019). These requirements have been traditionally met by the use of fishmeal and fish oil as the main protein and fatty acid sources, although plant protein ingredients, such as soybean meal, have also been included in feedstuffs (Ayala *et al*., 2020). However, in recent years, microalgae species and specific strains of cyanobacteria have emerged as a raw material of extraordinary interest in aquaculture (Shah *et al*., 2018; Han *et al*., 2019). Owing to their chemical composition, species of the genus *Arthrospira* are considered excellent candidates that have been successfully used as ingredients in feeds for several fish species (Mahmoud *et al*., 2018; Liu *et al*., 2019; Niccolai *et al*., 2019; Rajasekar *et al*., 2019). Despite this, the relatively high cost of microalgae, compared to conventional ingredients, limits their use in commercial aquafeeds (Yarnold *et al*., 2019). Even though cheaper than other microalgae species commercially available, the current price of freeze-dried *A. platensis* (around 15 USD kg^{-1}) is, by far, above the average price of fishmeal (in the region of 1.6-2.0 USD kg^{-1}), and therefore it is not yet feasible to replace fishmeal as the main ingredient in aquaculture feeds. Hence, the interest of microalgae as potential functional additive in aquafeeds, instead of a major dietary ingredient, is increasing considerably (Galafat *et al*., 2020).

On the other hand, microalgae inclusion in aquafeeds becomes more difficult in the case of early life stages because fish larvae cannot handle dietary components like juveniles. The scarce enzyme activity during these stages hinders digestion processes (Khoa *et al*., 2019). Namely the limited luminal protease activity affects directly protein digestion, a factor that has been pointed out as a major limitation to the utilization of diets by fish larvae (Cahu & Infante, 2001; Cai *et al*., 2015). In this regard, this work raises the question of whether protein hydrolysates obtained from microalgae could be used as dietary ingredient in weaning diets for marine fish larvae. Earlier, Galafat *et al*. (2020) corroborated the potentially positive physiological effects of *Arthrospira* sp. protein hydrolysates as bioactive additive in diets for juvenile gilthead seabream (*Sparus aurata*). According to this work, an enzymatic pre-treatment could release low molecular weight bioactive peptides and free amino acids that could be easily absorbed by enterocytes, this leading to earlier maturation of digestive organs, as well as to improved nutrient digestibility and acceptability by the animal (Srichanun *et al*., 2014). Beside this, a range of molecules that are known to be bioactive or so-called "nutraceutical" effects. These bioactive compounds might trigger host immune reactions in response to pathogen surface molecules, thus improving anti-inflammatory and antimicrobial abilities of the host (Novak *et al*., 2009), apparently by raising both the non-specific and the specific immunity of fish (Sahoo & Mukherjee, 2001). This fact might well be critical in early life stages in which the specific immunity is not well established, and the immune response of fish depends on non-specific activities (Uribe *et al*., 2011). It has been recently described an immunity communication between gut and liver in which more specific immune proteins and transcript were detected in gut and more non-specific immune molecules in liver (Wu *et al*., 2016). The potential role of microalgae enzymatic hydrolysates in aquafeeds on the abovementioned physiological phenomena at early stages of fish development remains unexplored. Therefore, this research is aimed at assessing the effects of low dietary inclusion levels of crude and hydrolysed *Arthrospira platensis* on fish growth performance, proximate composition, fatty acid composition, muscle

lipid oxidation, digestive functionality, and innate immune activities in gilthead seabream fry.

III.2.2. MATERIAL AND METHODS

III.2.2.1. Microalgae biomass

Crude biomass of *Arthrospira platensis* (crude protein: 65% dry weight, DW; crude lipid: 5%, DW) was provided by Biorizon Biotech (Almería, Spain). The term microalgae is used to refer to prokaryotic species such as cyanobacteria *A. platensis* (Cyanophyceae) with microscopic size. Cyanobacterium *Spirulina platensis* and *A. platensis* are considered as synonyms to each other as indicated by Guiry & Guiry (2018). The microalgal protein hydrolysate was produced from the crude raw biomass after performing an enzymatic hydrolysis following the method described by Saadaoui *et al*. (2019), as modified by Galafat *et al.* (2020). Briefly, a sludge containing 150 g L^{-1} microalgae biomass was incubated with 0.2% w/w mixture of commercial proteases (Alcalase 2.4L® and Flavourzyme 1000L® from Novozymes A/S, Bagsvaerd, Denmark) under controlled conditions (pH 8.0 and 50 °C under continuous stirring) during 4 h. Immediately after the hydrolysis, the reaction mixture was heated at 80 °C for 15 min in order to inactivate commercial proteolytic enzymes. The hydrolysate was then kept at 4 °C until use.

III.2.2.2. Experimental diets

Five iso-nitrogenous (59%, DW) and iso-lipidic (18.0%, DW) experimental diets were formulated; two of them contained 5 and 10% w/w microalgae raw biomass (designated as C-5 and C-10, respectively); two other experimental groups included 5 and 10% w/w hydrolysed biomass (designated as H-5 and H-10, respectively), and a fifth diet, microalgae-free, was used as the control batch (CT). The formulation and chemical composition of the experimental diets are shown in Tables 1, 2 and 3. The experimental diets were designed and manufactured by CEIMAR-University of Almería (Service of Experimental

Diets) (Almería, Spain) using standard aquafeed processing procedures. Particle size of the microdiets elaborated ranged from 0.3 to 0.8 mm.

Table 1. Ingredients and proximate composition (g kg^{-1} DW) of the experimental diets.

Ingredients	**CT**	**C-5**	**C-10**	**H-5**	**H-10**
Fishmeal LT94[1]	685.0	641.0	597.0	641.0	597.0
A. platensis meal[2]		50.0	100.0		
A. platensis hydrolysate[3]				50.0	100.0
Attractant premix[4]	80.0	80.0	80.0	80.0	80.0
Wheat gluten[5]	50.0	50.0	50.0	50.0	50.0
Soybean protein concentrate[6]	20.0	20.0	20.0	20.0	20.0
Fish oil	53.0	55.0	58.0	55.0	58.0
Soybean lecithin[7]	40.0	40.0	40.0	40.0	40.0
Choline chloride	2.0	2.0	2.0	2.0	2.0
Wheat meal[8]	17.0	9.0		9.0	
Betaine	2.0	2.0	2.0	2.0	2.0
Vitamins and minerals premix[9]	30.0	30.0	30.0	30.0	30.0
Vitamin C	1.0	1.0	1.0	1.0	1.0
Binder (alginate)	20.0	20.0	20.0	20.0	20.0
Proximate composition (% DW)					
Crude protein	58.9	59.1	59.3	59.3	59.4
Crude lipid	18.1	18.0	17.9	18.4	18.3
Ash	16.0	16.0	15.7	15.8	16.1
Crude fiber	2.1	1.9	2.3	2.0	2.2
NfE[10]	4.9	5.0	4.8	4.5	4.0

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. [1](protein: 69.4%; lipid: 12.3%), Norsildemel (Bergen, Norway); [2](protein: 60.5%; lipid: 5.6%); [3]Liquid product containing 150 g microalgae meal L^{-1}; [4](50% squid meal, 25% shrimp meal, 25% krill meal); [5](protein: 76.0%; lipid: 1.9%); [6](protein: 50.0%; lipid: 1.0%); [7]Lecico P700 IP (Lecico GmbH, Germany) [8](protein: 12.0%; lipid: 2.0%); [9]Vitamin & Mineral Premix: Vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2,000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E , 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500

mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain); [10]NfE: Nitrogen free extract calculated as 100 – (% crude protein + % ether extract + % ash + % crude fiber). DW: dry weight.

Table 2. Fatty acid composition (% of total fatty acids) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
14:0	3.0	3.0	3.1	2.8	2.9
16:0	16.0	16.8	16.5	16.8	16.4
18:0	2.8	2.7	2.7	2.7	2.8
18:1n-9	19.0	19.2	19.3	19.5	19.3
18:2n-6	12.6	12.9	12.7	13.4	13.0
18:3n-3	2.0	2.0	1.9	1.8	1.9
20:4n-6, ARA	0.9	0.9	0.9	0.9	0.9
20:5n-3, EPA	7.5	7.3	7.2	6.8	7.1
22:6n-3, DHA	10.8	10.6	10.4	10.3	10.5
SFA	21.9	22.5	22.2	22.3	22.1
HUFA	21.9	21.4	21.6	20.9	21.4
n-3	24.5	23.8	23.8	23.0	23.7
n-6	13.4	13.8	14.1	14.3	13.9
n-9	5.9	5.7	5.8	5.4	5.6

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. DW: dry weight. *SFA*: saturated fatty acids; *HUFA*: highly unsaturated fatty acids.

Table 3. Amino acids profile of *A. platensis* biomass and the experimental diets (g 100 g^{-1} DW).

	A. platensis	**CT**	**C-5**	**C-10**	**H-5**	**H-10**
NEAA						
Ala	5.0	3.6	3.2	3.7	3.3	3.6
Asp	6.5	5.3	4.9	4.7	4.9	4.8

Cys	0.3	0.3	0.2	0.3	0.2	0.3
Glu	8.9	8.9	7.9	8.8	8.0	8.5
Gly	3.3	3.6	3.2	3.5	3.2	3.4
Pro	4.9	5.1	4.5	4.7	4.5	4.8
Ser	3.4	2.6	2.4	2.6	2.4	2.5
Tyr	3.2	2.0	1.9	1.9	1.9	2.0
EAA						
Arg	4.1	3.4	3.2	3.5	3.2	3.4
His	1.2	1.3	1.3	1.2	1.2	1.3
Ile	3.1	2.1	2.0	2.4	2.0	2.3
Leu	5.1	4.0	3.8	4.1	3.8	4.1
Lys	4.2	5.6	5.2	5.4	5.1	5.4
Met	0.9	1.6	1.4	1.5	1.4	1.5
Phe	3.6	2.6	2.4	2.5	2.4	2.6
Thr	3.3	2.4	2.1	2.3	2.2	2.2
Val	3.4	2.5	2.3	2.8	2.4	2.7
EAA/NEAA	0.9	0.8	0.8	0.9	0.8	0.9

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. *EAA*: essential amino acids, *NEAA*: non-essential amino acids. DW: dry weight.

III.2.2.3. Fish and experimental design

Larval rearing took place at the Planta de Cultivos Marinos facility of the Instituto Español de Oceanografía (IEO, Puerto de Mazarrón, Murcia, Spain). Previously, fertilized eggs of gilthead seabream (*Sparus aurata*) were stocked in a 500 L cylindrical-conical incubator with gentle aeration until hatching. The incubator was supplied with ultraviolet-irradiated seawater at 19 ± 0.5 °C, same as spawning water temperature. Then, hatched larvae were transferred to a 5000-L tank where they began to be fed with rotifers (*Brachionus plicatilis*) once the mouth opening occurred (5 days post-hatching, dph) until 27 dph, at a density of 20 rotifers mL^{-1}. From 20 to 27 dph, *Artemia* nauplii at a concentration of 1 - 3 nauplii mL^{-1} were introduced in the tank, and *Artemia* metanauplii from 26 dph until the weaning period was completed (50 dph). From 40 to 50 dph, larvae were progressively weaned through a co-feeding

regime based on *Artemia* metanauplii and the inert commercial diet (Gemma Wean 0.2, Skretting). The amount of *Artemia* was progressively reduced from 3 to 0.5 *Artemia* mL^{-1}, whereas inert feed supply increased. After weaning, gilthead seabream larvae were transferred to 170 L tanks (510 larvae $tank^{-1}$; 3 larvae L^{-1}) in an open circulation system five days before the beginning of the feeding trial. UV sterilized seawater (38‰ salinity; 20 - 23°C) was provided into the tank system at an exchange rate of 150 L h^{-1}. Supplemental aeration was provided in order to maintain dissolved oxygen above 6.5 mg L^{-1}, and photoperiod was fixed on a 14 h light/10 h dark cycle (450 lx). Ammonia (< 0.1 mg L^{-1}), nitrite (< 0.2 mg L^{-1}), and nitrate (< 50 mg L^{-1}) were determined once weekly.

From 55 dph onwards, larvae (~ 24 mg, average initial body weight) were exclusively fed with the experimental diets to apparent visual satiation (*ad libitum*) six times daily. The different dietary treatments (CT, C-5, C-10, H-5 and H-10) were randomly assigned to the experimental tanks. Each dietary treatment was tested in triplicate (5 feeding treatments × 3 tanks per treatment) for 40 days. Feeding rate and feed size were equal in all tanks, and those were adjusted according to fish age, larval weight, and water temperature, following the recommendations for gilthead seabream fry provided by Skretting España (Burgos, Spain).

III.2.2.4. Fish sampling

At the beginning, larvae (55 dph) were individually weighed and measured after 12-h fasting. During the feeding trial, fasted fish from each tank were randomly sampled at 75 and 95 dph. In each sampling point, 100 fish per tank were weighed, measured and sacrificed by anaesthetic overdose (50 ppm clove oil) according to the requirements of the Council Directive 2010/63/UE. One pool of complete intestines (15 animals) from fish previously fasted for 6 h was prepared from each tank. The biological material was then processed to obtain crude extracts prior to determine digestive enzyme activities. In brief, intestines were manually homogenized in distilled water at 4 °C to a final

concentration of 0.5 g mL^{-1}. Supernatants were obtained after centrifugation (16,000 ×*g* for 12 min at 4 °C) and stored at −20 °C until further analysis. For proximate composition analysis, one hundred of 95 dph-fry carcasses (without viscera) per tank were freeze-dried. In addition, muscle samples (1 g) of a pool of ten fish per tank were used for lipid oxidation determinations, and the excess sample was freeze-dried for fatty acid composition. The intestines of three specimens from each tank were collected at 95 dph for examination by transmission (TEM) and scanning (SEM) electron microscopy. Finally, also at 95 dph, the liver of three fish from each tank were extracted, weighed, and individually kept at −80 °C until processing for the analysis of immune-related parameters.

III.2.2.5. Growth performance, nutrient utilization and somatic indices

Growth performance was assessed by different parameters according to the following formulae: daily gain (DG, g day^{-1}) = (W_f − W_i) / days; specific growth rate (SGR, % d^{-1}) = (Ln (W_f) − Ln (W_i) $days^{-1}$) × 100; condition factor (K) = (W_f (SL^3)$^{-1}$) × 100, where W_f was the final weight (g), W_i was the initial weight (g) and SL was the standard length. Somatic indices were also calculated: Hepatosomatic Index (HSI, %) = (liver weight (g) whole body weight (g)$^{-1}$) × 100, and Viscerosomatic Index (VSI, %) = (visceral weight (g) whole body weight (g)$^{-1}$) × 100.

III.2.2.6. Proximal composition and fatty acid profile

Chemical analysis of feeds and carcasses was carried out following the methods by AOAC (2000) for dry matter and ash, whereas crude protein (N × 6.25) was determined by using elemental analysis (C:H:N) with a Fisons EA 1108 analyser (Fisons Instruments, Beverly, MA, USA). Total lipid content was analysed following the procedures described by Folch (1957). Fatty acid profile of feeds and muscle samples was determined following the method of Rodríguez-Ruiz *et al*. (1998), using a gas chromatograph. For amino acid

analysis, microalgal biomass and experimental diets were hydrolysed (20 mg in 1 mL HCl 6M) at 110 °C during 24 h under an inert atmosphere (N_2). After that, 50 µL of the hydrolysate were mixed with 50 µL of 6 M NaOH. Then 100 µL of internal standard (2.5 mM norleucine) and 800 µL sodium citrate loading buffer (pH 2.2) were added and mixed by vortex for 5 s, and then filtered (0.2 µm). A sample (20 µL) of this mixture was analysed with an amino acid analyser Biochrom 30+ amino acid analyser (Biochrom LTD Cambridge, UK) according to the manufacturer's protocol.

III.2.2.7. Muscle lipid oxidation

Lipid oxidation in muscle samples was estimated by measuring thiobarbituric acid-reactive substances (TBARS) following the method of Buege & Aust (1978). Samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000×g, 20 min, 4 °C). Supernatants were mixed in a 1:5 ratio (v/v) with 2-thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6-di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl), heated for 15 min and centrifuged (3,600 ×g, 10 min, 4 °C). The absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

III.2.2.8. Digestive enzyme activities

Total alkaline protease activity was determined according to the method described by Alarcón *et al.* (1998) using buffered 5 g L^{-1} casein (50 mM Tris-HCl, pH 9.0) as substrate. The amount of enzyme releasing 1 µg tyrosine per minute was defined as one unit of activity, considering an extinction coefficient for tyrosine of 0.008 μg^{-1} cm^{-1} mL^{-1}, measured spectrophotometrically at 280 nm. Trypsin and chymotrypsin activities were measured spectrophotometrically at 405 nm using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-nitroanilide) as substrate, according to Erlanger *et al.* (1961), and 0.2 mM SAPNA (N-succinyl-

$(Ala)_2$-Pro-Phe-p-nitroanilide), following the method described by Del Mar *et al*. (1979), respectively. Leucine aminopeptidase activity was quantified using 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer (pH 8.8) as substrate according to Pfleiderer (1970). Alkaline phosphatase activity was determined using buffered p-nitrophenyl phosphate (pH 9.5) as substrate, following the method described by Bergmeyer (1974). For trypsin, chymotrypsin and leucine aminopeptidase activities, the amount of enzyme that released 1 μmol of p-nitroanilide (pNA) per minute was defined as one unit of activity, considering as extinction coefficient 8,800 M cm^{-1}, measured spectrophotometrically at 405 nm. For alkaline phosphatase, one unit of activity was defined as the amount of enzyme that released 1 μg of nitrophenyl per min (extinction coefficient 17,800 M cm^{-1} at 405 nm).

III.2.2.9. Ultrastructural analysis of the intestinal mucosa

Intestine samples from the anterior region were collected for evaluation by electron microscopy. Samples for scanning electron microscopy (SEM) were previously washed with 1% S-carboxymethyl-L-cysteine (Sigma) for 20 seconds in order to remove the epithelial mucus, and fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h. Then, samples were washed and dehydrated in graded ethanol. Critical point drying of samples (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain) was carried out by using absolute ethanol as intermediate fluid and CO_2 as transition fluid. After that, dried samples were mounted on supports and fixed with graphite (PELCO® Colloidal Graphite, Ted Pella INC., Ca, USA) and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). All samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). Samples for transmission electron microscopy (TEM) were fixed in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5 (4 h, 4 °C). Then, intestine sections were washed with PBS for 20 min, subjected to a post-fixation step with 20 g L^{-1} osmium tetroxide, and dehydrated by consecutive

immersion (20 min each) in ethanol solution of gradients from 50% to 100% (v/v). Samples were embedded for two hours in 1:1 Epon resin: absolute ethanol mixture under continuous shaking, and then included in pure Epon resin during 24 h, and polymerized at 60 °C. Finally, the ultrathin cuts were placed on 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C transmission electron microscopy (Carl Zeiss, Barcelona, Spain) at 100 Kv. Visualization fields were recorded at × 16,000 magnification.

SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software. *Microvilli* length (ML) and *microvilli* diameter (MD) as well as the number of *microvilli* within 1 µm distance (Vizcaíno *et al*., 2014) were determined in TEM micrographs. SEM images were used to obtain several measurements of enterocyte apical area (EA). Finally, data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno *et al*. (2014).

III.2.2.10. Antimicrobial activities in liver homogenates

Liver samples, stored at –80 °C, were homogenized (1 mg of tissue mL^{-1}) in phosphate buffer saline (PBS, pH 7,4) and protease, antiprotease, peroxidase, lysozyme and bactericidal activities were analysed in extracts. Protease activity was determined by measuring the hydrolysis of azocasein, according to the protocol by Charney & Tomarelly (1995) modified as described in Chaves-Pozo *et al*. (2019). Results were expressed as relative values, considering 100% protease activity that observed for a protease standard solution (10 µL of 2 mg mL^{-1} proteinase K (AppliChem) in PBS) under the specified assay conditions. Results were then expressed as % $µg^{-1}$ of tissue. The antiprotease activity was determined by the ability to inhibit proteinase K activity using a modification of the protocol described in Ellis (1990) as described elsewhere (Chaves-Pozo *et al*., 2019). The percentage of inhibition of proteinase K activity for each sample was calculated as 100 – (% of protease activity). Results were expressed

as % μg^{-1} of tissue. Peroxidase activity was measured in extracts according to Quade *et al*. (1997) and Chaves-Pozo *et al*. (2019). One unit was defined as the amount of activity producing an absorbance change of 1, and the activity was expressed as units (U) μg^{-1} of tissue. The lysozyme was measured using a modification of the turbidimetric method described by Parry *et al*. (1965), using 0.3 mg mL^{-1} freeze-dried *Micrococcus lysodeikticus* (Sigma M-3770) in phosphate citrate buffer (0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2) as substrate. The reduction in absorbance at 450 nm was measured immediately every 30 s during 15 min at 22°C in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. Results were expressed as units (U) μg^{-1} of tissue. Bactericidal activity was determined by evaluating their effects on the bacterial growth of *Vibrio harveyi*, as explained elsewhere (Sunyer *et al*., 1995, Chaves-Pozo *et al*., 2019). Results were corrected with the absorbance measured in each sample at the initial time point and expressed as % μg^{-1} of tissue.

III.2.2.11. Statistical analysis

Data were presented as mean ± SD. The effects of the different dietary treatments were determined by one-way ANOVA considering a significance level of 95% to indicate statistical difference ($p < 0.05$), followed by a generalized lineal statistical model (GLM analysis). Significant differences were determined using Tukey's multiple comparison test. Data with nonparametric distribution were analysed using Kruskal-Wallis one-way analysis. Also, discriminant analysis (DA) was conducted in digestive enzyme activities and morphometrical analysis of TEM and SEM images. The estimation of the validity of the discriminant function was based on the significance of Wilk's Lambda and the percentage of correct assignment to a certain diet. All statistical analyses were performed with Statgraphics Plus 4.0 (Rockville, Maryland, USA) software.

III.2.3. RESULTS

III.2.3.1. Growth performance and proximate composition

The evolution of fish growth during the feeding trial is shown in Figure 1. Dietary inclusion of *A. platensis* biomass, either crude or hydrolysed, did not affect fish growth or nutrient utilization. In fact, final body weight (FBW), daily gain (DG), specific growth rate (SGR) and Fulton's body condition factor (K) were similar in all the experimental groups (Table 4). Regarding somatic indices, hepatosomatic index (HSI) tended to decrease in fish fed on hydrolysed *Arthrospira*-supplemented diets, although significant differences with respect to CT group were only observed for H-10 lot ($p < 0.05$). Viscerosomatic index (VSI) remained unaffected.

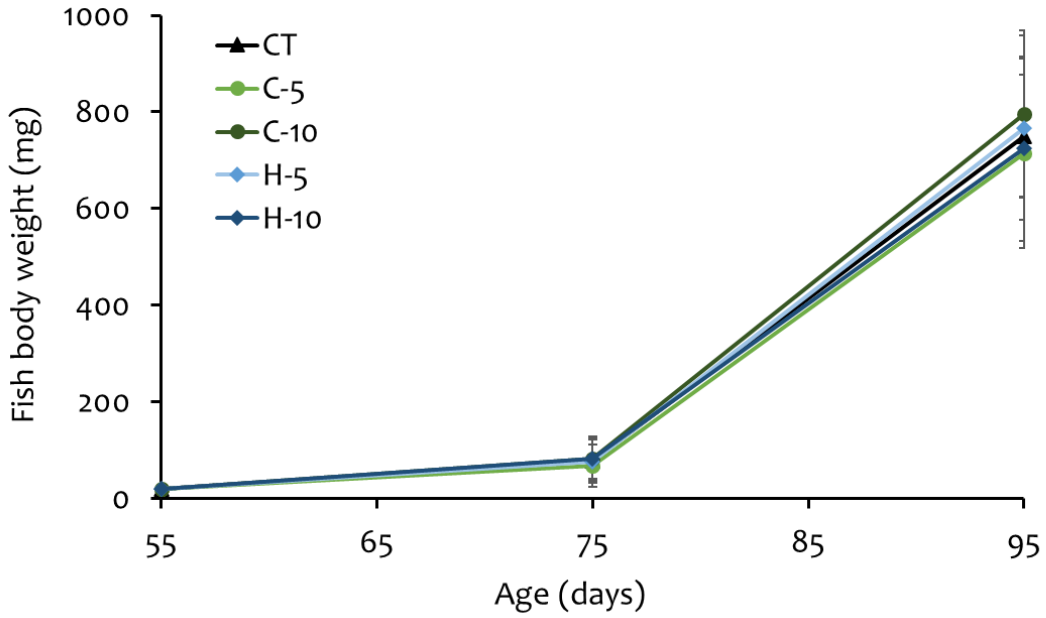

Figure 1. Final body weight of gilthead seabream fry fed with the experimental diets for 40 days. Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

The proximate composition of eviscerated fish is shown in Table 5. The inclusion of *Arthrospira* biomass, crude or hydrolysed, did not modify protein, lipid, or ash contents of gilthead seabream fry ($p < 0.05$).

Table 4. Carcass proximate composition (% of DW) of gilthead seabream at the end of the feeding trial.

	Protein	**Lipid**	**Ash**
CT	66.17 ± 0.16	15.24 ± 0.27	3.76 ± 0.02
C-5	65.81 ± 1.54	15.46 ± 0.28	3.65 ± 0.08
C-10	67.17 ± 0.04	15.23 ± 0.19	3.67 ± 0.21
H-5	65.08 ± 0.12	15.43 ± 0.37	3.63 ± 0.05
H-10	67.39 ± 0.02	15.53 ± 0.48	3.59 ± 0.04
p-value	*0.0882*	*0.7804*	*0.2192*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. DW: dry weight.

Table 5. Growth performance and nutrient utilization parameters of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*p-value*
Growth performance						
Final body weight (FBW, mg)	749.78 ± 126.28	714.23 ± 196.81	795.19 ± 173.62	766.79 ± 190.87	723.78 ± 190.95	*0.276*
Fulton's Condition Factor (K)	1.41 ± 0.12	1.36 ± 0.08	1.37 ± 0.03	1.48 ± 0.07	1.34 ± 0.04	*0.300*
Daily Gain (DG, mg day^{-1})	19.93 ± 2.94	16.10 ± 4.58	17.98 ± 4.04	17.31 ± 4.44	16.32 ± 0.44	*0.277*
Specific Growth Rate (SGR, % d^{-1})	8.17 ± 0.40	8.01 ± 0.65	8.29 ± 0.53	8.19 ± 0.59	8.05 ± 0.62	*0.215*
Somatic indices						
Hepatosomatic Index (HSI, %)	2.42 ± 0.42^{b}	2.29 ± 0.61^{b}	2.10 ± 0.63^{b}	1.95 ± 0.65^{ab}	1.77 ± 0.36^{a}	*<0.001*
Viscerosomatic Index (VSI, %)	6.30 ± 1.20	6.39 ± 1.12	6.23 ± 1.06	5.82 ± 1.00	6.22 ± 1.27	*0.296*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate tanks (n=3). Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

III.2.3.2. Fatty acid profile

Muscle fatty acid composition of gilthead seabream fry is shown in Table 6. All the experimental groups showed similar saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid contents ($p > 0.05$). PUFAs were the predominant lipid fraction (32.68 – 33.42%), followed by MUFAs (30.77 – 31.18%) and SFAs (22.97 – 24.90%). Considered individually, muscle content of myristic acid (C14:0) and palmitic acid (C16:0) was significantly lower in fish fed on H-10 and H-5 diets, respectively, compared to the rest of dietary treatments evaluated. However, fish fed with crude *A. platensis*-supplemented diets (C-5 and C-10) showed the highest values of palmitic acid. Regarding polyunsaturated fatty acids, the inclusion of microalgae hydrolysates (H-5 and H-10 diets) yielded a significant decrease in eicosapentaenoic acid (EPA, 20:5n-3) content in comparison with CT group ($p < 0.05$), whereas docosahexaenoic acid (DHA, 22:6n-3) tended to increase in these experimental groups, although no significant differences were observed. As a result, fish fed on diets containing microalgae hydrolysates (H-5 and H-10) showed lower *EPA*/*DHA* ratios ($p < 0.05$).

Table 6. Muscle fatty acid composition (% fatty acids) of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*p-value*
14:0	2.40 ± 0.07^{b}	2.39 ± 0.04^{b}	2.40 ± 0.07^{b}	2.33 ± 0.09^{ab}	2.14 ± 0.01^{a}	*0.029*
16:0	16.86 ± 0.12^{b}	17.25 ± 0.05^{c}	17.59 ± 0.26^{c}	16.27 ± 0.14^{a}	16.71 ± 0.01^{b}	*0.002*
18:0	4.99 ± 0.02	4.89 ± 0.14	4.90 ± 0.06	4.37 ± 0.02	4.57 ± 0.02	*0.594*
16:1n-7	4.59 ± 0.08	4.75 ± 0.18	4.78 ± 0.11	4.89 ± 0.04	4.87 ± 0.02	*0.153*
18:1n-9	17.82 ± 0.07	17.97 ± 0.16	18.24 ± 0.32	12.21 ± 0.12	18.16 ± 0.15	*0.254*
18:1n-7	3.26 ± 0.03	3.29 ± 0.03	3.25 ± 0.07	3.26 ± 0.01	3.24 ± 0.01	*0.729*
18:2n-6	10.81 ± 0.01^{a}	11.32 ± 0.17^{b}	11.70 ± 0.22^{b}	11.63 ± 0.06^{b}	11.72 ± 0.03^{b}	*0.003*
18:3n-3	1.44 ± 0.01	1.50 ± 0.02	1.53 ± 0.06	1.52 ± 0.02	1.45 ± 0.02	*0.087*
20:1n-9	5.10 ± 0.06	4.88 ± 0.07	4.81 ± 0.31	4.82 ± 0.06	4.57 ± 0.19	*0.180*
20:4n-6, ARA	0.46 ± 0.01	0.39 ± 0.01	0.42 ± 0.03	0.36 ± 0.02	0.38 ± 0.05	*0.069*
20:5n-3, EPA	5.13 ± 0.13^{c}	5.22 ± 0.06^{c}	4.99 ± 0.01^{c}	3.81 ± 0.03^{b}	3.44 ± 0.04^{a}	*<0.001*
22:5n-3	1.72 ± 0.04^{b}	1.70 ± 0.04^{ab}	1.66 ± 0.01^{ab}	1.60 ± 0.00^{a}	1.62 ± 0.01^{a}	*0.020*
22:6n-3, DHA	12.61 ± 0.34^{ab}	12.11 ± 0.39^{a}	11.94 ± 0.22^{a}	12.45 ± 0.12^{ab}	13.21 ± 0.01^{b}	*0.026*
SFA	24.25 ± 0.17	24.53 ± 0.15	24.90 ± 0.39	22.97 ± 0.25	23.41 ± 0.04	*0.489*
MUFA	30.77 ± 0.07	30.89 ± 0.44	31.08 ± 0.81	31.18 ± 0.16	30.85 ± 0.01	*0.845*
PUFA	33.35 ± 0.51	33.41 ± 0.43	33.42 ± 0.01	32.68 ± 0.06	33.07 ± 0.12	*0.605*
Other FA	9.94 ± 0.29^{ab}	9.42 ± 0.08^{ab}	8.64 ± 0.92^{a}	11.43 ± 0.35^{b}	10.83 ± 0.17^{b}	*0.010*
n-3	20.90 ± 0.51^{b}	20.53 ± 0.46^{ab}	20.12 ± 0.18^{ab}	19.39 ± 0.17^{a}	19.71 ± 0.06^{ab}	*0.029*
n-6	11.27 ± 0.00^{a}	11.71 ± 0.18^{ab}	12.11 ± 0.25^{b}	11.99 ± 0.07^{b}	12.11 ± 0.02^{b}	*0.007*
n-9	22.92 ± 0.12	22.85 ± 0.23	23.04 ± 0.63	23.04 ± 0.18	22.73 ± 0.05	*0.519*

n-3/n-6	1.85 ± 0.05	1.75 ± 0.07	1.66 ± 0.05	1.62 ± 0.02	1.63 ± 0.01	*0.595*
EPA/DHA	0.41 ± 0.00^{c}	0.43 ± 0.01^{d}	0.42 ± 0.01^{cd}	0.31 ± 0.00^{b}	0.26 ± 0.00^{a}	*<0.001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$). *SFA*: saturated fatty acids; *HUFA*: highly unsaturated fatty acids; *PUFA*: polyunsaturated fatty acids.

III.2.3.3. Muscle lipid oxidation (TBARS)

Results of muscle TBARS are shown in Figure 2. Overall, fish fed on microalgae-supplemented diets showed a significant decrease in TBARS values compared to CT group ($p < 0.05$), especially in C-10, H-5 and H-10 experimental groups.

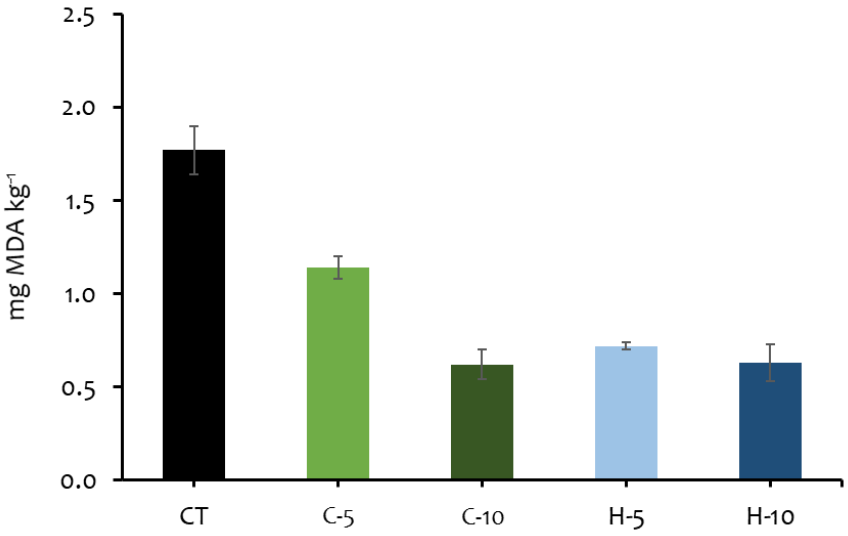

Figure 2. Muscle thiobarbituric acid-reactive substances (TBARS) content of gilthead seabream fry after the 40-day feeding trial. Values with different lowercase letter indicate significant differences among dietary treatments (mean ± SD, n = 12; $p < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

III.2.3.4. Digestive functionality

Intestinal enzyme activities were measured at two time points throughout the feeding trial (Figure 3). In general, the results obtained revealed that the dietary inclusion of crude and hydrolysed *A. platensis* had no adverse impact on the digestive enzymes evaluated, regardless of the dietary inclusion considered. Overall, the activity level of the different digestive enzymes evaluated increased significantly with larval age regardless of dietary treatment used ($p < 0.001$) (Table 7). At 75 dph, an increase of trypsin and

chymotrypsin activity levels was found in groups fed with crude and hydrolysed microalgae compared to control (CT) ($p > 0.001$). Moreover, results showed that the use of the hydrolysates (H-5 and H-10) increased the chymotrypsin and total alkaline protease activity levels compared to fish fed with crude microalgal biomass (chymotrypsin, $p = 0.001$; total alkaline protease, $p = 0.012$). Regarding brush border enzymes, the inclusion of the microalgae biomass increased significantly both leucine aminopeptidase ($p = 0.015$) and alkaline phosphatase ($p = 0.004$) activities. Fish fed on C-5 and H-10 diets showed higher leucine aminopeptidase activity than CT group. For alkaline phosphatase, all the experimental groups fed on *A. platensis* supplemented diets, especially H-10 group, displayed higher activity than fish receiving CT diet. At the end of the feeding trial (95 dph) only chymotrypsin, and leucine aminopeptidase activities increased owing to the inclusion of the microalga. C-5 and H-10 showed higher chymotrypsin activity levels than those observed in the control fish, whereas the increase of leucine aminopeptidase activity ($p < 0.05$) was observed in most groups fed on microalgae-supplemented diets, except C-10 group.

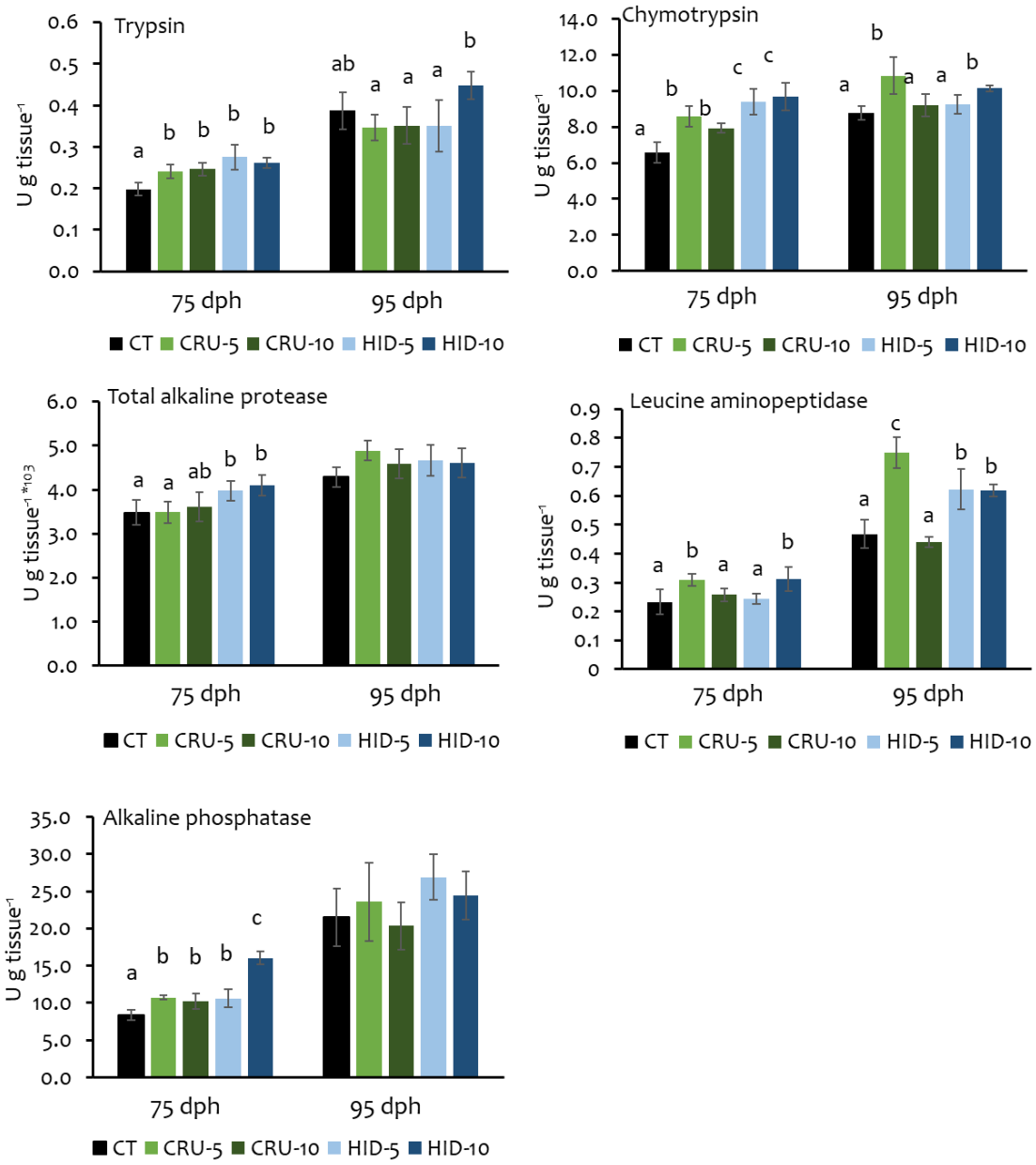

Figure 3. Enzyme activities measured in intestinal extracts of *S. aurata* fry fed with the experimental diets for 40 days. Values are mean ± SD (n=9, nine pools of intestines per dietary treatment). Values in the same day with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Table 7. Evaluation of different factors on digestive enzyme activities in the intestinal extracts of gilthead seabream at the end of the feeding trial.

	Age	Microalgae	Inclusion level	Hydrolysis treatment
Total alkaline protease	<0.001	0.0381	0.8467	0.2237
Trypsin	<0.001	0.419	0.3013	0.1461
Chymotrypsin	<0.001	<0.001	0.6056	0.0484
Leucine aminopeptidase	<0.001	0.0169	0.2618	0.7807
Alkaline phosphatase	<0.001	0.0952	0.5947	0.1142

Data are mean ± SD (n=9). Factors are: i) Age of fish larvae; ii) Microalgae (with or without microalgae inclusion), iii) Inclusion level (microalgae dietary inclusion level), and iv) Hydrolysis treatment (crude biomass vs hydrolysed biomass).

On the other hand, TEM and SEM images showed an intestinal mucosa with normal appearance in all the fish fed with the experimental diets. In general, a regular and densely packed *microvilli* on the enterocyte surface was observed in all the specimens without any sign of structural damage or *microvilli* alterations (Figures 4A and 4B).

Morphometric analysis of TEM and SEM images revealed that the dietary inclusion of both crude and hydrolysed *A. platensis* caused a significant effect on *microvilli* length, *microvilli* diameter, enterocyte apical area and absorption surface ($p < 0.001$) (Table 8). Overall, fish fed on microalgae-supplemented diets showed significantly increased *microvilli* length and *microvilli* diameter compared to fish of CT group, not least owing to the use of the microalgal hydrolysate ($p < 0.001$), regardless the dietary inclusion level. In addition, H-10 fed fish showed the highest values of enterocyte apical area (EA) and enterocyte absorption surface (TAS).

Table 8. Microvillar morphology of the anterior intestine of gilthead seabream fry fed with the experimental diets for 40 days.

	ML (μm)	MD (μm)	EA (μm²)	TAS (μm²)
CT	1.84 ± 0.16^{a}	0.12 ± 0.01^{a}	27.04 ± 3.21^{b}	1012.01 ± 157.51^{a}
C-5	2.06 ± 0.13^{b}	0.14 ± 0.01^{c}	22.56 ± 3.70^{a}	1089.77 ± 194.24^{a}
C-10	1.88 ± 0.19^{a}	0.14 ± 0.01^{c}	22.65 ± 3.64^{a}	1021.84 ± 148.08^{a}
H-5	2.09 ± 0.13^{b}	0.15 ± 0.01^{d}	22.92 ± 3.39^{a}	1079.58 ± 158.57^{a}
H-10	2.89 ± 0.43^{c}	0.13 ± 0.01^{b}	29.84 ± 2.94^{c}	1930.06 ± 311.07^{b}
p-value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. ML: *microvilli* length; MD: *microvilli* diameter; EA: enterocyte apical area; TAS: enterocyte absorption surface. Values are mean ± SD of 60 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

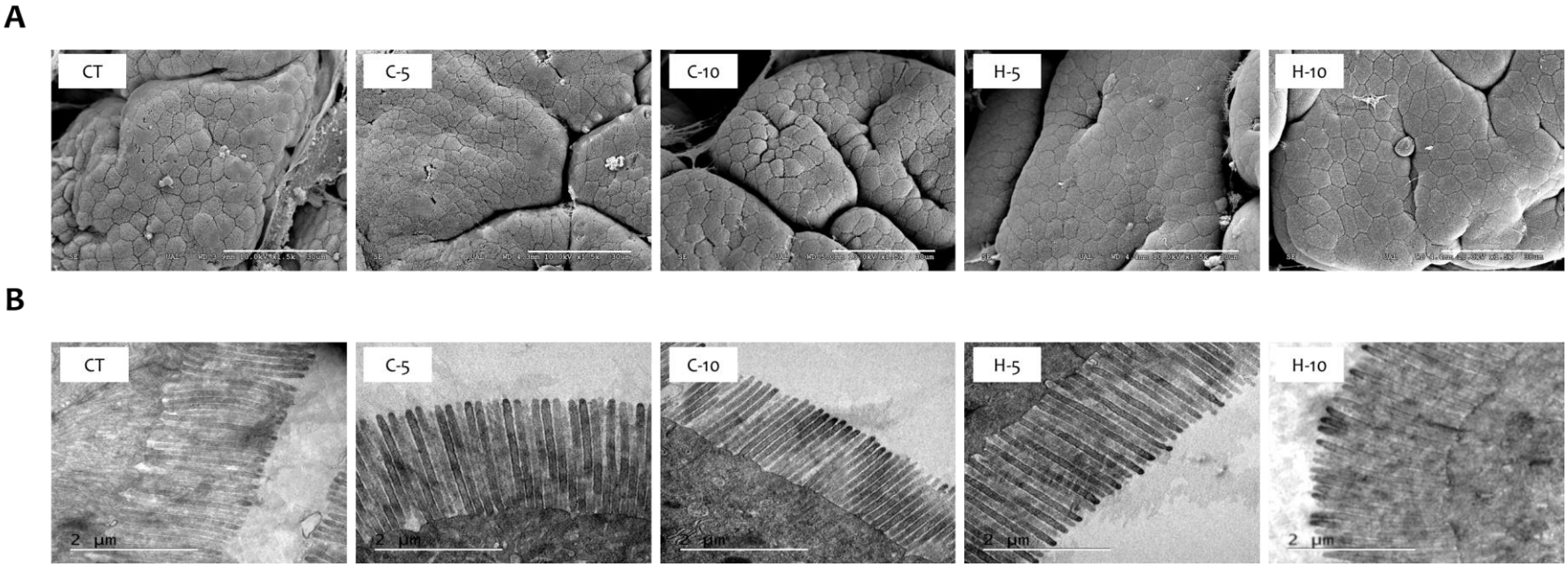

Figure 4. Comparative SEM (A) and TEM (B) micrographs from the anterior intestine of *S. aurata* fry at the end of the feeding trial. CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet (SEM bar: 30 µm; TEM bar: 2 µm).

Values of digestive enzyme activities and ultrastructural analysis of intestinal mucosa were analysed by discriminant analysis (DA) (Figure 5, Table 9). Grouping was recognized on the basis of the diet ingested by fish. DA confirmed that function 1 could discriminate clearly diet H-10, which was plotted much further to the right than the other dietary treatments. The second function provided certain discrimination between the CT diet and the microalgae-supplemented diets, but not nearly as clear as that provided by the first function.

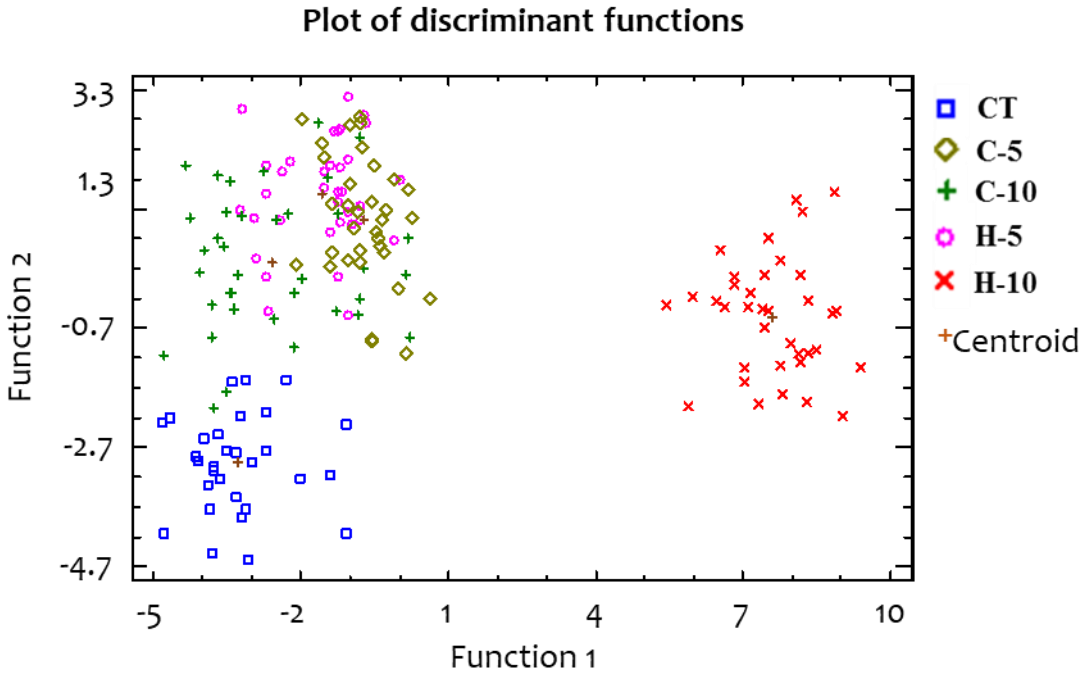

Figure 5. Plot of the first two discriminant functions established by the discriminant analysis (DA) of digestive functionality of *S. aurata* fry fed with the experimental diets.

Table 9. Summary of the results in the discriminant analysis (DA) of the different dietary treatments considering digestive enzyme activities and ultrastructural analysis of the intestinal mucosa as predictor variables.

Discriminant function	Eigenvalue	Relative percentage	Canonical correlation	Wilk´s λ	χ^2	Degree of freedom	*p-value*
1	8.31	81.45	0.945	0.0358	760.81	16	*<0.001*
2	1.83	17.98	0.805	0.3334	250.99	9	*<0.001*

III.2.3.5. Immune system activities

The immune activities analysed in liver extracts did not show significant differences between treatments ($p > 0.05$) (Table 10). These results indicate that the microalgae-supplemented diets did not cause any negative impact on the innate immune activities in the liver of gilthead seabream fry. On the contrary, protease activity tended to increase in fish fed on C-5 and H-10 diets, and lysozyme and bactericidal activities tended to increase in H-10 diet, although these changes were only significant for lysozyme activity (H-10 group), likely owing to the great variability observed between specimens.

Table 10. Antimicrobial activities in liver extracts of gilthead seabream fry fed with the experimental diets for 40 days.

	Antiprotease (% g tissue^{-1})	**Protease (% µg tissue^{-1})**	**Peroxidase (U µg tissue^{-1})**	**Lysozyme (U µg tissue^{-1})**	**Bactericidal activity (% µg tissue^{-1})**
CT	0.09 ± 0.04	0.02 ± 0.01	0.68 ± 0.06	0.05 ± 0.01^{a}	0.32 ± 0.12
C-5	0.09 ± 0.04	0.46 ± 0.24	0.86 ± 0.10	0.06 ± 0.01^{a}	0.48 ± 0.16
C-10	0.07 ± 0.02	0.03 ± 0.01	0.74 ± 0.07	0.05 ± 0.01^{a}	0.42 ± 0.09
H-5	0.08 ± 0.01	0.01 ± 0.00	0.67 ± 0.07	0.04 ± 0.00^{a}	0.37 ± 0.07
H-10	0.07 ± 0.03	0.34 ± 0.18	0.84 ± 0.11	0.16 ± 0.06^{b}	0.72 ± 0.21
p-value	*0.9851*	*0.1979*	*0.4330*	*0.0194*	*0.1564*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of 9 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

III.2.4. DISCUSSION

The dietary inclusion of microalgae hydrolysates emerges as a novel strategy in aquaculture nutrition. The enzymatic hydrolysis of microalgae biomass may increase extraction yields of high-value products or improve the bioavailability of intracellular components (Galafat *et al*., 2020; Siddik *et al*., 2021), and therefore an improvement on fish growth parameters derived from its dietary inclusion could be expected. However, the results obtained in this work revealed that the dietary inclusion of crude and hydrolysed *A. platensis* biomass had no significant effect on body growth parameters after a 40-day feeding trial. These results are in agreement with those reported by Galafat *et al*. (2020), who pointed out similar effects in gilthead seabream juveniles fed on *Arthrospira* sp. protein hydrolysate up to 4% inclusion level. Similarly, Ayala *et al*. (2020) did not observe differences in growth rates and feed intake in gilthead seabream juveniles attributable to the dietary inclusion of *N. gaditana* either raw or hydrolysed. On the contrary, other authors reported that low dietary inclusion of different microalgae species, such as *Tetraselmis suecica, Phaeodactylum* sp., *Nannochloropsis* sp., or *Chlorella* sp., in microdiets led to improved growth performance in gilthead seabream and Senegalese sole fry (Vizcaíno *et al*., 2016; Peixoto *et al*., 2021). These disparate results confirm that microalgae have a species-specific and dose-dependent effect on growth performance and nutrient utilization of farmed fish.

Regarding somatic indices, the dietary inclusion of hydrolysed *A. platensis* decreased hepatosomatic index (HSI), especially with the highest inclusion level. This reduction of HSI has been previously documented in several studies (Vizcaíno *et al*., 2016; Tulli *et al*., 2012), and seems to be related to the existence of a direct relationship with lipid metabolism. Thus, some microalgae compounds are apparently able to modulate to a certain extent lipid metabolism by increasing the efficiency of lipid mobilization from liver to muscle, especially in phases of rapid growth (Knutsen *et al*., 2019). However, the results obtained in this work indicate that the dietary inclusion of crude or hydrolysed *A. platensis* did not modify gilthead seabream fry body

composition, in line with earlier reports on juveniles of the same (Galafat *et al*., 2020), and other farmed fish species (Sorensen *et al*., 2017; Valente *et al*., 2019). The influence of diets on fish body composition can also be assessed by means of the muscle fatty acid profile (Fernandes *et al*., 2012). According to previous studies (Tibaldi *et al*. 2015; Cardinaletti *et al*., 2018; He *et al*., 2018), certain degree selective retention of specific fatty acids in the muscle of fish fed with microalgae-supplemented diets has been observed, especially EPA and DHA. Thus, muscle EPA contents were lower in fish fed on microalgae hydrolysate diets (H-5 and H-10) than those measured in the respective experimental diets, a fact that seems to be related to the modulating effect on lipid metabolism previously mentioned and with a higher catabolic use of this specific fatty acid (Tocher, 2003). Both EPA and DHA play a key role in fish development, and their dietary deficiency is related to adverse effects on fish growth and survival (Glencross, 2009; Tocher *et al*., 2010). In this work, EPA/DHA ratios in all the experimental diets ranged from 0.66 to 0.69, similar to the optimum (0.67) for gilthead seabream (Rodriguez *et al*., 1997), although this factor did not affect growth performance and nutrient utilization at the end of the feeding trial, as it has been observed in previous works (Vizcaíno *et al*., 2016; Atalah *et al*., 2007).

On the other hand, a significant reduction in MDA levels was observed in muscle of fry fed the microalgae-supplemented diets. This phenomenon might well be directly related to enhanced scavenging activity against reactive oxygen species, resulting in lower muscle lipid oxidation (Teimouri *et al*., 2019). Microalgae contain a wide range of bioactive compounds like astaxanthin and xanthophylls able to prevent oxidative stress, as previously observed in fish fed on diets supplemented not only with *Arthrospira* sp. (Abdelkhalek *et al*., 2015; Teimouri *et al*., 2019; Galafat *et al*. 2020), but also with other microalgae species (Qiao *et al*., 2019; Batista *et al*., 2020). It is worth mentioning that low dietary inclusion of *Arthrospira* hydrolysate (H-5) caused a notable reduction in muscle MDA level; in fact, similar to that observed in fish fed with 10% crude microalgal biomass. This could be explained by the fact that enzymatic hydrolysis might have facilitated the release of antioxidant compounds that

otherwise are less available within the intact cells (Liu *et al*., 2019; Afify *et al*., 2018).

In addition to the effects on growth and fish chemical composition, the nutritional value of first-feeding diets, combined with other biotic and abiotic factors, play a key role on the ontogeny of the digestive tract in marine fish larvae (Yufera *et al*., 2000; Zambonino-Infante & Cahu, 2001). Indeed, imbalanced diets can lead to a disruption in maturation processes, such as a reduction in pancreatic secretory capacity (Zambonino-Infante *et al*., 2008). The dietary inclusion of microalgae and cyanobacteria has been previously evaluated in different farmed fish species, and roughly, no detrimental effects have been described on proteolytic enzyme activity (Vizcaíno *et al*., 2016; Gong *et al*., 2019; Galafat *et al*., 2020). In this work, partial replacement of fish meal with crude and hydrolysed *A. platensis* did not impair the pancreatic secretion of proteolytic enzymes, quite the contrary, given that fish fed on microalgae-supplemented diets showed higher trypsin and chymotrypsin activity levels than fish fed a fishmeal:fish oil (FM:FO) diet, especially in the case of fish fed with the microalgae hydrolysate. Both enzymes play a key role in early life stages, since digestion relies mainly on these pancreatic enzymes until a gastric digestion was attained (Hamre *et al*., 2013; Khoa *et al*., 2019). These enzymes are modulated by dietary protein content, so that the increase in activity observed could be related to an increase in the availability of peptides and amino acids, which may stimulate the pancreatic secretion (Zambonin-Infante *et al*., 2008; Hamre *et al*., 2013). Indeed, Gisbert *et al*. (2018) evidenced differences in chymotrypsin activity in fish related to differences in the distribution of peptide molecular weight among hydrolysates obtained from fish, yeast and pig blood. Regarding brush border enzymes, our results revealed a lack of negative effects on leucine aminopeptidase and alkaline phosphatase activities in fish fed on *A. platensis* supplemented feeds. Fish from these experimental groups, especially H-5 and H-10, showed significantly increased activities for both enzymes, which seems to be related to adequate maturation of the intestine (Zambonino-Infante & Cahu, 2001).

Despite this, the improvement in fish digestive enzyme activities did not translate into increased growth performance. This finding might be explained by two different hypotheses; on the one hand, the increase in enzyme activity levels could be associated with a compensation mechanism against dietary changes (Santigosa *et al*., 2008); and on the other, it could be related to a saturation of the peptide transport system in the intestinal brush-border membrane. In fact, the use of protein hydrolysates may induce an overload of short peptides, and/or impaired utilization of the small peptides and free amino acids, which are used for energy production rather than for growth (Yúfera & Moyano, 2018).

In addition to the effects on the digestive enzyme activities, inadequate dietary and environmental conditions may interfere with the complex regulation mechanisms involved in epithelial development, disrupting epithelial integrity (Rønnestad *et al*., 2013). According to previous studies, the dietary inclusion of different species of microalgae exerts positive effects on the apical brush border integrity, such as increased *microvilli* length, enterocyte apical area, and increased absorption surface in enterocytes (Galafat *et al*., 2020; Vizcaíno *et al*., 2016; Araújo *et al*., 2016). In line with these studies, the results obtained revealed that the inclusion of *A. platensis* biomass caused favourable effects on gut morphology of gilthead seabream fry, especially in the case of the hydrolysed microalgae. Hence, changes observed in *microvilli* length and *microvilli* diameter might improve the contribution of the intestinal mucosa as a natural barrier, whereas increased total absorption surface observed in fish fed on 10% hydrolysed biomass might enhance the absorptive capacity of the intestinal mucosa (Vizcaíno *et al*., 2014).

Regarding the innate immune system, it has been reported that the dietary inclusion of crude microalgae could modulate slightly protease activity, peroxidase and antibacterial activities, but not lysozyme activity (Chaves-Pozo *et al*., 2014), while stimulated cellular immune activities were reported in some cases (Cerezuela *et al.*, 2012). In this study, no detrimental effects on the immune status of gilthead seabream larvae could be attributed to *A. platensis*-

supplemented diets, but on the contrary, fish fed on 10% hydrolysed microalgae showed increased lysozyme activity levels, this fact suggesting better defence against infectious diseases. However, the discrepancies observed among different studies assessing the impact of microalgae on the activity of the innate immune system suggest both a species-specific and dose-specific effect on the results reported.

In conclusion, the results obtained in this study indicate that *A. platensis* hydrolysate can be used as dietary ingredient in starter feeds for gilthead seabream fry. The use of enzymatically hydrolysed microalgae up to 10% inclusion level did not improve body growth parameters, but yielded positive effects on gut functionality, muscle lipid peroxidation, and lysozyme activity in liver, which seems to be associated with an increase in the nutritional and functional properties of this biomass compared with the raw microalgae. These positive effects confirm that this supplement can be included as a functional ingredient in starter feeds for gilthead seabream.

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III.3. EVALUATION OF AN *Arthrospira* sp. ENZYME HYDROLYSATE AS DIETARY ADDITIVE IN GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES

III.3.0. ABSTRACT

The aim of this work was to evaluate the effects of the dietary inclusion of *Arthrospira* sp. enzyme hydrolysate on gilthead seabream (*Sparus aurata*) juvenile in a 128-day feeding trial. Algal hydrolysate was tested at low inclusion level, namely 2 and 4%, against a control diet without *Arthrospira* sp. supplementation. At the end of the feeding trial, fish body weight was recorded for growth evaluation. No significant differences were found among the experimental groups regarding growth performance or nutrient utilization, despite the fact that those animals fed with diets enriched with *Arthrospira* displayed higher trypsin, chymotrypsin, and leucine aminopeptidase enzyme activities, compared to fish fed with control diet. The ultrastructural study of the intestinal mucosa also revealed increased *microvilli* length and absorptive capacity in fish with *Arthrospira* sp. diets, especially at 4% inclusion level. *Arthrospira* supplementation was also responsible for lower lipid oxidation in muscle tissue, as well as for remarkable colour differences in skin, compared to control animals. These results suggest, overall, that low dietary supplementation with this microalgal hydrolysate might improve nor only the intestinal ultrastructure and functionality but also muscle pigmentation and antioxidant capacity of juvenile gilthead seabream.

III.3.1. INTRODUCTION

The interest in microalgae has increased strongly in the last years, given that they have valuable potential for reducing the dependence on unsustainable conventional raw ingredients in aquafeeds (Shah *et al*., 2018). The use of microalgae in aquaculture can be approached from two perspectives: on one hand, taking into account their nutritive value as protein and lipid sources, and on the other, considering that microalgae also have plenty of substances with potential bioactive effects. Abundant literature on the first consideration is available, but it is likely that the main constraint for extensive utilization of microalgae consists of the fact that any satisfactory alternative feed ingredient must be able to supply comparable nutritional value at competitive cost. To date, this is far from being achieved, given that any large-scale practical utilization of microalgae relies on a significant reduction in production costs. With regard to the second aspect mentioned above, growing interest is currently being paid to the fact that microalgae can accumulate useful metabolites, normally at relatively low concentration, with potentially bioactive effects. Thereby, the interest in microalgae as potential nutraceutical additive in aquafeeds is increasing considerably (Chakraborty & Hancz, 2011; Cardinaletti *et al*., 2018; Shah *et al*., 2018).

Numerous studies have reported that microalgae can be used as dietary ingredient or additive in aquafeeds without exerting negative impacts on fish growth and nutrient utilization (De Cruz *et al*., 2018; Perez-Velazquez *et al*., 2018) and even yielding valuable effects on lipid metabolism (Robin & Vincent, 2003), fish gut functionality (Vizcaíno *et al*., 2016; 2018), and oxidative status and lipid utilization in different fish species (Kiron, 2012; Teimouri *et al*., 2013; Roy & Pal, 2015; Amer, 2016). In addition, positive effects have been reported in rainbow trout (*Oncorhynchus mykiss*) related to pigmentation attributes (Teimouri *et al*., 2013) and lipid peroxidation (Teimouri *et al*., 2016; 2019).

The genus *Arthrospira* (filamentous Cyanobacteria) is known for its high protein content, up to 70% on dry matter basis (Santigosa *et al*., 2011; Macias-Sancho *et al*., 2014; Ansarifard *et al*., 2018), with amino acid profiles comparable

to those found in some reference feed proteins (Becker, 2007). *Arthrospira* is also rich in polyunsaturated fatty acids (PUFAs), mainly gamma-linolenic acid (18:3n6) (Ronda *et al*., 2012), as well as in vitamins (A and B12), minerals, and pigments with acknowledged antioxidant activity, such as carotenoids (Pugh *et al*., 2001; El-Sheekh *et al*., 2014; Adel *et al*., 2016; Velasquez *et al*., 2016), phycobilins, and phycocyanins (Mahmoud *et al*., 2018; Takyar *et al*., 2019). Thus, the dietary inclusion of *Arthrospira* has been evaluated in different fish species (Hussein *et al*., 2013; Kim *et al*., 2013; Teimouri *et al*., 2013; Velasquez *et al*., 2016; De Cruz *et al*., 2018; Perez-Velazquez *et al*., 2018), these studies reporting, overall, a lack of negative effects on growth performance or nutrient utilization but even favourable impacts on fish physiology. However, the potential effects of *Arthrospira* on fish growth and objective quality parameters of Mediterranean fish species remain virtually unknown.

However, microalgae also display certain disadvantages from a nutritional point of view, such as the structure and composition of their cell wall, which is a protective barrier that reduces the bioavailability of the intracellular nutrients (Wu *et al*., 2017). The efficiency of marine animals to digest the cell walls depends on the carbohydrate composition, on how they are linked to each other, and on the existence of suitable digestive enzymes. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases, but carnivorous fish do not, and this fact should be taken into consideration when it comes to formulating aquafeeds. Consequently, it may be reasonable to think that any strategy aimed at improving the bioavailability of the inner compounds, not only of *Arthrospira* but of any other species, might enable to include microalgae at low inclusion level in aquafeeds. Several procedures have been evaluated with the aim of releasing inner components of microalgae (Tibbetts *et al*., 2017; Agboola *et al*., 2019; Teuling *et al*., 2019), but when it comes to large-scale cell lysis, enzymatic hydrolysis is one of the most promising strategies, not least owing to its economic viability. By following this procedure, even low inclusion level of enzyme-hydrolysed microalgae in aquafeeds might well improve the physiological aspects in fish in a manner similar to including higher amounts of raw microalgae (Tchorbanov &

Bozhkova, 1988). To our knowledge, despite the potential of this procedure to increase nutrient bioavailability and functional properties, the use of microalgae enzymatic hydrolysates in aquafeeds remains unexplored. Thus, the production of microalgal hydrolysates is a promising strategy that deserves further research efforts.

Protein hydrolysates are believed to be more effective than intact protein or free amino acids from a nutritional point of view. The enzymatic hydrolysis of proteins results in the formation of a mixture of free amino acids, di-, tri-, and oligopeptides, and enhances the occurrence of polar groups and the solubility of hydrolysate compounds. The dietary use of protein hydrolysates of different origins in some species of farmed fish has proved several positive bioactive effects, such as antioxidant, antimicrobial, or anti-inflammatory, and beneficial effects on the functionality of the intestinal mucosa (Leduc *et al*., 2018; Zamora-Sillero *et al*., 2018). In the case of algae protein, enzymatic hydrolysis could release low molecular weight bioactive peptides and free amino acids, which might enable not only increased bioavailability but also lead to potential positive physiological effects (Morris *et al*., 2007; Chalamaiah *et al*., 2012; Montone *et al*., 2018; Wang *et al*., 2018).

In this piece of research, we hypothesise that *Arthrospira* sp. enzyme hydrolysate might improve some parameters related to growth performance, muscle lipid oxidation, skin pigmentation, and digestive functionality of juvenile gilthead seabream when added a low dietary inclusion level. The overall objective of this study is focused specifically on the assessment of the potential effects of low level of microalgae protein hydrolysate as functional additive in practical diets for juvenile fish of this Mediterranean species.

III.3.2. MATERIALS AND METHODS

III.3.2.1. *Arthrospira* sp. hydrolysate

Arthrospira sp. hydrolysate was produced starting from a sludge containing up to 150 g L^{-1} of microalgae biomass after performing an enzymatic hydrolysis

with a mixture of commercial proteases under controlled conditions (pH 8.0 and 50°C under continuous stirring) for 4 h providing 0.2% w/w proteases (Alcalase 2.4 L and Flavourzyme 1000 L from Novozymes A/S, Denmark), following a modification of the method described by Saadaoui *et al*. (2019). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson Unit (AU) per gramme. One AU is the amount of enzyme, which, under standard conditions, digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one mequivalent of tyrosine released per minute. Flavourzyme 1000 L is a protease complex of *Aspergillus oryzae* that contains both endo- and exoprotease activities. It has an activity of 1.0 leucine aminopeptidase (LAPU) unit g^{-1}. One LAPU is the amount of enzyme that hydrolyses 1 µmol of leucine-p-nitroanilide per minute. Immediately after the hydrolysis, the reaction mixture was heated at 80 °C for 15 min in order to inactivate the proteolytic enzymes. Total free amino acids were quantified spectrophotometrically at 340 nm using L-leucine as standard (Church *et al*., 1983). In brief, triplicate samples of 50 µL were withdrawn from microalgal protein hydrolysate, and 50 µL of 20% trichloroacetic acid (TCA) were added with the purpose of stopping the enzyme reaction. Afterwards, protein precipitates were discarded by centrifugation (12,000 rpm, 15 min at 4 °C), and the supernatants were stored at – 20 °C until further analysis. Finally, SDS-PAGE (Laemmli, 1970) for crude *Arthrospira* sp. meal and its protein hydrolysate was performed in order to identify the protein fractions and their molecular weight.

III.3.2.2. Experimental diets

Three isonitrogenous (450 g crude protein kg^{-1}) and isolipidic (170 g crude lipid kg^{-1}) experimental feeds were formulated, control without microalgae (CT), plus AH-2 and AH-4, containing 2% and 4% (DM basis) *Arthrospira* sp. hydrolysate, respectively. The formulation and chemical composition of the

experimental diets are shown in Table 1. Before adding fish oil and diluted choline chloride, feed ingredients were finely ground and mixed in a vertical helix mixer (Sammic 13 M-11, 5-L capacity, Sammic SA, Spain) for 20 min. Then the algae hydrolysate was added, and water content was adjusted to provide 400 mL per kg of ingredient mixture to obtain a homogenous dough. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JS Conwell Ltd., New Zealand) in order to obtain 2- and 3- mm pellets. The feeds were dried in a 12-m^3 drying chamber with forced-air circulation (Airfrio, Spain) at 30°C for 24 h and stored at –20 °C until use. An attractant premix was added (50 g kg^{-1}) to improve feed palatability (according to Barroso *et al.*, 2013). The experimental diets were produced by LifeBioencapsulation SL (Spin-off, Universidad de Almería, Spain).

III.3.2.3. Feeding trial and sampling

Feeding trial was carried out at the Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, Universidad de Cádiz, Puerto Real, Spain). All experimental procedures complied with the Guidelines of the European Union (Directive 2010/63/UE) regarding the use of laboratory animals. The competent Ethical Committee approved the experimental procedures involving the use of fish (Junta de Andalucía, reference number 06/02/2020/011). A total of 180 gilthead seabream juveniles (20 g average body weight) were selected and randomly distributed in 9 tanks (triplicate tanks per dietary treatment) of 75-L capacity (400 g average biomass per tank). Fish were fed with a commercial diet (Skretting España, 45% crude protein, 19% crude lipid) during a 15-day acclimation period. Afterwards, experimental diets were offered twice per day (9:00 and 17:00) at 2% of the biomass, until triplication of the initial body weight. The 128- day feeding trial was carried out in a flow-through filtered (1 µm) seawater system sterilized by UV, under constant temperature (19.0 ± 1.1°C), salinity (35 ± 1 ‰), and natural photoperiod (light:dark, LD; from 11:13 h in February to 13:11 h in May).

Table 1. Ingredient composition and proximate composition (g kg^{-1} on dry matter basis) of the experimental diets.

Ingredients	**CT**	**AH-2**	**AH-4**
Fishmeal[1]	374.2	358.4	340.6
Arthrospira sp. hydrolysate (g dry matter)[2]		20	40
Attractant premix[3]	50	50	50
Wheat gluten[4]	95	95	95
Soybean meal[5]	165	165	165
Fish oil	72.8	73.9	74.9
Soybean oil	28	28	28
Wheat flour[6]	170	164.7	161.5
Betaine	5	5	5
Vitamins and minerals premix[7]	20	20	20
Binder (guar gum)[8]	20	20	20
Proximate composition (%)			
Crude protein	449.9	450.3	449.5
Crude lipid	169.7	170.1	170.3
Ash	70.8	69.2	67.2
Crude fibre	34.7	34.6	34.5
N*f*E[9]	274.8	275.9	278.5

CT control diet, AH-2 2% *Arthrospira* hydrolysate-supplemented diet, AH-4 4% *Arthrospira* hydrolysate-supplemented diet.

[1] Protein, 69.4%; lipid, 12.3%; Norsildemel (Bergen, Norway).

[2] Liquid product containing 150 g microalgae meal L^{-1}.

[3] 50% squid meal, 25% shrimp meal, 25% krill meal.

[4] Protein, 76.0%; lipid, 1.9%.

[5] Protein, 50.0%; lipid, 1.0%.

[6] Protein, 12.0%; lipid, 2.0%.

[7] Vitamin and mineral premix: vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K_3 (menadione sodium bisulphite), 2500 mg; vitamin B_1(thiamine hydrochloride), 3000 mg; vitamin B_2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B_6 (pyridoxine hydrochloride), 2000 mg; vitamin B_9 (folic acid), 1500 mg; vitamin B_{12} (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (LifeBioencapsulation premix).

[8] EPSA (Sevilla, Spain).

[9] N*f*E nitrogen-free extract calculated as 100 – (% crude protein + % ether extract + % ash + % crude fibre).

The water in all the tanks was oxygen saturated (> 90% O_2 saturation) with air stones. Water ammonia (< 0.1 mg L^{-1}), nitrite (< 0.2 mg L^{-1}), and nitrate (< 50 mg L^{-1}) were determined with commercial kits (SERA GmbH, Heinsberg, Germany).

Fish were individually weighed every 2 weeks after a 24-h fasting period in order to determine growth and feed utilization parameters. At the end of the feeding trial, 16 fish per tank were killed by anaesthetic overdose (200 mg L^{-1} clove oil) followed by spine severing. Sampled fish were dissected, and the digestive tract and dorsal muscle were removed. Dorsal muscle samples were carefully washed, dried, and packaged in transparent sterile polyethylene bags and stored in a cold room (4 ± 1°C). Muscle samples were withdrawn from each dietary treatment at day 1 and 8 of cold storage. Colour parameters were determined on the right side of the anterior dorsal skin of fish, and then a portion of muscle tissue (5 g) was used for lipid oxidation determination (TBARS). The rest of individual muscle samples were freeze-dried and stored at −20 °C for further analysis of muscle proximate composition. For digestive enzyme activity determinations, intestines from nine fish per tank were randomly pooled to obtain three enzymatic extracts from each experimental tank. Intestine samples were homogenized in distilled water at 4°C (0.5 g mL^{-1}). Supernatants were obtained after centrifugation (11,200 ×g, 12 min, 4°C) and stored in aliquots at −20 °C until further use. Total soluble protein was determined using bovine serum albumin as standard (according to Bradford, 1976). Finally, the intestines of three specimens from each tank were collected for examination by transmission (TEM) and scanning (SEM) electron microscopy.

III.3.2.4. Growth performance, nutrient utilization, and somatic indices

Growth performance was assessed by different parameters according to the following formulae: Daily Gain (DG, g day^{-1}) = (W_f − W_i) $days^{-1}$; Specific Growth Rate (SGR, %) = ((Ln W_f − Ln W_i) $days^{-1}$ × 100; Condition Factor (K) = (W_f (SL^3)$^{-1}$) × 100, where W_f was the final weight (g), W_i was the initial weight (g),

and SL was the standard length. Nutrient utilization indices were estimated as follows: feed conversion ratio (FCR) = total feed intake on dry basis (g) weight gain $(g)^{-1}$ and protein efficiency ratio (PER) =WG total protein ingested $(g)^{-1}$, where WG was the weight gain (g).

III.3.2.5. Proximate composition

Proximate analysis of feeds and muscle samples were carried according to AOAC (2000) for dry matter and ash, whereas crude protein (N × 6.25) was determined by using elemental analysis (C:H:N) (Fisons EA 1108 analyzer, Fisons Instruments, USA). Total lipid content was analysed following the procedure described by Folch (1957).

III.3.2.6. Skin colour determinations

Instrumental colour was measured in triplicate on the right side of the dorsal fish skin by *L**, *a**, and *b** system (CIE 1978), using a Minolta chroma meter CR-400 (Minolta, Japan). The lightness (*L**, on a 0–100 point scale from black to white), redness (*a**, estimates the position between red, positive values, and green, negative values), and yellowness (*b**, estimates the position between yellow, positive values, and blue, negative values) were recorded.

III.3.2.7. Muscle lipid oxidation

Lipid oxidation in muscle samples was estimated by thiobarbituric acid-reactive substances (TBARS) at 1 and 8 dpm (days post-*mortem*). TBARS were measured in muscle samples according to the method of Buege & Aust (1978). Samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000 ×g, 20min, 4°C). Supernatants were mixed in a 1:5 ratio (v/v) with 2-tiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6-di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl). The mixture was heated for 15 min and centrifuged (3600 ×g, 10 min, 4°C), and the absorbance of supernatants was

measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

III.3.2.8. Digestive enzyme activities

Total alkaline protease activity in the digestive extracts was spectrophotometrically determined using 5 g L^{-1} casein in 50 mM Tris-HCl (pH 9.0) as substrate, according to Alarcón *et al*. (1998). One unit of total protease activity was defined as the amount of enzyme that released 1 µg of tyrosine per minute in the reaction mixture, considering an extinction coefficient or tyrosine of 0.008 μg^{-1} mL^{-1} cm^{-1}, measured at 280 nm. Trypsin and chymotrypsin activities were determined by using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-p-nitroanilide) as substrate, according to Erlanger *et al*. (1961), and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-p-nitroanilide) according to Del Mar *et al*. (1979); both substrates dissolved in 50 mM Tris-HCl and 10 mM $CaCl_2$ buffer (pH 8.5). Leucine aminopeptidase activity was quantified according to Pfleiderer (1970), using 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer, pH 8.8, as substrate. For trypsin, chymotrypsin, and leucine aminopeptidase activities, one unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 µmol of p-nitroanilide (pNA) per minute, measured spectrophotometrically at 405 nm, considering an extinction coefficient of 8,800 M cm^{-1}. Alkaline phosphatase activity was determined by using p-nitrophenyl phosphate in 1 M diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5, as substrate, following the method described in Bergmeyer (1974). For alkaline phosphatase, one unit of enzyme activity was defined as the amount of enzyme that releases 1 µg of nitrophenyl per min, considering a molar extinction coefficient of 17,800 M cm^{-1} for p-nitrophenol measured at 405 nm.

III.3.2.9. Ultrastructural study of the intestinal mucosa

Intestine samples were collected for electron microscopy evaluation. Samples for scanning electron microscopy (SEM) were previously washed with 1% S-carboxymethyl-L-cysteine (Sigma Chem.) for 20 s, with the aim of removing the epithelial mucus. Afterwards, the samples were fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h, after which samples were washed and dehydrated in graded ethanol. Then samples were critical point dried with absolute ethanol as intermediate fluid and CO_2 as transition fluid (CDP 030 Critical point dryer, Leica Microsystems, Spain). After drying, specimens were mounted on supports and fixed with graphite (PELCO Colloidal Graphite, Ted Pella INC., USA) and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). Samples for transmission electron microscopy (TEM) were fixed (4 h, 4°C) in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5. Next, intestine sections were washed with PBS for 20 min, and then, a post-fixation step with 20 g L^{-1} osmium tetroxide was carried out. Then, samples were dehydrated by consecutive immersion (20 min each) in ethanol solution of gradients ranging from 50 to 100% (v/v). Next, samples were embedded for 2 h, in 1:1 mixture of Epon resin and 100% (v/v) ethanol under continuous shaking, and then they were included in pure Epon resin and polymerized at 60°C. Finally, the ultrathin sections were placed on a 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C TEM at 100 Kv (Carl Zeiss, Spain). Visualization fields were recorded at ×16,000 magnification. SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software (University of Texas Health Science Center, San Antonio, TX, USA). *Microvilli* length (ML) and *microvilli* diameter (MD) and the number of *microvilli* within 1 µm distance (Vizcaíno *et al*., 2014) were determined in TEM micrographs. SEM images were used to obtain several measurements of enterocyte apical area (EA). Finally,

data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno *et al*. (2014).

III.3.2.10. Statistical analysis

All assays were repeated at least three times with three replicates. Data were expressed as mean ± SE. Comparison of means was carried out by one-way ANOVA with a 5% level of probability ($p < 0.05$) followed by a multiple comparison test. Data in percentage (%) were arcsine$^{(1/2)}$-transformed, checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). When the data did not meet the ANOVA assumptions, a Kruskal-Wallis one-way analysis of variance on ranks was used. When the Kruskal-Wallis test showed significance, and Dunn's method of multiple comparisons was used to compare individual medians. All statistical analyses were performed with Statgraphics Plus 4.0 (USA) software.

III.3.3. RESULTS

III.3.3.1. Characterization of the protein hydrolysate of *Arthrospira* sp.

Figure 1 shows the proteinograms of raw *Arthrospira* sp. meal and its protein hydrolysate. Raw meal showed a complex protein profile made up of several fractions with a wide range from 13 to 86 kDa. However, microalgal protein hydrolysate shows only two protein fractions (57 and 39 kDa) in the range of molecular weight visualized in the proteinogram. In addition, quantification of total free amino acids revealed that *Arthrospira* sp. hydrolysate contained higher level of free amino acids (84.06 ± 3.23 mg leucine equivalents 100 mg^{-1} protein) than those found in the raw microalgae biomass (31.5 ± 3.09 mg leucine equivalents 100 mg^{-1} protein).

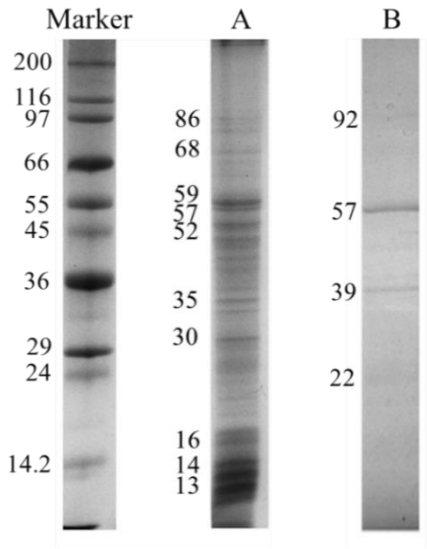

Figure 1. SDS-PAGE of the raw *Arthrospira* sp. biomass (A) and its protein hydrolysate (B). Numbers at the left of proteinogram indicate the molecular weight (kDa) of the main proteins identified. Marker: 5 µL of wide range molecular weight marker (S-84445, Sigma, St. Louis, USA) ranging from 6.5 kDa (aprotinin, bovine lung) to 200 kDa (myosin, porcine heart).

III.3.3.2. Growth performance and nutrient utilization

Growth of gilthead seabream juveniles fed experimental diets for 128 days is shown in Figure 2.

All dietary groups showed similar final body weight, DG, and SGR, without significant differences ($p > 0.05$), although mean values were slightly lower in AH-2 and AH-4 groups. Similarly, no significant differences were observed in FCR and PER mean values (Table 2).

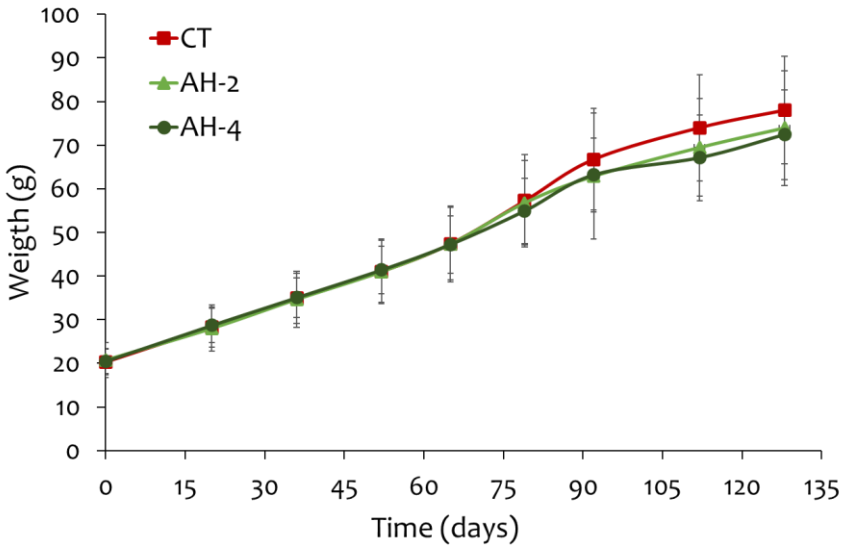

Figure 2. Time course of changes in body weight of fish fed with the experimental diets. *CT*: control, *AH-2*: 2% *Arthrospira* hydrolysate, *AH-4*: 4% *Arthrospira* hydrolysate.

Table 2. Growth performance and nutrient utilization parameters of gilthead seabream juveniles fed with the experimental diets during the 128-day feeding trial.

	CT	**AH-2**	**AH-4**	*p-value*
Growth and nutrient utilization				
Initial body weight (g)	20.32 ± 0.48	20.80 ± 0.52	20.51 ± 0.36	*0.7701*
Final body weight (g)	79.09 ± 1.75	75.80 ± 1.38	74.06 ± 1.15	*0.0529*
Fulton's condition factor	1.80 ± 0.03	1.81 ± 0.05	1.77 ± 0.04	*0.9404*
Daily gain (DG, g day^{-1})	0.46 ± 0.06	0.43 ± 0.01	0.42 ± 0.02	*0.5803*
Specific Growth Rate (SGR)	1.05 ± 0.13	0.99 ± 0.04	0.98 ± 0.05	*0.8948*
Feed Conversion Ratio (FCR)	0.47 ± 0.03	0.41 ± 0.04	0.42 ± 0.05	*0.5789*
Protein efficiency ratio (PER)	2.15 ± 0.15	2.49 ± 0.24	2.45 ± 0.33	*0.6054*

CT: control, AH-2: 2% *Arthrospira* hydrolysate, AH-4: 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate tanks.

III.3.3.3. Muscle proximate composition

Muscle chemical composition is shown in Table 3. Protein content increased significantly in fish fed with diets supplemented with *Arthrospira* hydrolysate, especially in AH-2 group, whereas a significant decrease in the lipid content

was observed in fish fed with AH-2 and AH-4 diets. Moisture and ash content were similar among dietary treatments.

Table 3. Muscle proximate composition (g kg^{-1} dry weight) and moisture (%) of gilthead seabream at the end of the feeding trial.

	CT	AH-2	AH-4	*p-value*
Total protein	745.31 ± 3.83 [a]	789.38 ± 0.51 [c]	772.19 ± 0.77 [b]	*0.035*
Total lipid	166.65 ± 2.36 [c]	141.72 ± 1.02 [a]	157.61 ± 0.57 [b]	*<0.001*
Ash	54.06 ± 3.32	55.23 ± 1.87	54.82 ± 2.13	*0.114*

CT: control, AH-2: 2% *Arthrospira* hydrolysate, AH-4: 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determination (n = 3). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$).

III.3.3.4. Instrumental skin colour determinations

Initial L* values were significantly higher in AH-2-fed fish, compared to CT and AH-4 groups (Table 4). After 8 days of cold storage, values remained stable in AH-2 and AH-4, whereas in CT significantly decreased ($p < 0.001$). CT presented a* negative values indicating a skin greenish coloration. However, values for AH-2 and AH-4 were positive which evidenced a slightly red coloration, though they decreased significantly at 8 dpm (p= 0.040 and <0.001). Skin b* values in CT were positive and sharply decreased during storage refrigeration. Nevertheless, values of AH-2 and AH-4 significantly increased, indicating a yellowish colour of the skin.

III.3.3.5. Muscle lipid oxidation (TBARS)

Muscle TBARS content in CT group showed significantly higher values (Table 4). Muscle lipid oxidation increased during cold storage (p = 0.015 and p = 0.019), although TBARS values were significantly lower at any sampling time in specimens fed with *Arthrospira* hydrolysate compared to CT fish (p = 0.019 and p = 0.015).

Table 4. Changes in skin colour and muscle TBARS content during cold storage in gilthead seabream fed the experimental diets for 128 days.

		CT	AH-2	AH-4	*p-value*
L*	1	76.4 ± 1.8^{aB}	83.3 ± 1.9^{b}	74.8 ± 1.3^{a}	0.009
	8	59.6 ± 2.6^{aA}	77.6 ± 2.4^{b}	76.5 ± 1.4^{b}	<0.001
	p	*<0.001*	*0.828*	*0.072*	
a*	1	-1.3 ± 0.4^{c}	2.3 ± 0.2^{bA}	3.5 ± 0.3^{aA}	<0.001
	8	-1.3 ± 0.2	1.9 ± 0.2^{B}	1.6 ± 0.3^{B}	0.353
	p	*0.409*	*0.040*	*<0.001*	
b*	1	5.6 ± 0.6^{B}	5.7 ± 0.8^{A}	4.3 ± 1.0^{aA}	0.169
	8	2.0 ± 0.4^{aA}	6.7 ± 0.6^{bB}	6.3 ± 0.7^{bB}	<0.001
	p	*0.002*	*0.027*	*0.030*	
TBARS	1	0.40 ± 0.01^{bA}	0.34 ± 0.01^{aA}	0.34 ± 0.02^{aA}	0.019
	8	0.50 ± 0.01^{cB}	0.44 ± 0.01^{aB}	0.42 ± 0.01^{aB}	0.005
	p	*0.012*	*0.015*	*0.019*	

CT: control, AH-2: 2% *Arthrospira* hydrolysate, AH-4: 4% *Arthrospira* hydrolysate. Values are mean ± SE (n=4), Superscript lowercase letters indicate differences ($p < 0,05$) attributable to diets (CT, AH-2 and AH-4). Superscripts capital letters indicate differences attributable to storage time (1 and 8 day).

III.3.3.6. Digestive enzyme activities

Trypsin, chymotrypsin, and leucine aminopeptidase activities significantly increased in fish fed with *Arthrospira* hydrolysate-supplemented diets ($p = 0.001$, $p = 0.001$, and $p < 0.001$, respectively) compared to control fish (Table 5). Fish fed with AH-4 showed the highest enzyme activity levels. Total alkaline protease and alkaline phosphatase activities did not differ among experimental groups ($p = 0.160$ and $p = 0.844$).

Table 5. Digestive enzymes activities (U g^{-1} tissue) measured in intestine of gilthead seabream juveniles fed experimental diets for 128 days.

	CT	AH-2	AH-4	*p-value*
Total alkaline protease	118.4 ± 11.26	95.6 ± 2.77	124.3 ± 14.72	*0.160*
Trypsin	27.8 ± 1.38^{a}	32.7 ± 0.72^{b}	37.9 ± 1.02^{c}	*0.001*

Chymotrypsin	25.4 ± 1.14 [a]	32.8 ± 1.39 [b]	44.9 ± 3.94 [c]	*0.001*
Leucine-aminopeptidase	0.22 ± 0.02 [a]	0.40 ± 0.01 [b]	0.38 ± 0.01 [c]	*<0.001*
Alkaline phosphatase	57.2 ± 3.77	54.5 ± 3.18	54.9 ± 3.03	*0.844*

CT: control, AH-2: 2% *Arthrospira* hydrolysate, AH-4: 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determination per tank (n = 9). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$).

III.3.3.7. Ultrastructural study of the intestinal mucosa

TEM and SEM observations evidenced that all specimens presented intestinal mucosa without any evidence of abnormality (Figure 3). Nevertheless, the morphometric analysis of the intestinal *microvilli* carried out on both TEM and SEM images evidenced a significant increase in *microvilli* length (ML) and *microvilli* diameter (MD) in fish fed with AH-4 diet. Enterocyte apical area values were similar in all dietary treatments ($p = 0.211$), but total enterocyte absorption surface (TAS) was significantly higher in fish fed with AH-4 diet compared to CT group ($p < 0.001$) (Table 6).

Table 6. *Microvilli* morphometric parameters of the anterior intestine of juvenile gilthead seabream fed with the experimental diets for 128 days.

	CT	**AH-2**	**AH-4**	*p*
ML (μm)	1.83 ± 0.05[a]	1.70 ± 0.05[a]	2.62 ± 0.05[b]	*< 0.001*
MD (μm)	0.13 ± 0.00[a]	0.13 ± 0.00 [a]	0.14 ± 0.00[b]	*0.012*
EA (μm^2)	23.36 ± 0.82	21.75 ± 0.51	21.18 ± 1.01	*0.211*
TAS (μm^2)	767.90 ± 20.28[a]	751.45 ± 20.85[a]	1347.44 ± 20.95[b]	*< 0.001*

CT: control; AH-2: 2% *Arthrospira* hydrolysate; AH-4: 4% *Arthrospira* hydrolysate. Values are mean ± SE (n = 50). ML: *microvilli* length; MD: *microvilli* diameter; EA: enterocyte apical area; TAS: total enterocyte absorption surface. Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$).

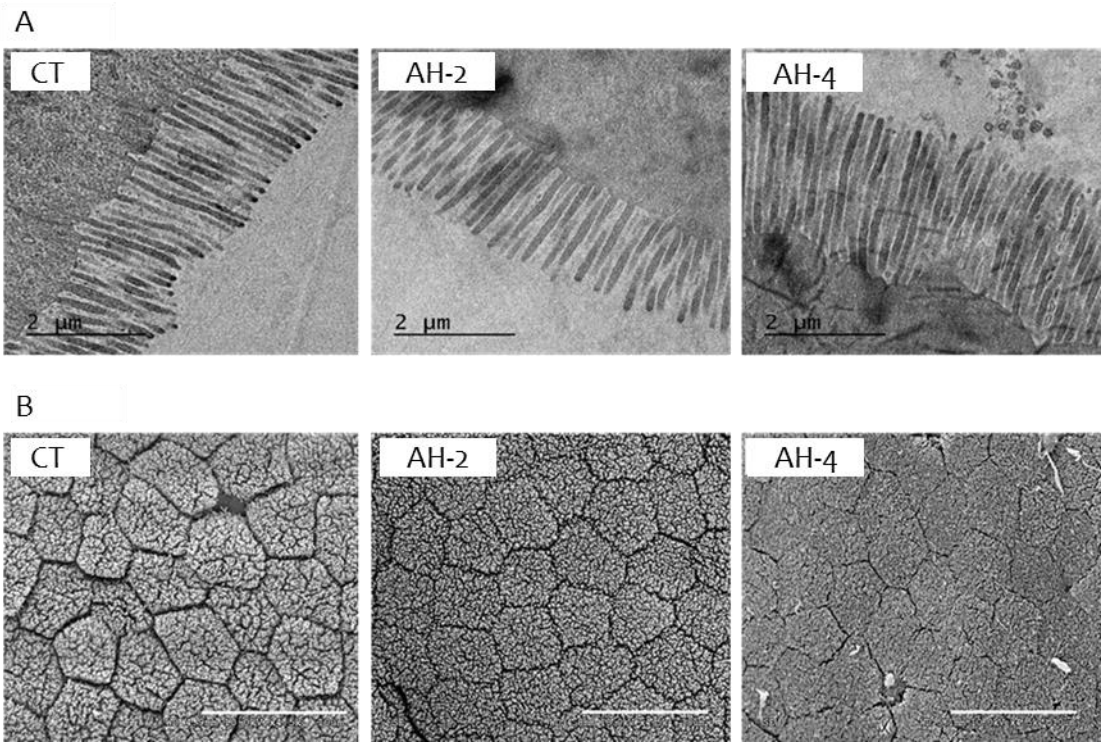

Figure 3. TEM (A) and SEM (B) micrographs from the anterior intestine of juvenile gilthead seabream fed the experimental diets. (TEM bar: 2 µm; SEM bar: 10 µm).

III.3.4. DISCUSSION

The use of *Arthrospira* hydrolysates in aquafeeds arises as a novel strategy aimed at increasing the nutritional and functional properties of the original raw biomass, by turning the proteins into low molecular weight peptides and free amino acids with higher bioavailability (Chalamaiah *et al*., 2012). The existence of low molecular mass molecules and free amino acids as a result of the enzymatic hydrolysis has been proposed as an interesting dietary supplement for aquacultured fish (Xu *et al*., 2016). Indeed, the potential beneficial effects derived from the use of protein hydrolysates in aquafeeds have been proven previously (Bui *et al*., 2014; Khosravi *et al*., 2015). However, to our knowledge, studies focused specifically on the assessment of microalgae hydrolysates for this purpose are not available and even less with regard to *Arthrospira* sp.

The dietary inclusion of *Arthrospira* sp. raw biomass in aquafeeds has been evaluated previously in different fish species with favourable results. Thus, Adel *et al*. (2016) and Yu *et al*. (2018) revealed that the incorporation of 10% *Arthrospira* in feeds yielded positive effects on growth performance in great sturgeon and coral trout (*Plectropomus leopardus*), respectively. Similarly, Kim *et al*. (2013) reported positive effects on fish performance with 5% *Arthrospira* inclusion level in feeds for parrot fish (*Oplegnathus fasciatus*). However, results obtained in our work revealed that the dietary inclusion of *Arthrospira* protein hydrolysate up to 4% did not increase fish performance after a 128-day feeding trial.

It is a well-known phenomenon that the skin of many species of commercial fish lacks colour and brightness in captivity, a feature directly linked to the consumer's acceptance of fish, which, accordingly, influences their market value. In this regard, different studies have described positive effects of the addition of *Arthrospira* at low inclusion level on the pigmentation attributes in different fish species (Kumprom *et al*., 2011; Teimouri *et al*., 2013; Abdulrahman & Ameen, 2014; Roohani *et al*., 2019). Our results indicate that juveniles fed with *Arthrospira*-supplemented diets showed a skin lighter, reddish, and yellowish, and these differences remained stable over 8 days of cold storage.

Similar results indicating intensified redness and yellowness in fish skin were found in golden carp (*Carassius auratus*; Kumprom *et al*., 2011), common carp (*Cyprinus carpio*; Abdulrahman & Ameen, 2014), and rainbow trout (*Oncorhynchus mykiss*; Teimouri *et al*., 2013), fed with *Arthrospira* at a low dietary inclusion level. This improvement in colorimetric parameters could be associated to the fact that most microalgae species are natural sources of pigments (Begum *et al*., 2016), which might play decisive role on the quality of the final product (Ginés *et al*., 2004). In this regard, changes in colour parameters observed could likely be attributed to the carotenoid content of *Arthrospira* (Lu *et al*., 2003; Teimouri *et al*., 2013), and thus, xanthophylls (mainly lutein and zeaxanthin) could explain the increased yellowness measured in the skin of fillets (Table 4).

Muscle lipid oxidation increased during the storage of fish fillets, as evidenced by the significant increase of TBARS. However, values for this parameter in muscle were significantly lower in the specimens fed with AH-2 and AH-4 diets (Table 4). The antioxidant capacity of *Arthrospira* sp. is wellknown, owing to the high content in different bioactive substances playing a key role in the inhibition of lipid peroxidation (Deng & Chow, 2010; Kim *et al*., 2013). Beyond their influence on colour parameters, xanthophylls have a potent antioxidant capacity against reactive oxygen species (ROS) (Hallerud, 2014) that could explain the reduced lipid peroxidation of muscle lipids found in those animals fed with AH diets. Moreover, it has also been described that *Arthrospira* sp. contains considerable amount of the enzyme superoxide dismutase that might decrease the rate of formation of free radicals, this resulting in lower muscle lipid oxidation at inclusion levels from 10 to 2.5% (Teimouri *et al*., 2016). Similar results were reported in clownfish (*Oplegnathus fasciatus*) (Kim *et al*., 2013) and in tilapia (Amer, 2016), attributed to the inclusion of *Arthrospira pacifica* and *Arthrospira* sp. as dietary additives.

The activity of digestive enzymes is not only a reliable indicator of the nutritional status of fish (Cahu & Infante 2001; Cara *et al*., 2007) but also a valuable tool for estimating the digestive and absorptive capacity of animals after a dietary treatment (Alarcón *et al*., 1998; Messina *et al*., 2019). The

existence of changes in the digestive-absorptive processes influenced by the dietary inclusion of microalgae has been previously assessed in aquacultured fish such as seabream (Vizcaíno *et al*., 2014; 2016), seabass (Messina *et al*., 2019), Senegalese sole (Vizcaíno *et al*., 2018), common carp (Ansarifard *et al*., 2018), or great sturgeon (Adel *et al*., 2016). The present study confirmed that dietary inclusion of *Arthrospira* hydrolysates increased the activity of some digestive enzyme activities, despite the low inclusion levels tested. Thus, trypsin and chymotrypsin activities increased significantly in fish fed with *Arthrospira*-supplemented diets, a fact that might have contributed to increasing the availability of substrates for muscle protein accretion. Vizcaíno *et al*. (2016) reported similar results in *Sparus aurata* fed with microalgae-supplemented diets, which might be related to the existence of compensatory mechanisms in fish against dietary changes. In line with the above, it has been reported that digestive protease and amylase activities increased after adding 3% dietary supplementation with plant protein hydrolysate in juvenile blunt snout bream *Megalobrama amblycephala* (Yuan *et al*., 2019). Regarding brush border enzymes, a significant increase in the activity of leucine aminopeptidase was observed in fish fed with the diets containing the microalgal hydrolysate (Table 5). Leucine aminopeptidase and alkaline phosphatase play a crucial role in the final stages of the digestive process, facilitating the absorption and transport of nutrients through the enterocytes (Infante & Cahu, 2001). In fact, both enzymes are used as indicators of the intestinal integrity (Wahnon *et al*., 1992) or as general markers of nutrient absorption (Silva *et al*., 2010). Previous studies proposed that the higher the activity levels of these enzymes, the better the efficiency of the digestive processes and the intestinal absorptive capacity (Infante & Cahu, 2001). However, Messina *et al*. (2019) reported that alkaline phosphatase activity was not affected when fishmeal was replaced by microalgae, indicating no major functional changes in the gut integrity of European seabass (*Dicentrarchus labrax*).

In addition to the activity of the digestive enzymes, the intestinal mucosa plays a key role in the digestive and absorptive processes (Sweetman *et al*.,

2008), as well as acting as a protective barrier against pathogenic microorganisms (Wilson & Castro, 2011). The study of the intestinal mucosa also enables to know the influence of dietary treatments on its structure and morphology. Several studies revealed that the dietary inclusion of plant protein ingredients, algae, or probiotics can lead to morphological changes in the structure of the digestive mucosa, which are linked to important consequences on the digestive physiology and the absorption capacity of the intestinal mucosa. This has been described in different fish species, such as gilthead seabream (Cerezuela *et al*., 2012; Vizcaíno *et al*., 2016), rainbow trout (Araújo *et al*., 2016), goldfish (*Carassius auratus*) (Omnes *et al*., 2015), or Senegalese sole (Vizcaíno *et al*., 2018). Until now, knowledge regarding the effects of protein hydrolysates on the intestinal structure is scarce. In the present study, the ultrastructural analysis revealed a significant effect of *Arthrospira* hydrolysate on the morphology of the intestinal mucosa, especially in those fish fed with 4% inclusion level. In agreement, it has been described that the dietary inclusion of microalgal hydrolysates can reduce mucosal barrier damage, as well as prevents colonic inflammation in mice (Wang *et al*., 2018). These authors evidenced that the oral administration of microalgae hydrolysates reversed the progression of dextran sulphate sodium-induced colitis and also prevented acute inflammation in that murine model. In the same way, the inclusion of 5% dietary shrimp hydrolysate resulted in larger intestinal *villi* and also modulated the transcriptomic response of the intestinal mucosa in European seabass (Leduc *et al*., 2018). Yuan *et al*. (2019) reported that 3% cottonseed meal protein hydrolysate increased the length of the intestinal *microvilli* in juvenile blunt snout bream (*Megalobrama amblycephala*). In our study, changes observed on *microvilli* length and *microvilli* diameter can be interpreted as an overall increase in enterocyte absorption surface and, consequently, an enhanced intestinal absorption capacity. This increased absorption area might have been responsible for higher amino acid uptake in the anterior intestine, this yielding higher protein accretion in muscle, especially in fish fed with 4% microalgae hydrolysate.

In conclusion, our results show that juvenile gilthead seabream fed with *Arthrospira* hydrolysate increased the activity of key digestive enzymes, improved the intestinal mucosa structure, and reduced the oxidation of muscle lipids. Thus, this supplement (especially when used at 4% inclusion level) could be useful for maintaining the overall condition status in juveniles of this fish species. The incorporation of microalgal hydrolysate as dietary additive seems promising for feeding *S. aurata* juveniles, not least due to the stimulating effect observed on the intestinal mucosa and as a natural alternative for the improvement of the skin colour in cultured fish. However, future studies should be focused on the intrinsic mechanism of their effects, as well as on the feasibility of its commercial use in aquafeeds at large scale.

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III.4. EVALUATION OF *Nannochloropsis gaditana* RAW AND HYDROLYSED BIOMASS AT LOW INCLUSION LEVEL AS DIETARY FUNCTIONAL ADDITIVE FOR GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES

III.4.0. ABSTRACT

Abundant research is being carried out in the last years aimed at exploring microalgal biomass as nutrient source for different species and developing stages of aquacultured fish. This sustainable resource has been proposed not only at high inclusion level as a partial substitute for fishmeal, but also as a functional additive at low level in feeds, owing to the richness in bioactive substances with potential interest in fish nutrition. Nevertheless, many microalgae species cultivated at industrial scale, such as *Nannochloropsis gaditana*, are characterized by having thick cell walls rich in cellulose, which together with the lack of constitutive cellulase activity in fish, might well limit the *in vivo* bioavailability of intracellular active compounds. Among the alternatives aimed at overcoming this limitation, cellulase enzyme hydrolysis is proposed as a convenient and practical solution. In this regard, *in vitro* hydrolysis of *N. gaditana* confirmed increased yields of soluble compounds compared to raw biomass, although the influence of the cellulase hydrolysates included at low level in feeds (2.5 and 5% w/w) on growth and proximate composition of juvenile gilthead seabream (*Sparus aurata*) didn't differ from feeds enriched with raw biomass after a 90-d feeding trial. The only clear improvement found in fish attributable to the cellulase pre-treatment was related to reduced oxidation of the lipid fraction. In addition, and irrespectively of the format in which the microalgal biomass was added, the lack of detrimental effects, together with the observed beneficial effects on certain physiological parameters (namely digestive mucosa structure and functionality, oxidative status of muscle lipids, and colour), suggest that *N. gaditana* used as additive in feeds might represent a valuable nutritional strategy for *Sparus aurata* juveniles.

III.4.1. INTRODUCTION

The interest in microalgae has emerged strongly in the last years, given that they stand valuable, and yet mostly untapped, potential for reducing the dependence on unsustainable conventional raw ingredients used in aquafeeds, namely fish meal (Yarnold *et al*., 2019). Nevertheless, due to several factors such as the current high production, their potential use is turning from the consideration as a main ingredient, to a functional ingredient at low inclusion level (Chakraborty & Hancz, 2011). The search for bioactive compounds is nowadays a thriving field in aquaculture research and consequently, considerable efforts aimed at improving not only fish growth, but also the general condition of the animals are being undertaken (Burr *et al*., 2005). This concept is based upon numerous reports indicating that many microalgae species are valuable sources of essential n-3 long chain polyunsaturated fatty acids (n3-PUFAs), vitamins, minerals, pigments, and polyphenols, among others (Sansone *et al*., 2000; Teimouri *et al*., 2013; Tibaldi *et al*., 2015; Shah *et al*., 2018). In this context, *Nannochloropsis gaditana*, by virtue of its richness in eicosapentanoic acid (EPA, C20:5n-3), pigments and other natural antioxidants and bioactive compounds (Kilian *et al*., 2011; Tibbetts *et al*., 2017, Cerón-García *et al*., 2018), is a promising candidate for commercial additive in practical aquafeeds (Li *et al*., 2014).

However, it has been reported in the literature that the theoretical nutritional potential of microalgae might not always be reflected straight on the animals, neither in terms of fish growth nor on physiological condition (Cerezuela *et al*., 2012, Cardinaletti *et al*., 2018). This fact might be explained owing to the existence of a cell wall that limits the bioavailability of inner microalgal compounds (Wu *et al*., 2017; Yong *et al*., 2020). Specifically, the existence of a cellulose-rich cell wall in certain genus, such as *Nannochloropsis*, together with the lack of digestive cellulase activity in fish, might well limit their further practical utilization.

Some strategies have been proposed in the literature in order to cope with such constraints, aimed at weakening microalgae cell walls prior to their

inclusion in aquafeeds (Tibbetts *et al*., 2017; Velazquez-Lucio *et al*., 2018; Teuling *et al*., 2019). Rather than chemical or enzymatic, most of the procedures described are based on physical treatments, which roughly have proven to increase significantly the digestibility and nutrient bioavailability of microalgae. Even if successful at laboratory scale, however, such methods of physical disruption may not end up in practical, scalable, and economically feasible procedures applicable to the feed processing industry, taking into account that additional costs should be added to the relatively high production costs of microalgal biomass. Consequently, there is still considerable scope for developing simple, economical, and cost-effective cell wall disruption protocols aimed at facilitating industrial up-scaling. The use of hydrolytic enzymes capable of weakening cell walls, prior to its incorporation into feeds, could overcome many limitations, especially taking into consideration that, as mentioned, cellulose abounds in numerous microalgae species, such as *N. gaditana*. Given that fibrolytic enzymes, not least cellulases, have a wide range of industrial applications, they are available at affordable prices, and consequently, any bioprocess including enzymes could be certainly scalable at industrial level.

Under this perspective, we hypothesize that the use of hydrolytic enzymes capable of weakening *N. gaditana* cell walls may represent a valuable strategy to improve the bioavailability of nutrients and bioactive compounds when added into gilthead seabream (*Sparus aurata*) experimental diets. To this end, *N. gaditana* was added at low inclusion level (2.5 and 5% w/w) in practical diets for juvenile fish, either crude or enzymatically hydrolysed. This is, the microalgal biomass was assessed as a potential functional additive rather than as a main ingredient. A 90-d feeding trial was carried out, and the occurrence of potential effects of the microalgae on fish growth, muscle composition, oxidative status, pigmentation and digestive structure and functionality were assessed.

III.4.2. MATERIALS AND METHODS

III.4.2.1. Microalgae biomass and enzyme hydrolysis

Nannochloropsis gaditana was cultured in tubular photobioreactors at the pilot plant (EU-H2020 SABANA facilities) of the Universidad de Almería (Spain) as reported by Menegol *et al*. (2019). The culture pH was maintained at 8 by the on-demand addition of CO_2. The culture was harvested daily by centrifugation (at a dilution rate of 0.3 d^{-1}) and then the concentrated biomass was freeze-dried. Raw microalgae biomass (approx. 15% dry matter) was freeze-dried and stored at -20 °C until further use. The proximal composition of *N. gaditana* meal is shown in Table 1.

Enzymatic hydrolysis was carried out by mixing the microalgal biomass, at a final concentration of 150 g dry weight L^{-1} in 50 mM sodium citrate buffer solution (pH 5.5), and incubated at 45 °C under continuous agitation for 5 h. Commercial cellulase (from *Aspergillus oryzae*, Sigma-Aldrich, Madrid, Spain) was added at an enzyme to microalgae ratio of 0.05 (50 g cellulase kg^{-1} dry microalgae). The estimation of the microalgae hydrolysis was carried out by monitoring the amount of reducing sugars (Miller, 1959), total amino acids (Church *et al*., 1983) released into the reaction vessel at different sampling times (0, 15, 30, 60, 90, 120, 180, 240, and 300 min). Total polyphenols (Singh *et al*. 2012) were also measured in reaction vessels at the beginning and at the end of the *in vitro* hydrolysis. A control assay was also carried out under the same experimental conditions, without the addition of cellulase enzyme.

Following the hydrolysis, the mixture was immediately used for manufacturing aquafeeds.

III.4.2.2. Experimental diets

Four isonitrogenous and isolipidic experimental diets containing *Nannochloropsis gaditana* biomass were elaborated at the CEIMAR-Universidad de Almería facilities (Service of Experimental Diets, http://www.ual.es/stecnicos_spe). Two inclusion levels (25 and 50 g kg^{-1} w/w),

and two microalgae formats (raw and enzymatically hydrolysed) were considered. Therefore, diets were designed as R25 and R50 for raw microalgae lots, and H25 and H50 for enzymatically-hydrolysed biomass. A microalgae-free diet was used as control (CT). The formulation and proximal composition of the experimental diets is shown in Table 1. In addition, fatty acid and amino acid profiles of each diet are presented in Table 2 and Figure 1, respectively. Feed ingredients were finely ground and mixed in a vertical helix mixer (Sammic 13M-11, 5-L capacity, Sammic SA, Azpeitia, Spain) for 20 min. Then the microalgae (crude or hydrolysed) were added at the specified inclusion level, and water content was adjusted to provide 400 mL per kg of the ingredient mixture, in order to obtain homogenous dough. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JS Conwell Ltd, New Zealand), provided with matrixes so as to obtain 2 and 3 mm-pellets, according to the size of fish. The feeds were dried with forced-air circulation (Airfrio, Almería, Spain) at 30 °C for 24 h, and then stored at -20 °C until use.

Table 1. Ingredient composition of the experimental diets.

Ingredients (% dry matter)	**CT**	**R25**	**R50**	**H25**	**H50**
Fish meal LT94[1]	15.0	15.0	15.0	15.0	15.0
Raw *Nannochloropsis gaditana*[2]	-	2.5	5.0	-	-
Hydrolysed *N. gaditana*	-	-	-	2.5	5.0
Squid meal[3]	2.0	2.0	2.0	2.0	2.0
CPSP90[4]	1.0	1.0	1.0	1.0	1.0
Krill meal[5]	2.0	2.0	2.0	2.0	2.0
Gluten meal[6]	15.0	15.0	15.0	15.0	15.0
Soybean protein concentrate[7]	40.0	38.8	37.3	38.8	37.3
Fish oil[8]	11.4	11.0	10.5	11.0	10.5
Soybean lecithin[9]	1.0	1.0	1.0	1.0	1.0
Wheat meal[10]	5.4	4.5	4.0	4.5	4.0
Choline chloride[11]	0.5	0.5	0.5	0.5	0.5
Betain[12]	0.5	0.5	0.5	0.5	0.5

Lysine[13]	1.5	1.5	1.5	1.5	1.5
Methionine[14]	0.6	0.6	0.6	0.6	0.6
Vitamin and mineral premix[15]	2.0	2.0	2.0	2.0	2.0
Vitamin C[16]	0.1	0.1	0.1	0.1	0.1
Guar gum[17]	2.0	2.0	2.0	2.0	2.0
Proximate composition (%)					
Crude protein	45.2	46.1	46.4	45.4	45.9
Crude lipid	15.2	15.9	15.5	15.8	15.8
Ash	11.8	11.9	12.2	12.0	11.7
Moisture	5.4	5.6	5.7	5.3	5.6

CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively.

1 69.4% crude protein, 12.3% crude lipid (Norsildemel, Bergen, Norway).

2 *N. gaditana* (44.5% crude protein, 33.3% carbohydrates, 4.5% ash, and 17.7% crude lipids).

3, 4, 5 Bacarel (UK).

6 78% crude protein (Lorca Nutrición Animal SA, Murcia, Spain).

7 Fish protein hydrolysate, 65% crude protein, 8% crude lipid (DSM, France).

8 AF117DHA (Afamsa, Spain).

9 P700IP (Lecico, DE).

10 Local provider (Almería, Spain).

11,12, 13,14 Lorca Nutrición Animal SA (Murcia, Spain).

15 Lifebioencapsulation SL (Almería, Spain). Vitamins (mg kg-1): vitamin A (retinyl acetate), 2,000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin E (Lutavit E50), 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine (Betafin S1), 50,000 mg. Minerals (mg kg-1): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg).

16 TECNOVIT, Spain.

17 EPSA, Spain.

Table 2. Fatty acid profile of *N. gaditana* meal and experimental diets (% of total fatty acids).

Fatty acids	*N. gaditana*	CT	R25	R50	H25	H50
14:0	5.60	3.11	3.09	3.13	3.15	3.17
16:0	22.40	21.60	21.51	21.49	22.03	21.70
18:0	21.30	5.95	5.77	5.59	5.89	5.67
16:1n-7		4.21	4.59	5.06	4.63	5.06
18:1n-7		2.45	2.42	2.35	2.45	2.36
18:1n-9		15.17	14.91	14.42	14.95	14.54
20:1n-9	4.01	1.40	1.42	1.62	1.42	1.38
18:2n-6		11.64	11.54	11.34	11.51	11.12
18:3n-3	3.70	1.61	1.50	1.51	1.44	1.41
16:2n-4		0.74	0.56	0.69	0.69	0.56
16:3n-4		0.94	0.83	0.93	0.97	0.82
18:4n-3		0.46	0.41	0.66	0.71	0.67
20:4n-6		0.31	0.27	0.27	0.31	0.28
20:4n-3	9.50	1.41	1.57	1.68	1.50	1.80
20:5n-3, EPA	33.40	6.10	6.52	7.10	6.47	7.15
22:5n-3		1.23	1.26	1.28	1.26	1.25
22:6n-3, DHA		17.14	16.31	15.60	16.10	15.77
Others		4.54	5.45	5.23	4.53	5.30
ΣSFA		30.65	30.38	30.21	31.07	30.53
ΣMUFA		23.23	23.34	23.45	23.45	23.34

ΣPUFA	38.14	39.91	39.44	39.29	38.45
Σn-3	27.95	27.56	27.83	27.48	28.05
Σn-6	11.95	11.83	11.60	11.82	11.40
n-3/n-6	2.34	2.33	2.40	2.33	2.46
EPA/DHA	0.36	0.40	0.46	0.40	0.45

[1]The statistical comparison was carried out among the experimental diets, excluding *N. gaditana* meal. Therefore, p-values illustrate the statistical significance of differences among CT, R25, R50, H25 and H50 diets. CT: control diet, R25 and R50: diets including 20 and 50 g kg^{-1} raw microalgal biomass, respectively, H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Different lowercase superscripts indicate significant differences among diets within each row. n.s.: not significant. Values (n=3) are mean ± SD. *SFA*: saturated fatty acids; *HUFA*: Highly unsaturated fatty acids; *PUFA*: Polyunsaturated fatty acids.

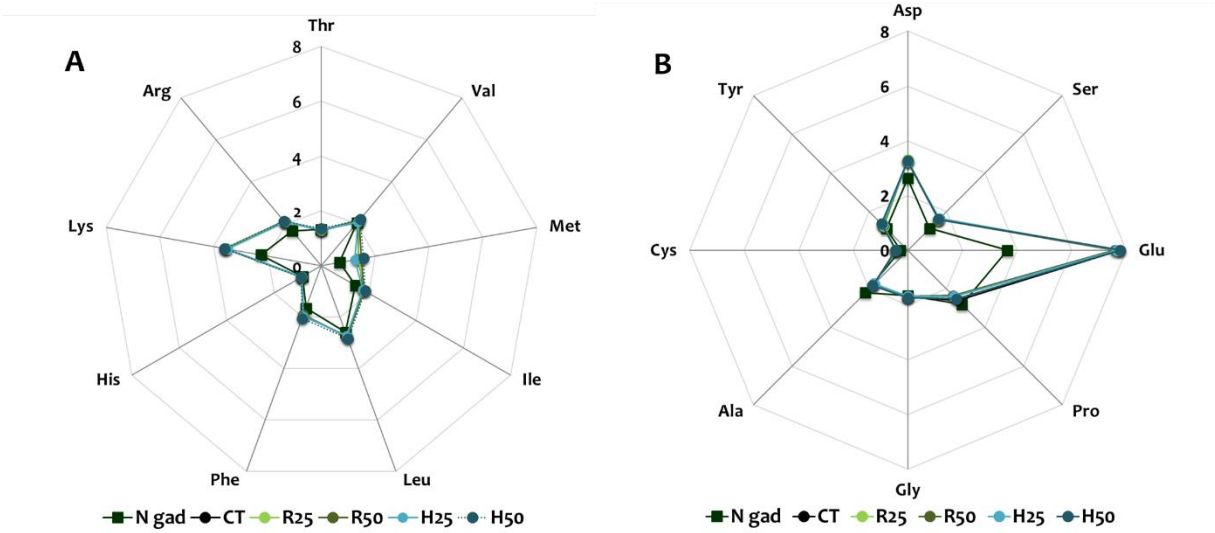

Figure 1. Amino acid profile of *N. gaditana* (N gad) meal and the experimental diets. A: essential amino acids; B: non-essential amino acids. Results (n=3) are expressed as % of total amino acids.

III.4.2.3. Fish maintenance and experimental design

The feeding trial was carried out at the Instituto Español de Oceanografía (IEO – CSIC; Centro Oceanográfico de Murcia, Mazarrón, Spain). All experimental procedures complied with the Guidelines of the European Union (Directive 2010/63/EU) and the Spanish regulations (Real Decreto 53/2013) on the protection of laboratory animals. Gilthead seabream juveniles (15.06 ± 1.40 g average initial body weight) were selected and randomly distributed in 15 tanks (triplicate tanks per dietary treatment) of 500 L capacity to reach an initial average biomass of 1200 g m^{-3} (40 fish $tank^{-1}$).

Fish were fed with CT diet (microalgae-free) during a 15-d acclimation period prior to the beginning of the feeding trial. Afterwards, the experimental diets: were offered thrice per day (9:00, 14:00 and 18:00) at 2% of the biomass, until quadruplicating of the initial body weight.

The 90-d feeding trial was carried out in an open flow circuit, keeping seawater renewal rate (37‰ salinity) at 500 L h^{-1} and ammonia and nitrite values (< 0.1 mg L^{-1}) suitable for gilthead seabream culture. Animals were kept under natural photoperiod and temperature was kept at 18 ± 0.5 °C. Light

intensity ranged from 100 to 150 lux. Tanks were equipped with aerators to maintain an adequate level of oxygenation (above 6 mg L^{-1}).

After 45 and 90 days of the feeding trial, twenty fish per tank (60 animals per dietary treatment and sampling time) were withdrawn at each sampling time, and killed by anaesthetic overdose (60 ppm clove oil) followed by spine severing. Immediately after slaughtering, instrumental colour parameters were determined on the right side of the anterior dorsal skin of fish. Then, sampled fish were dissected, and the digestive tract and dorsal muscle were removed. A portion of muscle tissue (5 g per fish) was stored at –80 °C for lipid oxidation determinations (TBARS). The rest of individual muscle samples were freeze-dried and stored at –20 °C for further analysis of proximate composition and fatty acids. For digestive enzyme activity determinations, intestines from five fish per tank were randomly pooled to obtain three enzymatic extracts from each experimental tank.

Finally, the anterior intestines of five specimens from each tank were collected for examination by optical, transmission (TEM) and scanning (SEM) electron microscopy.

III.4.2.4. Proximate composition, fatty acid and amino acid analysis

Proximate analysis (dry matter, ash, and crude protein, N × 6.25) of feeds and muscle samples were determined according to AOAC (2000) procedures. Lipids were extracted following Folch *et al*. (1957) methodology using chloroform/methanol (2:1 v/v) as solvent, and total lipid content was calculated gravimetrically. Fatty acid (FA) profiles of *N. gaditana*, feeds and muscle samples were determined by gas chromatography (Hewlett Packard, 4890 Series II, Hewlett Packard Company, Avondale, PA) following the method described in Rodríguez-Ruiz *et al*. (1998), using a modification of the direct transesterification method described by Lepage and Roy (1984) that requires no prior separation of the lipid fraction. Amino acid profiles of *N. gaditana* and feeds were determined by ion exchange chromatography with post column derivatization with Ninhydrin (Biochrom 30+ amino acid analyser, Biochrom

LTD Cambridge, UK) after hydrolysis of the samples (20 mg mL^{-1} HCl 6M, 110 °C, 24 h, under N2 atmosphere), using norleucine as standard.

III.4.2.5. Digestive enzyme activities

For intestinal extracts, samples were homogenized in distilled water (0.5 g mL^{-1}) at 4 °C. Supernatants were obtained after centrifugation (16,000 g, 12 min, 4 °C) and stored in aliquots at -20 °C until further use. Total soluble protein was determined according to Bradford (1976) using bovine serum albumin as standard.

Total alkaline protease activity in intestinal extracts was measured spectrophotometrically following the procedure described by Alarcón *et al.* (1998), using 5 g L^{-1} casein in 50 mM Tris-HCl (pH 9.0) as substrate. One unit of total protease activity was defined as the amount of enzyme that released 1 µg of tyrosine per min in the reaction mixture, considering an extinction coefficient of 0.008 μg^{-1} mL^{-1} cm^{-1} for tyrosine, measured at 280 nm wavelength. Trypsin and chymotrypsin activities were assayed using 0.5 mM BAPNA (N-α-benzoyl-DL-arginine-4-p-nitroanilide) as substrate according to Erlanger *et al.* (1961), and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-p-nitroanilide) according to Del Mar *et al.* (1979), respectively, in 50 mM Tris-HCl, 10 mM $CaCl_2$ buffer, pH 8.5. Leucine aminopeptidase activity was determined by using 2 mM L-leucine-p-nitroanilide in 100 mM Tris-HCl buffer, pH 8.8, as substrate (Pfleiderer, 1970), and alkaline phosphatase was assayed using 450 mM p-nitrophenyl phosphate in 1 M diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5 (Bergmeyer, 1974) as substrate. For trypsin, chymotrypsin, and leucine aminopeptidase activities, one enzyme activity unit (UA) was defined as the amount of enzyme releasing 1 µmol of p-nitroanilide (pNA) per minute, considering as extinction coefficient 8,800 M cm^{-1}, measured spectrophotometrically at 405 nm. For alkaline phosphatase, one activity unit was defined as the amount of enzyme that released 1 µg of nitrophenyl per min considering an extinction coefficient of 17,800 M cm^{-1} for p-nitrophenol,

measured also at 405 nm. All assays were performed in triplicate, and specific enzymatic activity was expressed as units (UA) g tissue^{-1}.

III.4.2.6. Histology of the intestinal mucosa

Intestine samples were fixed in phosphate-buffered formalin (4 % v/v, pH 7.2) for 24 h, then dehydrated and embedded in paraffin according to standard histological techniques, as described in Vizcaíno *et al*. (2018). Samples were cut in transversal sections (5 µm), and the slides were stained with haematoxylin-eosin (H&E). The preparations were examined under light microscope (Olympus ix51, Olympus, Barcelona, Spain) equipped with a digital camera (CC12, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Images were analysed with specific software (Image J, National Institutes of Health, USA). The length of mucosal folds and total enterocyte height were determined in intestinal samples (30 independent measurements per treatment).

III.4.2.7. Ultrastructure of the intestinal mucosa

Intestine samples for scanning electron microscopy (SEM) were washed with 1% S-carboxymethyl-L-cysteine (Sigma Chem.) for 20 seconds, with the aim of removing the epithelial mucus, prior to fixation. Then, specimens were fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h; next excess glutaraldehyde was removed by washing samples in 0.1 mol L^{-1} cacodylate buffer, pH 7.2, and then dehydrated with a series of increasing concentrations of ethanol (50% to 100% v/v). Samples were critical point dried in absolute ethanol as intermediate fluid, and CO_2 as transition fluid (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain). After drying, specimens were mounted on aluminium stubs, immobilized with graphite (PELCO® Colloidal Graphite, Ted Pella INC., Ca, USA), and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were screened with a scanning electron microscope (HITACHI S-3500, Hitachi High-Technologies Corporation, Japan).

Samples for transmission electron microscopy (TEM) were fixed (4 h, 4 °C) in 25 g L^{-1} glutaraldehyde, 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5. Next, intestine sections were washed with PBS for 20 min and then, a post-fixation step with 20 g L^{-1} osmium tetroxide was carried out. Samples were dehydrated by consecutive immersion in increasing concentrations of ethanol, embedded for two hours, in 1:1 mixture of Epon resin and 100% (v/v) ethanol under continuous shaking, and then, included in pure Epon resin, and let polymerize at 60 °C. Finally, ultrathin cuts were obtained from resin blocks, and placed on a 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C TEM at 100 Kv (Carl Zeiss, Barcelona, Spain). Visualization fields were recorded at x 16,000 magnification.

SEM and TEM visualization fields were recorded and digital images were analysed using UTHSCSA ImageTool software (University of Texas Health Science Center, San Antonio, TX). *Microvilli* length (ML) and *microvilli* diameter (MD) were determined in TEM micrographs according to (Vizcaíno *et al*. 2014). SEM images were used to obtain several measurements of enterocyte apical area (EAA). Finally, data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno *et al.* (2014).

III.4.2.8. Lipid oxidation

Lipid oxidation was estimated by thiobarbituric acid-reactive substances (TBARS) analysis in muscle and liver according to the method of Buege & Aust (1978). Briefly, samples (2 g each) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000 g, 20 min, 4 °C) and supernatants were mixed in a ratio 1:5 (v/v) with 2-thiobarbituric acid (TBA) reagent (0.375% w/v TBA, 15% w/v TCA, 0.01% w/v 2,6-dibutyl hydroxytoluene (BHT) and 0.25 N HCl). The mixture was heated for 15 min and then centrifuged (3,600 g, 10 min, 4 °C), and the absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of

malonyl dialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

III.4.2.9. Instrumental colour determination

For all fish samples colour was measured on skin dorsal portion by L*, a*, and b* system (CIE, 1986), using a Minolta Chroma meter CR400 device (Minolta, Osaka, Japan). The parameter lightness (L*, on a 100-point scale from black to white), redness (a*, assesses the position between red, positive values, and green, negative values), and yellowness (b*, assesses the position between yellow, positive values, and blue, negative values) were recorded.

III.4.2.10. Statistics

The effect of the categorical variables "pre-treatment" and "doses", as well as their interactions, were determined for each numeric parameter studied by fitting a generalized lineal statistical model (GLM analysis) that relates measured parameters to predictive factors, using specific software (SPSS 25, IBM Corporation Inc.). Least square means were tested for differences using Fisher's least significant difference (LSD) procedure. Unless otherwise is specified, a significance level of 95% was considered to indicate statistical differences ($p < 0.05$). When measurements were expressed as a percentage (e.g., fatty acids profile), arcsine transformation of their square root was carried out in order to normalize data prior to the statistical analysis.

III.4.3. RESULTS

III.4.3.1. Microalgae hydrolysis

The concentration of reducing sugars in the reaction vessels increased throughout the *in vitro* assay owing to the addition of the commercial cellulase enzyme (Figure 2A). Results indicate that the hydrolysis of the enzyme-treated (5% cellulase) microalgae biomass reached final values in the region of 8 g glucose equivalents (GE) per 100 g microalgae dry mass. This value was about

4-fold the amount of reducing sugars released from untreated raw algae (control), which accounted for stable values about 2 - 2.5 g GE throughout the complete assay (300 min).

Analogously, the total amount of amino acids released (Figure 2B) during the assay indicated that cellulase hydrolysis increased significantly ($p < 0.05$) their concentration in the reaction vessels, compared to controls. Under the assay conditions, final concentration of free amino acids in enzyme-treated batches reached 12 g 100 g protein^{-1}, compared to 6 - 7 g free amino acids 100 g protein^{-1} measured in controls (Figure 3).

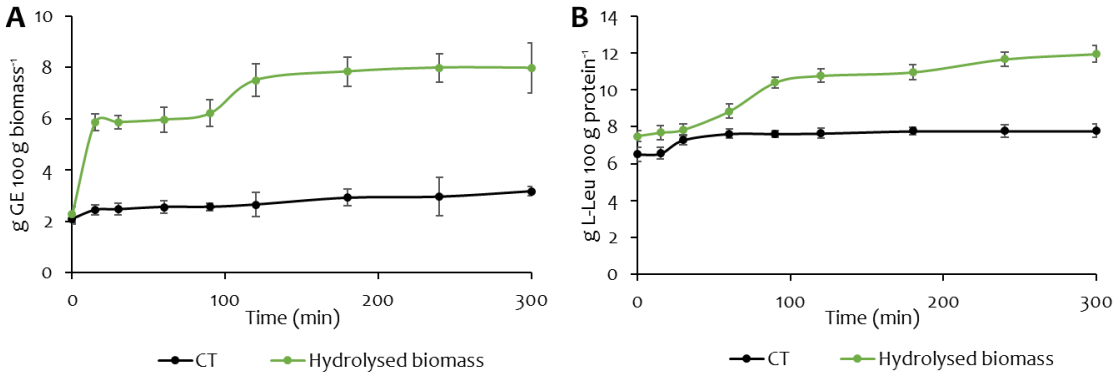

Figure 2. Time-course of the concentration in reducing sugars (A, expressed as D-glucose equivalents 100 g dry biomass^{-1}) and total free amino acids (B, expressed as g L-leucine 100 g protein^{-1}) measured from raw and cellulose-hydrolysed biomass of *N. gaditana* during the *in vitro* assay. Values (n=6) are mean ± SD.

The quantification of total polyphenols at the beginning and at the end of the enzymatic assay revealed a notable increase of these substances when the algal biomass was enzymatically treated. The final concentration of total phenolics in supernatants was significantly higher ($p < 0.05$) in cellulase-treated *N. gaditana* (reaching 70 mg gallic acid equivalents (GAE) 100g dry microalgae^{-1}), than that measured in controls (40 mg GAE 100 g^{-1}) (Figure 3). In absence of cellulase, no significant differences were observed in total phenolics measured to the reaction vessel after the 5 h incubation period.

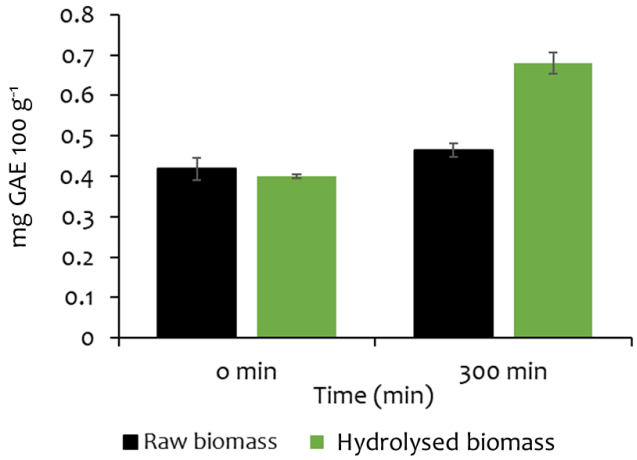

Figure 3. Total phenolics released from raw and cellulase-hydrolysed *N. gaditana* biomass at the beginning and at the end of the *in vitro* hydrolysis. Results are represented as mg gallic acid equivalents (GAE) 100 g microalgal dry biomass^{-1}. Data (n=6) are mean ± SD. n.s.: not significant.

III.4.3.2. Growth, muscle proximate composition and fatty acid profile

Experimental diets were well accepted by the fish, and feed intake was similar in all groups. Mortality was below 1%. During the experimental period, no differences were observed regarding growth parameters among experimental lots at the end of the feeding trial (Table 3). Final body weight (approx. 50 g) almost quadruplicated initial values for this parameter (approx. 15 g). No significant differences were observed for any of the parameters of muscle proximate composition. Although protein content was similar among dietary treatments, this parameter tended to increase slightly owing to the inclusion of raw microalgae. On the other hand, although not significantly, microalgae-enriched diets tended to decrease total muscle lipid content compared to CT diet, no matter the microalgae concentration or treatment considered.

Table 3. Fish biometric parameters and muscle proximate composition at the end of the feeding trial (90 days).

	CT	R25	R50	H25	H50	*p*
Final BW (g)	49.10 ± 5.69	51.30 ± 4.75	49.50 ± 5.69	49.90 ± 5.69	50.2 ± 5.69	*n.s.*
FCR	1.07 ± 0.09	1.04 ± 0.09	1.01 ± 0.19	1.01 ± 0.09	1.01 ± 0.19	*n.s*
SGR (% d^{-1})	1.47 ± 0.09	1.49 ± 0.09	1.54 ± 0.09	1.54 ± 0.09	1.52 ± 0.09	*n.s*
Protein (%)	17.68 ± 0.28	18.36 ± 0.34	18.29 ± 0.33	17.99 ± 0.48	17.86 ± 0.27	*n.s.*
Lipid (%)	7.33 ± 0.87	7.05 ± 0.77	7.19 ± 0.75	7.21 ± 0.81	7.18 ± 0.74	*n.s.*
Ash (%)	1.81 ± 0.06	1.76 ± 0.04	1.75 ± 0.05	1.75 ± 0.06	1.77 ± 0.11	*n.s.*
Moisture (%)	72.59 ± 0.64	72.61 ± 0.66	71.91 ± 0.79	71.9 ± 0.73	72.18 ± 0.49	*n.s.*

BW: final body weight. FCR: feed conversion rate. SGR: specific growth rate. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Different lower-case superscripts indicate significant differences among diets within each row ($p < 0.05$). n.s.: not significant. Values are mean ± SD. For proximate composition n=15. For biometric parameters n=90.

Overall results on muscle fatty acid profile (FA) indicated that the inclusion of raw or hydrolysed microalgae yielded significant changes in FA profile (Table 4), especially with regard to MUFAs and PUFAs, which displayed opposing tendencies. Thus, microalgae-enriched diets reduced total MUFAs compared to CT, not least when raw biomass was considered. Regarding individual MUFAs, all of them showed the same tendency. On the other hand, increased total PUFAs in muscle was observed in fish fed on microalgae-containing diets. It is worth mentioning that both EPA and DHA paralleled such increase.

Table 4. Effects of the dietary inclusion of *N. gaditana* on fatty acid profile of *S. aurata* muscle after a 90-d feeding trial (% of total fatty acids).

Fatty acids	**CT**	**R25**	**R50**	**H25**	**H50**	*p-value*
14:0	2.00 ± 0.01^{d}	1.51 ± 0.00^{a}	1.47 ± 0.02^{a}	1.78 ± 0.04^{b}	1.86 ± 0.02^{c}	*0.020*
16:0	16.54 ± 0.14^{a}	16.57± 0.15^{a}	16.83 ± 0.15^{ab}	16.68 ± 0.12^{a}	16.96 ± 0.26^{b}	*0.015*
18:0	4.70 ± 0.04^{a}	5.51 ± 0.00^{c}	5.52 ± 0.04^{c}	5.10 ± 0.05^{b}	5.10 ± 0.08^{b}	*0.022*
16:1n-7	4.69 ± 0.02^{c}	3.58 ± 0.02^{a}	3.82 ± 0.05^{b}	4.63 ± 0.05^{c}	4.40 ± 0.02^{c}	*0.033*
18:1n-7	2.69 ± 0.02^{b}	2.53 ± 0.12^{ab}	2.53 ± 0.01^{b}	2.69 ± 0.11^{b}	2.44 ± 0.09^{a}	*0.036*
18:1n-9	19.64 ± 0.18^{e}	14.76 ± 0.31^{a}	15.76 ± 0.14^{b}	17.68 ± 0.07^{c}	18.35 ± 0.09^{d}	*0.024*
20:1n-9	1.49 ± 0.05^{b}	1.32 ± 0.14^{ab}	1.28 ± 0.09^{a}	1.36 ± 0.09^{ab}	1.48 ± 0.05^{b}	*0.006*
18:2n-6	8.37 ± 0.08^{b}	8.06 ± 0.03^{a}	8.14 ± 0.08^{a}	8.50 ± 0.16^{b}	8.52 ± 0.07^{b}	*0.014*
18:3n-3	0.97 ± 0.01^{b}	0.86 ± 0.02^{a}	0.86 ± 0.02^{a}	1.03 ± 0.02^{b}	1.05 ± 0.08^{b}	*0,014*
16:4n-3	0.59 ± 0.01^{c}	0.49 ± 0.02^{a}	0.51 ± 0.06^{a}	0.55 ± 0.01^{b}	0.56 ± 0.01^{b}	*0.019*
18:4n-3	0.60 ± 0.01^{c}	0.43 ± 0.01^{a}	0.44 ± 0.02^{a}	0.55 ± 0.05^{bc}	0.54 ± 0.01^{b}	*0.019*
20:4n-6	1.72 ± 0.02^{a}	2.20 ± 0.01^{c}	2.37 ± 0.03^{d}	1.78 ± 0.06^{ab}	1.88 ± 0.11^{b}	*0.033*
20:4n-3	0.52 ± 0.00	0.53 ± 0.01	0.50 ± 0.01	0.51 ± 0.02	0.53 ± 0.01	*n.s.*
20:5n-3, EPA	4.76 ± 0.03^{a}	5.63 ± 0.04^{c}	5.72 ± 0.09^{c}	5.23 ± 0.11^{b}	5.25 ± 0.04^{b}	*0,014*
22:5n-3	2.22 ± 0.02^{c}	2.12 ± 0.01^{a}	2.12 ± 0.09^{a}	2.14 ± 0.05^{ab}	2.19 ± 0.03^{ab}	*0.031*
22:6n-3, DHA	22.84 ± 0.08^{a}	23.75 ± 0.07^{c}	23.96 ± 0.16^{c}	23.42 ± 0.10^{b}	23.56 ± 0.26^{bc}	*0.001*
Other FA	5.17 ± 0.38^{a}	9.52 ± 0.51^{d}	7.76 ± 0.36^{c}	5.61 ± 0.25 ^{ab}	4.70 ± 0.40^{b}	*< 0.001*
∑SFA	23.24 ± 0.18	23.58 ± 0.16	23.67 ± 0.16	23.70 ± 0.23	23.92 ± 0.37	*n.s*

∑MUFA	28.50 ± 0.26^{d}	22.19 ± 0.31^{a}	23.39 ± 0.06^{b}	26.35± 0.17^{c}	26.67± 0.16^{c}	*0.032*
∑PUFA	41.82 ± 0.17^{a}	43.62 ± 0.02^{c}	44.10 ± 0.12^{d}	43.16 ± 0.16^{b}	43.53 ± 0.17^{bc}	*< 0.001*
∑n-3	31.73 ± 0.19^{a}	33.33 ± 0.03^{b}	33.59 ± 0.12^{bc}	32.87 ± 0.03^{c}	33.13 ± 0.20^{b}	*< 0.001*
∑n-6	10.09 ± 0.08^{a}	10.28 ± 0.09^{b}	10.50 ± 0.10^{b}	10.29 ± 0.13^{b}	10.40 ± 0.09^{b}	*0.026*
n-3/n-6	3.15 ± 0.02^{a}	3.24 ± 0.01^{b}	3.20 ± 0.01^{b}	3.19 ± 0.02^{b}	3.19 ± 0.03^{ab}	*< 0.001*
EPA/DHA	0.21 ± 0.00^{a}	0.24 ± 0.00^{c}	0.24 ± 0.01^{c}	0.22 ± 0.01 ^{b}	0.22 ± 0.00^{b}	*< 0.001*

CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Values with different lowercase superscript within each row indicate significant differences in muscle lipids attributed to dietary treatments ($p < 0.05$). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. Values (n=15) are expressed as average ± SD. n.s.: not significant.

III.4.3.3. Digestive enzyme activities

Taken as a whole, the supplementation with *N. gaditana* increased the enzyme activities measured in intestinal extracts (Table 5). This effect was particularly evident for trypsin and total alkaline protease activities, irrespectively of microalgae concentration ($p < 0.05$) and sampling time (45 and 90 days), compared to CT fish. Chymotrypsin activity was influenced by the dietary treatments only at the end of the feeding trial (90 days). Similarly, Leucine aminopeptidase (LAP) activity did not differ among the experimental groups after 45 days, but higher values were observed after 90 d for all the dietary treatments compared to CT batch. With regard to alkaline phosphatase, this activity was higher only in fish fed with the hydrolysed microalgae, regardless of the dose considered. However, no clear tendency could be observed for this enzyme activity after 90 d.

Within each inclusion level, only alkaline phosphatase activity was increased owing to the previous hydrolysis of the biomass, and hence, for the rest of enzyme activities measured, raw biomass yielded, consistently higher values after 45 or 90 days. It was also observed that significant dose-dependent effect was observed for raw biomass (R25 vs. R50), not least for total alkaline, trypsin and chymotrypsin activities at both sampling times, but this effect was not noticed for the hydrolysed microalgae.

Table 5. Measurements of enzymes in the intestinal extracts of *S. aurata* juveniles fed with the experimental diets during 90 days.

Sampling time	Enzyme activity	**CT**	**R25**	**R50**	**H25**	**H50**	*p*-value
45 d	Total alkaline protease	491.55 ± 50.47^{a}	567.54 ± 23.68^{b}	589.56 ± 24.92^{b}	553.14 ± 37.69^{b}	560.90 ± 28.50^{b}	*<0.001*
	Trypsin (x10^{-3})	16.60 ± 2.30^{a}	33.02 ± 1.76^{c}	35.77 ± 2.22^{d}	28.71 ± 1.58^{b}	28.90 ± 1.26^{b}	*<0.001*
	Chymotrypsin	2.49 ± 0.17^{a}	2.45 ± 0.13^{a}	3.05 ± 0.32^{b}	2.34 ± 0.20^{a}	2.32 ± 0.13^{a}	*<0.001*
	Leucine aminopeptidase (x10^{-3})	0.43 ± 0.04	0.41 ± 0.04	0.41 ± 0.04	0.44 ± 0.04	0.42 ± 0.04	*n.s.*
	Alkaline phosphatase	11.00 ± 0.36^{a}	10.44 ± 0.51^{a}	10.78 ± 0.40^{a}	13.25 ± 0.40^{b}	13.38 ± 0.27^{b}	*<0.001*
90 d	Total alkaline protease	676.46 ± 38.27^{a}	909.67 ± 57.50^{b}	1015.58 ± 22.37^{c}	849.97 ± 49.89^{b}	826.73 ± 29.72^{b}	*<0.001*
	Trypsin (x10^{-3})	22.85 ± 2.03^{a}	46.57 ± 1.75^{c}	53.31 ± 2.57^{d}	36.78 ± 2.44^{b}	34.46 ± 2.30^{b}	*<0.001*
	Chymotrypsin	2.10 ± 0.10^{a}	3.21 ± 0.15^{c}	3.79 ± 0.11^{d}	2.81 ± 0.27^{b}	2.80 ± 0.11^{b}	*<0.001*
	Leucine aminopeptidase (x10^{-3})	0.37 ± 0.04^{a}	0.52 ± 0.05^{c}	0.41 ± 0.05^{ab}	0.51 ± 0.04^{c}	0.43 ± 0.07^{b}	*<0.001*
	Alkaline phosphatase	14.60 ± 0.81^{a}	15.22 0.97^{a}	16.59 ± 0.49^{ab}	17.25 ± 0.92^{b}	15.09 ± 0.78^{a}	*<0.001*

Values in the same column with different lowercase indicate significant differences ($p < 0.05$) owing to dietary treatments. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Values (n=15) are expressed as mean ± SD. n.s.: not significant.

III.4.3.4. Intestinal mucosa histology

The histological characteristics of intestinal sections from fish receiving the experimental dietary treatments at the end of the feeding trial are shown in Figure 4, and results of the image analysis of haematoxylin-eosin preparations is summarized in Table 6. Neither evidence of lipid droplet accumulation in the intestinal epithelium nor inflammatory changes in the *lamina propria* were observed. Consequently, no apparent damage attributable to any of the dietary treatments was found. Enterocytes presented well-organized brush border, aligned nucleus, homogenous supranuclear vacuolization and adequate cell shape (columnar and high). Intercellular spaces were not visible between enterocytes, and goblet cells were evenly dispersed throughout the epithelium.

Image analysis of preparations indicate that no significant differences were found in fold length or enterocyte height among dietary treatments, although animals receiving R50 and H50 diets showed lamina propria significantly thinner than the rest of experimental batches.

Table 6. Measurements in histological preparations of the intestinal mucosa of *S. aurata* juveniles fed with the experimental diets during 90 days.

Diets	Fold length (µm)	Enterocyte height (µm)	*Lamina propria* (µm)
CT	940.82 ± 216.24	46.94 ± 7.47	13.76 ± 3.62^{b}
R25	1173.12 ± 414.47	57.21 ± 8.26	13.06 ± 2.50^{b}
R50	1193.48 ± 269.95	46.95 ± 3.95	6.35 ± 1.40^{a}
H25	1053.49 ± 205.41	45.61 ± 4.94	11.23 ± 1.95^{b}
H50	1013.00 ± 239.95	50.01 ± 8.33	7.63 ± 2.39^{a}
p-value	*n.s.*	*n.s.*	*< 0.001*

Values in the same column with different lowercase indicate significant differences ($p < 0.05$) owing to dietary treatments. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolyzed microalgae, respectively. Values (n=15) are expressed as mean ± SD. n.s.: not significant.

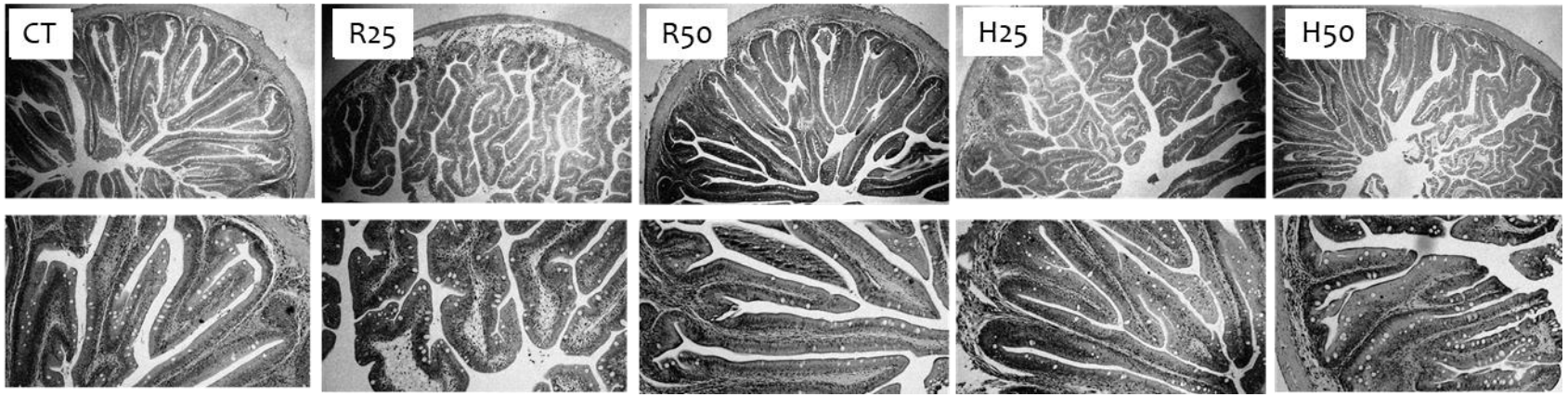

Figure 4. Light microscopy details of intestine sections of *S. aurata* juveniles fed on the experimental diets for 90 days. H&E stain, magnification x100 and ×400, respectively. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively.

III.4.3.5. Ultrastructure of the intestinal mucosa

TEM and SEM observations indicated that none of the experimental diets caused any perceptible damage on the enterocyte brush border ultrastructure (Figure 5), since specimens from animals from all the experimental groups presented a well-defined and organized intestinal brush border membrane. Moreover, no intercellular spaces were visible in the apical zone of the epithelium. Image analysis showed that, compared to controls, all the microalgae-enriched diets tended to increase the parameters studied (Table 7), although significant differences were observed only for 5% inclusion level. Hence, H50-fed animals exhibited significantly longer *microvilli* (ML) and enterocyte apical area (EA); this resulting in significantly increased total absorption area (TAS), whereas R50 treatment accounted for higher *microvilli* diameter (MD) and EA.

Table 7. *Microvilli* morphological parameters obtained from ultramicrographs of the anterior intestine of *S. aurata* juveniles fed with the experimental diets during 90 days.

Diets	ML (µm)	MD (µm)	EAA (µm²)	TAS (µm²)
CT	2.07 ± 0.59^{a}	0.11 ± 0.01^{a}	19.36 ± 3.57^{a}	587.63 ± 88.60^{a}
R25	2.16 ± 0.15^{a}	0.10 ± 0.01^{a}	20.75 ± 4.20^{a}	620.47 ± 55.46^{a}
R50	2.59 ± 0.20^{ab}	0.13 ± 0.01^{b}	26.14 ± 4.03^{b}	692.94 ± 81.93^{a}
H25	2.28 ± 0.13^{ab}	0.10 ± 0.01^{a}	20.45 ± 4.02^{a}	772.71 ± 38.01^{ab}
H50	2.73 ± 0.14^{b}	0.11 ± 0.01^{a}	25.92 ± 4.03^{b}	917.96 ± 56.62^{b}
p-value	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*

ML: *microvilli* length; MD: *microvilli* diameter; EA: enterocyte apical area; TAS: total enterocyte absorption surface. Values in the same column with different lowercase superscripts indicate significant differences ($p < 0.05$) owing to dietary treatments. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Values (n=15) are expressed as mean ± SD.

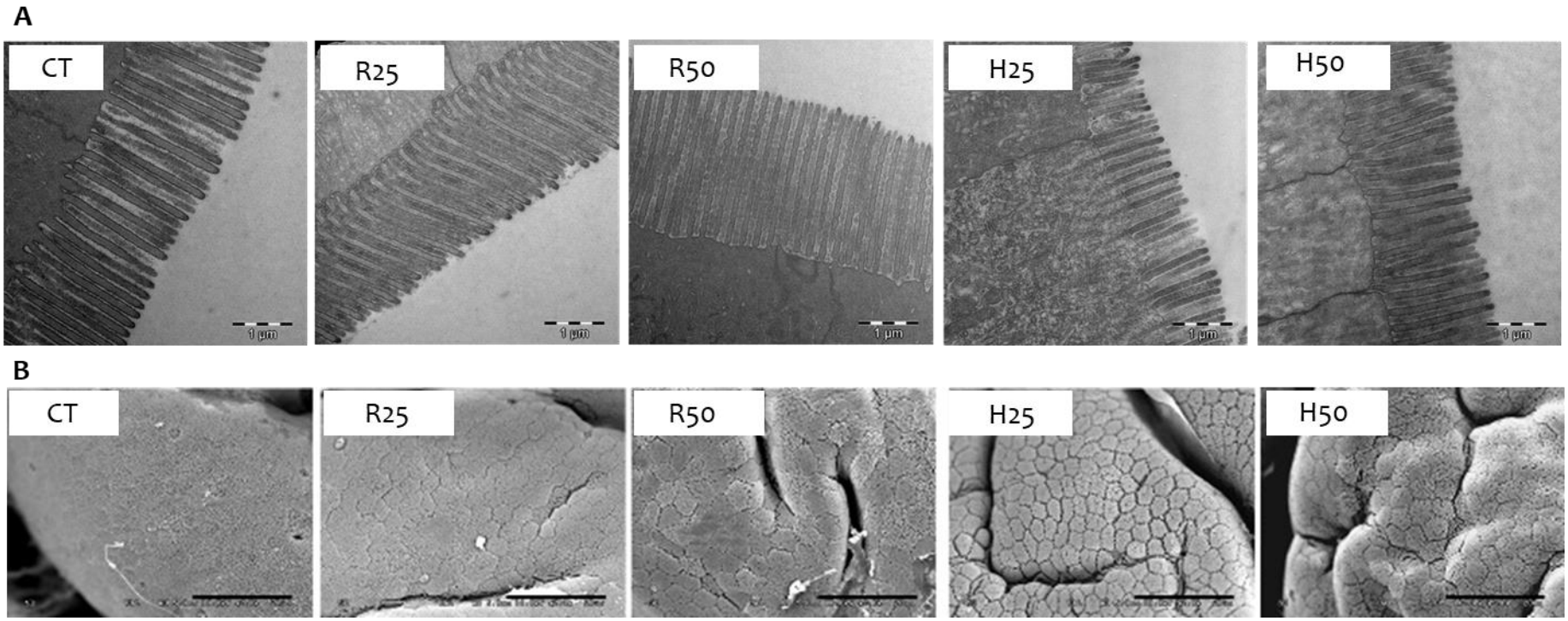

Figure 5. Transmission (A) and scanning (B) electron microscopy micrographs from the anterior intestinal region of juvenile *S. aurata*. 90 days. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively.

III.4.3.6. Muscle and liver lipid oxidation (TBARS)

TBARS assay yielded significantly higher values for this parameter in CT batch (Table 8) than in the rest of experimental diets, both in muscle and liver. The inclusion of algal biomass decreased significantly lipid oxidation, not least when *N. gaditana* biomass was previously hydrolysed (H25 and H50). The results also indicate a dose-dependent effect, showing lower TBARS values those samples from fish fed on 50 g kg^{-1} diets, regardless of tissue or sampling time considered.

III.4.3.7. Instrumental colour determinations

At 45 days of the feeding trial, skin L* values were similar in all lots, remaining this trend through the experimental period (Table 9). Skin a* parameter presented negative values in all specimens. Nevertheless, values for fish fed any experimental diets supplemented with *N. gaditana* biomass were lower that CT group, indicating a more greenish coloration. On the other hand, algae biomass as additive affected b* parameter, showing a yellowish pigmentation of the skin, mainly in R25 and R50 specimens at 90 days.

Table 8. Estimation of lipid oxidation (TBARS) in muscle and liver of juvenile fish fed on the different experimental diets.

	Time	CT	R25	R50	H25	H50	*p-value*
Muscle	45 d	2.17 ± 0.37^{c}	1.68 ± 0.14^{bc}	1.42 ± 0.12^{b}	1.31 ± 0.14^{ab}	1.04 ± 0.13^{a}	*<0.001*
	90 d	2.01 ± 0.39^{b}	1.71 ± 0.05^{b}	1.15 ± 0.23^{ab}	1.26 ± 0.20^{ab}	0.92 ± 0.09^{a}	*<0.001*
Liver	45 d	4.62 ± 0.43^{d}	3.86 ± 0.39^{c}	3.19 ± 0.17^{b}	2.77 ± 0.19^{a}	2.52 ± 0.18^{a}	*<0.001*
	90 d	4.64 ± 0.19^{e}	3.73 ± 0.26^{d}	3.03 ± 0.09^{c}	2.67 ± 0.20^{b}	2.15 ± 0.07^{a}	*<0.001*

Values in the same row with different lowercase superscripts indicate significant differences ($p < 0.05$) owing to dietary treatments. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Values (n=15) are mean ± SD. TBARS stands for thiobarbituric acid reactive substances, expressed as mg malonyldialdehyde (MDA) kg $tissue^{-1}$.

Table 9. Instrumental colour determinations on the skin surface of juvenile fish fed with the different experimental diets.

Time	Colour parameters	CT	R25	R50	H25	H50	*p-value*
45 d	*L**	73.74 ± 4.22	74.98 ± 3.24	73.36 ± 3.44	72.40 ± 5.19	72.13 ± 5.33	*n.s.*
	*a**	- 1.00 ± 0.39^{a}	- 1.88 ± 0.44^{b}	- 1.85 ± 0.40^{b}	- 1.89 ± 0.39^{b}	- 1.80 ± 0.32^{b}	*0.047*
	*b**	5.25 ± 1.40^{a}	7.79 ± 1.81^{ab}	8.54 ± 1.04^{b}	7.09 ± 1.09^{ab}	7.88 ± 1.36^{ab}	*0.033*
90 d	*L**	89.75 ± 1.41	87.91 ±3.24	87.91 ±3.25	88.07 ±2.06	87.37 ±1.45	*n.s.*
	*a**	- 1.48 ± 0.16^{a}	- 2.01 ± 0.38^{b}	- 2.16 ± 0.11^{b}	- 1.89 ± 0.12^{b}	- 1.94 ± 0.08^{b}	*0.021*
	*b**	5.53 ± 1.13^{a}	8.16 ± 0.46^{b}	8.28 ± 0.46^{b}	8.02 ± 0.92^{ab}	7.25 ± 1.07^{ab}	*< 0.001*

Values in the same row with different lowercase superscripts indicate significant differences ($p < 0.05$) owing to dietary treatments. CT: control diet. R25 and R50: diets including 25 and 50 g kg^{-1} raw microalgal biomass, respectively. H25 and H50: diets including 25 and 50 g kg^{-1} hydrolysed microalgae, respectively. Values (n=30) are mean ± SD. n.s.: not significant. Parameters L*, a* and b* as defined in M&M section.

III.4.4. DISCUSSION

Microalgae are drawing the attention of nutritionists in the last years not only owing to their potential as macronutrients, but also as a valuable source of bioactive compounds (e.g. PUFAs, vitamins, minerals, pigments, polyphenols, etc.), many of which have been described, but many others remain to be identified. Different studies have pointed out that these substances can exert positive effects on different aspects of fish physiology, even if added at low inclusion level in feeds (Kiron *et al*., 2012; Becker, 2013). Although it is doubtful that such low supplementation could influence decisively fish growth or proximal composition, however, evidences also suggest that other characteristics of fish might well result benefited (Batista *et al*., 2020).

Nannochloropsis gaditana is currently cultivated at industrial or semi-industrial scale (Heredia *et al*., 2021; Kavitha *et al*., 2021), a reason which, together with its content in EPA, pigments and other natural antioxidants and bioactive compounds (Lubian, 1982; Tibbetts *et al*., 2017; Sales *et al*., 2021) is gaining the interest as additive in aquafeeds. However, the existence of a thick cell wall rich in cellulose (Cho *et al*., 2013; Scholz *et al*., 2014; Velazquez-Lucio *et al*., 2018) might hinder the bioavailability of many of those relevant substances when *N. gaditana* is included in practical feeds. This limitation has been highlighted specially for process-scale lipid extraction (Günerken *et al*., 2015), and thereby, considerable effort is being put into developing affordable procedures aimed at increasing the recovery of inner compounds from microalgae cells.

These include disruption by physical, chemical or enzymatic methods, all of them with pros and cons (Mendes Pinto *et al*., 2001; Tibbetts *et al*., 2017; Gomes *et al*., 2020). Although the use of physical methods has become the most common approach (Agboola *et al*., 2019), not least owing to their success in extracting the lipid fraction, however, the costs of the disruption processes can outweigh the benefits of higher yields (Alhattab *et al*., 2019; Singh & Dhar, 2019).

In this regard, an emerging cost-effective alternative might involve the breakage of cell walls by hydrolysis with cellulase enzymes, taking into consideration the existence of thick cell walls rich in cellulose. The following may be cited as advantages of this procedure: i) owing to the wide variety of industrial applications, cellulases are reasonably inexpensive; ii) no complex equipment is needed to perform the hydrolysis; iii) negative impacts on thermolabile compounds are not expected; and iv) given the specificity of the catalytic action on cellulose, the remaining released compounds would not be hydrolysed by the enzymatic pre-treatment.

Under this perspective, this study evaluated in a 90-d feeding trial the effects of a cellulase pre-treatment on *Nannochloropsis gaditana* biomass prior to its incorporation into feeds for gilthead seabream juveniles. It was expected that nutrient bioavailability would increase as a result of the enzyme treatment, and according to the results of the *in vitro* assay (Figures 2 and 3), the increased release of reducing sugars, free amino acids, soluble protein and polyphenols taking place in the reaction vessels seem to confirm this hypothesis.

Even if the enzyme treatment increased *in vitro* bioavailability, however, no impact on fish growth was observed for any of the experimental batches in our study after the 90-d feeding trial. These results are in line with previous studies on this microalgae genus (Qiao *et al*., 2019, in turbot, *Scophthalmus maximus*; Sørensen *et al*., 2017, in salmon, *Salmo salar*; Walker and Berlinsky, 2011, in Atlantic cod, *Gadus morhua*). Although some reports pointed to improved fish growth owing to microalgae inclusion in diets (Abdel-Tawwab & Ahmad, 2009; Cerezuela *et al*., 2012; Vizcaíno *et al*., 2014; 2016; 2018; Norambuena *et al*., 2015), considerably higher inclusion levels were evaluated. Undoubtedly, the low inclusion levels considered have accounted for this limited effect, taking into account that the microalgal biomass did not change total protein, amino acid or fatty acid composition of diets (Table 2 and Figure 1).

With regard to fish muscle composition, overall, no differences attributable to the experimental diets were observed, in agreement with previous studies

on *N. gaditana* enriched diets (Qiao *et al*., 2019; Vizcaíno et al., 2018; Sales *et al*., 2021). Only slight, but not significant differences in lipid and protein contents were measured among the experimental groups (Table 3). This tendency has been described in previous studies on the nutritional effects of microalgae, and thus, Galafat *et al*. (2020) reported a significant increase in muscle protein content of fish fed enzymatically hydrolysed *Arthrospira platensis* added at an inclusion level of 2%, as well as a significant decrease in total lipids when added at an inclusion level of 4%. Reduced muscle lipid storage has also been reported not only for microalgae species (Hussein *et al*., 2013; El-Sheekh *et al*., 2014; Vizcaíno *et al*., 2014, 2016), but also for macroalgae (Ortiz *et al*., 2006; Yildirin *et al*., 2009; Sáez *et al*., 2020). These findings altogether suggest the existence of bioactive compounds in algae capable of influencing protein and lipid metabolism, although the nature of such substances or the underlying mechanisms involved in such effects have not been ascertained yet.

Whilst no quantitative differences in muscle lipid content were observed, however, qualitative differences were found in this analytic component. It is known that fish muscle lipids reflect dietary FA profiles (Grigorakis *et al.,* 2002; 2007; Yildiz *et al*., 2007), but the significant increase in muscle n-3-PUFAs observed in this work for microalgae-containing diets (Table 4) can't be attributed to differences in dietary FA as a result of microalgae inclusion (Table 2). *N. gaditana* is rich in EPA (33% of total FA), and consequently, FA profiles of the microalgae-supplemented diets, either raw or hydrolysed (Table 4), were affected. This effect was dose-dependent, but not influenced by the enzymatic pre-treatment of the biomass. Nevertheless, the dose-dependent effect mentioned for EPA in microalgae-enriched diets didn't yield a similar tendency in muscle. Moreover, even if the enzymatic treatment yielded higher EPA content in muscle compared to CT batch, fish fed on the hydrolysed microalgae feeds (H25 and H50) didn't improve EPA content in muscle compared to raw *N. gaditana* (R25 and R50). It could be expected that the hydrolytic treatment would have released higher amounts of EPA from the microalgae cells. However, the opposite was observed, a phenomenon that might well be explained by the fact that released PUFAs could be more susceptible to

structural damage than those remaining within the microalgae cells. In other words, intact cell walls might have acted as a sort of "natural microcapsule" for EPA.

All the experimental batches fed with the supplemented diets yielded significantly higher DHA muscle content than control fish. As mentioned, this fact can't be explained by differences in this FA in the experimental diets (Table 2); on the contrary, control diet showed the highest DHA figures, but yielded the lowest content in fish muscle. In this regard, previous studies have also reported certain selective retention of structural FA owing to the addition of both macro and microalgae (Hussein *et al*., 2013; Vizcaíno *et al*., 2014, 2016; Kousoulaki *et al*., 2016; Sáez *et al*., 2020). In short, the results suggested that *N. gaditana*, even at the low inclusion level studied, was responsible for some selective retention of n-3 PUFA in muscle, whilst the opposite effect was observed with regard to MUFAs (Table 4).

On the other hand, microalgae are acknowledged as valuable source of pigments and phenolic compounds with antioxidant capacity (Koyande *et al*., 2019 ; Almendinger *et al*., 2021; Sáez *et al*., 2021), many of which remain unidentified (Sansone *et al*., 2020).

Due to the interest of the pharmaceutical industry in pigments, these substances have received more attention than phenolics, but some authors suggested that both groups of substances might contribute similarly to the antioxidant activity of microalgae (Almendinger *et al*., 2021). Nevertheless, the relative contribution of phenolics and pigments to the antioxidant capacity of most microalgae species remains to be ascertained (Goiris *et al*., 2012). *N. gaditana* contains chlorophylls, β-carotene, violaxanthin y vaucheriaxanthin, as well as trace amounts of astaxanthin (Cerón-García *et al*., 2018; Sales et al., 2021), which might explain our results pointing to higher antioxidant response in muscle and liver of fish supplemented with the microalgal biomass, being this effect dose-dependent. Teimouri *et al*. (2016; 2019) also described this effect as a result of the inclusion of microalgae in feeds, and more specifically, Qiao *et al*. (2019) reported lower TBARS values both in liver and serum in

Scophtalmus maximus juveniles fed on 5% *N. gaditana* diets. A recent study (Sales *et al*., 2021) has shown that purified extracts of the unsaponifiable fraction of *N. gaditana*, rich in carotenoids, included in feeds to partially replace fish oil yielded potent antioxidant effects in muscle of *Sparus aurata* juveniles.

Interestingly, and in agreement with the *in vivo* antioxidant effects on muscle and liver lipids (Table 8), the cellulase pre-treatment of the microalgal biomass was responsible for a significant increase of the antioxidant effect, compared to untreated *N. gaditana*, this suggesting increased release and further bioavailability of some inner bioactive compounds contained in the cells. Galafat *et al*. (2020) also found lower TBARS values in muscle of *Sparus aurata* juveniles fed with *Arthrospira* sp. protease hydrolysates at low inclusion level (2 and 4%). In agreement, and with regard to phenolics, *N. gaditana* contains certain amount of these substances in raw biomass, in line with previous studies (Kherraf *et al*., 2017; Haoujar *et al*., 2019), which might explain the potent antioxidant effects found on fish lipids in our study. Noticeably, total phenolics measured in the reaction vessels increased as a result of the cellulase treatment (Figure 3).

Although no studies have been accessed in the literature in which the microalgae biomass were pre-treated enzymatically, other strategies (namely mill grinding) aimed at disrupting microalgae cellulosic cell walls have also reported higher yields of compounds with antioxidant capacity (Almendinger *et al*., 2021). However, physical treatments, even if valuable when it comes to increasing the yield of microalgae main compounds (i.e. protein and lipid fractions), might jeopardize the chemical integrity of thermolabile minor compounds (Schafberg *et al.*, 2020), and consequently, impair their functional activity.

Given the susceptibility of pigments, not least carotenoids, to different factors (temperature, oxygen, light, acidic pH, etc., Schieber *et al*., 2016), and even if the extraction procedures increase the releasing of inner compounds, it should also be born in mind that microalgal biomass, as part of the ingredient mixture, will be extruded during the elaboration of the experimental diets, a

process involving high pressure and temperature. Consequently, doubts could arise related to the integrity and the subsequent *in vivo* bioavailability of the compounds released *in vitro*. Previous research suggests that the resulting balance of disrupting strategies is favourable to the enrichment of aquafeeds (Schafberg *et al*., 2020), and our results coincide with that idea, but given the diversity and complex nature of the antioxidant substances involved, this specific issue deserves further research.

Though instrumental colour measurements at early stages of the productive cycle have no interest in practical terms of fish quality assessment, they can still provide valuable information about pigment deposition and antioxidant effects in growing fish tissues. The favourable influence of microalgae on fish colour parameters found in our study (increased a* and b*, Table 9) has been documented previously (Teimouri *et al*., 2013; Cardinaletti *et al*., 2018; Galafat *et al*., 2020; Kousoulaki *et al*., 2020; Sales *et al.*, 2021). Although tendencies observed for pigmentation suggest that raw microalgae intensified the effects compared to hydrolysed biomass (Table 9), however, no significant differences attributable to the enzymatic treatment were found. This might well be explained again by the fact that some pigments contained in the hydrolysed biomass might have been damaged to a higher extent than those from raw biomass due to feed processing.

The activity of digestive enzymes is acknowledged not only as a marker of their digestive and absorptive capacity (Alarcón *et al*., 1998), but also as a reliable indicator of the nutritional status of aquacultured fish. More specifically, the activity of some brush border membrane enzymes, such as leucine aminopeptidase (LAP) and alkaline phosphatase, reveals the integrity and the absorptive capability of the intestinal mucosa (Silva *et al*., 2010). Disparate results have been reported on the effects of raw microalgae on digestive enzyme activities, and thus, Qiao *et al*. (2019) found increased trypsin activity in juvenile turbot supplemented with *N. gaditana* biomass at 7.5% inclusion level after a 10-week feeding trial. On the contrary, Jorge *et al.* (2019) observed no effects on total alkaline protease, trypsin, α-amylase and lipase activities in response to dietary *N. gaditana* supplementation, although the low

inclusion levels considered (0.5, 1, and 1.5%) together with the short duration of the feeding trial (37 d) might well have accounted for such lack of effects. Few studies are available assessing the physiological consequences of microalgae enzyme hydrolysates on such activities. Galafat *et al*. (2020) described higher trypsin and LAP activities as a result of protease hydrolysates of *Arthrospira* sp. at low inclusion level (2 and 4%) in *Sparus aurata* juveniles. The results obtained in this study indicate that *N. gaditana* supplementation, even at the low inclusion levels tested, overall increased the enzyme activities assayed compared to control fish, irrespectively of sampling time (45 or 90 d), or biomass pre-treatment (Table 5). It is also worth mentioning that the favourable effects of the experimental diets on intestinal digestive activities observed in this work concur, generally speaking, with the histological (Table 6) and ultrastructural (Table 7) determinations carried out on the intestinal mucosa, especially at the highest concentration assayed (5%).

Concerning the enzyme pre-treatment considered in this work, roughly, no decisive effects were observed in terms of fish growth, muscle composition, or digestive functionality, but the remarkable influence of this treatment on the oxidative status of fish lipids could result in beneficial effects on other parameters linked to the health status of aquacultured fish, a fact that deserves further investigation as well. It should also be born in mind that any feeding trial under controlled conditions and short duration has evident limitations in terms of further applicability on-farm, owing to the numerous additional factors involved in the operation of long-term production cycles in commercial fish farms.

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IV. GENERAL DISCUSSION

This Doctoral Thesis broaches the study of the potential of crude and hydrolysed microalgal biomass as a functional additive in the elaboration of practical diets for seabream larvae and juvenile fish.

Nowadays, aquaculture is the fastest growing animal production industry. This production stands above wild fisheries, providing 52% of the aquatic food for human consumption (Fiorella *et al*., 2021; FAO, 2020; Yue & Shen, 2021). This increase in the aquaculture requires more sustainable feeding strategies, such as the use of alternative novel ingredients, capable to reduce the current dependence of aquaculture on feeds obtained from wild-caught fish. In this regard, the use of algae, and especially microalgae, in aquaculture nutrition is a burgeoning research field. From a nutritional point of view, algal biomass can be used as a main ingredient as a sustainable source of protein and lipids, as well as a dietary additive due to its abundance in bioactive compounds like pigments, polysaccharides, polyphenols and vitamins. Indeed, there is plenty of literature pointing to potential beneficial effects on health and general status of fish owing to the dietary inclusion of several microalgae species (Shah *et al*., 2018). However, microalgae are not exempt from certain drawbacks, such as the high variability in their nutrient composition, as well as the existence in many species of a recalcitrant cell wall that might well limit their digestibility; limitations that must be taken into account before including this resource in practical diets for aquaculture.

As pointed previously, there are numerous studies aimed at assessing the zootechnical and physiological effects of the dietary inclusion of microalgae in fish nutrition; however, the characterization of algal biomass, especially in terms of protein and amino acid availability, as well as protein digestibility by marine fish, is still limited. In this context, **CHAPTER 1** focuses on the *in vitro* evaluation of the protein bioavailability of different marine and freshwater microalgae species and cyanobacteria, with the aim of assessing their potential as dietary ingredients in aquafeeds. The results obtained indicate that all the microalgae and cyanobacteria evaluated had a high protein content ranging from 25% to 61% in dry matter basis. In addition, all of them showed a similar

amino acid profile, able to provide most of the essential amino acids to fulfil the requirements of farmed fish, so that their inclusion can assure adequate fish growth (Wilson, 2003). In addition to the protein and amino acid profile, the presence of antinutritive factors able to inhibit the digestive proteases of fish was also assessed. The results obtained revealed that high inclusion of microalgae would be needed for reaching noticeable inhibition values. From a physiological point of view, an hypothetical feed supplemented with 15% microalgae would produce less 10% inhibition, an effect that can be easily compensate by fish (Haard *et al*., 1996; Santigosa *et al*., 2010). On the other hand, a species-specific *in vitro* protein digestibility simulation was carried out as a preliminary evaluation of microalgal biomass. This *in vitro* procedure has been previously used for evaluating other potential ingredients for aquafeeds, and it may provide valuable information about the digestive capacity of fish proteases to hydrolyse microalgae proteins (Alarcón *et al*., 2002; Vizcaíno *et al*., 2019). Roughly, the results obtained suggest a high bioavailability of microalgae protein, although significant differences were observed among species on their susceptibility to be hydrolysed. Thus, values obtained for CPD, total amount of free amino acids, as well as the quantitative profiles of essential and non-essential amino acids released (*EAAR*/*NEAAR*), revealed significant differences in the susceptibility of microalgae protein to the activity of the digestive enzymes of gilthead seabream juveniles. Both the characteristics of the cell walls, and the the particular digestive physiology of fish might have accounted for such disparity (Kamalam *et al*., 2017; Bernaerts *et al*., 2018). This fact corroborates the value of this preliminary analysis as an useful tool for the evaluation and selection of alternative protein ingredients for aquafeed formulation (Vizcaíno *et al*., 2019).

Haven made this consideration, the following chapters focused on the *in vivo* assessment of the effects of the cyanobacteria *Arthorspira platensis* and the microalgae *Nannochloropsis gaditana* included in practical diets for gilthead seabream by performing different feeding trials. Additionally, these feeding trials evaluated not only the inclusion of the crude microalgae, but also the

incorporation of enzymatic hydrolysates as a strategy for increasing the nutritional and functional value of the raw biomass.

Overall, the *in vivo* trials revealed the lack of negative effects on fish growth and nutrient utilization owing to the dietary inclusion of crude and hydrolysed *A. platensis* (**CHAPTER 2** and **3**) and *N. gaditana* (**CHAPTER 4**).

By comparing these results with others previously reported in the literature (Vizcaíno *et al*., 2016; Ayala *et al*., 2020; Yu *et al*., 2018; Peixoto *et al*., 2021), it seems to be clear that the dietary inclusion of microalgae has disparate effects on fish growth and nutrient utilization. This fact confirms the species-specific and dose-dependent nature of the effects of microalgae on growth performance and nutrient utilization in farmed fish.

With regard to the effects of microalgae inclusion on muscle composition, **CHAPTER 2** revealed that the dietary inclusion of crude or hydrolysed *A. platensis* did not modify gilthead seabream fry body composition. However, results obtained in **CHAPTER 3** showed certain modifications in muscle protein and lipid contents in fish fed with the diets containing *A. platensis* protein hydrolysate, in agreement with similar findings in previous studies (Roohani*et al*., 2019). This phenomenon seems to be attributable to the presence in microalgae of some bioactive compounds with the ability to modulate lipid metabolism, leading to increased efficiency of lipid mobilization from liver to muscle, especially in phases of rapid growth (Knutsen *et al*., 2019). On the other hand, **CHAPTER 2** also provides information about the influence of microalgae dietary inclusion on muscle fatty acid profile. Thus, the results obtained in this chapter revealed a decrease in muscle EPA contents in fish fed on diets supplemented with *A. platensis* protein hydrolysate, which could be related to the modulating effect on lipid metabolism mentioned above, as well as to a higher catabolic use of this specific fatty acid (Tocher, 2003). Furthermore, in both **CHAPTER 2** and **CHAPTER 4**, the dietary inclusion of *A. platensis* and *N. gaditana*, respectively, induced an increase in DHA content in fish fed with crude and hydrolysed microalgae-supplemented diets, this finding suggesting

a selective retention of DHA, which has been described previously for sea bass and Senegalese sole (Haas *et al*., 2016; Rodiles *et al*., 2014, Sáez *et al*., 2020).

Microalgae are also considered a valuable source of natural pigments, since they contain a wide range of bioactive compounds, such as astaxanthin and xanthophylls, able to induce positive effects on skin colour pigmentation (Koyande *et al*., 2019; Teimouri *et al*., 2019; Batista *et al*., 2020). In this context, in **CHAPTER 3** is described that the dietary inclusion of hydrolysed *A. platensis* exerts positive effects on skin instrumental colour parameters. Thus, fish fed on *A. platensis* supplemented diets displayed a more luminous, reddish and yellowish skin than those fish fed on the microalgae-free diet, a fact that could end up in increased consumer's acceptance of fish. Regarding the antioxidant properties of microalgae, the results indicate a significant reduction in muscle lipid oxidation in all fish batches fed with microalgae-supplemented diets, regardless the microalgae species and irrespectively of the hydrolytic pre-treatment of the biomass included in feeds (**CHAPTERS 2, 3, 4**). However, it is worth mentioning that the enzymatic pre-treatment of the microalgal biomass was responsible for a significant increase of the antioxidant effect, compared to untreated *A. platensis* and *N. gaditana*, this suggesting increased release and further bioavailability of some inner bioactive compounds contained in the cells (Liu *et al*., 2019; Afify *et al*., 2018), but this specific issue needs further research, given the diversity and varied nature of the antioxidant substances involved.

Feed efficiency depends on the physiological capacity of fish to digest and transform the ingested nutrients. In this regard, the evaluation of both pancreatic and brush border enzymes can be used as indices not only of fish nutritional condition, but also of adaptation capability to dietary changes (Vizcaíno *et al*., 2016). These enzymes play a key role in fish development, so that any change in their activity could reveal an impact on fish growth and proper utilization of nutrients. Nevertheless, contrasting results have been reported on the effects of raw microalgae on digestive enzyme activities (Vizcaíno *et al*., 2014; Qiao *et al*., 2019; Jorge *et al*., 2019). In this work, the results obtained in **CHAPTERS 2, 3** and **4** indicate an increase in the activity of

pancreatic enzymes (trypsin, chymotrypsin and total alkaline protease) in fish fed on microalgae-supplemented diets, regardless the microalgae considered. Furthermore, in the case of *Arthrospira* biomass (**CHAPTERS 2** and **3**), the inclusion of the enzymatically hydrolysed microalgae caused significant increase in the activity of these digestive enzymes, compared to raw biomass. Similarly, an improvement in the activity of brush border enzymes (leucine aminopeptidase and alkaline phosphatase) was also observed in those fish fed on microalgae-supplemented diets. These enzymes play a key role at the final stages of the digestion of dietary protein, paving the way to both amino acid absorption and transport through the enterocytes (Infante & Cahu, 2001), altogether increasing the overall efficiency of the digestive and absorptive processes (Vizcaíno *et al*., 2016).

In addition to the effects on the digestive enzyme activities, inadequate dietary and environmental conditions may result in altered epithelial development, or in disruption of the epithelium integrity (Rønnestad *et al*., 2013). In this context, it is worth mentioning that the positive effects of the dietary inclusion of *A. platensis* and *N. gaditana* on the intestinal digestive activities observed in this work concur with the histological and ultrastructural determinations carried out on the intestinal mucosa. As has been described in previous studies, the dietary inclusion of microalgae biomass caused favourable effects on the gut morphology of gilthead seabream.

Overall, beneficial changes were observed in microvilli length, enterocyte apical area and absorption surface in enterocytes, which together with the absence of any sign of damage, such as lipid droplet accumulation in the intestinal epithelium, inflammatory changes in the *lamina propria*, or alterations in the intestinal brush border membrane, suggest altogether that the inclusion of the microalgal biomass is well tolerated by the animals (**CHAPTER 2**, **3** and **4**). The observed modifications of the epithelium, indeed, could be associated with increased absorption capacity of the intestinal mucosa, as well as with the reinforcement of the *microvilli* as a physical barrier against potential pathogens (Wilson & Castro, 2011).

In conclusion, the present study provides useful species-specific information of microalgae and cyanobacteria as potential ingredients for aquafeeds. On the whole, the adequate protein profile and their susceptibility to be hydrolysed by *S. aurata* digestive enzymes confirmed that microalgae biomass is a valuable alternative ingredient to be used in aquafeeds formulation. Despite the dietary inclusion of microalgae had no impact on fish growth or nutrient utilization, it has been corroborated that their inclusion yields positive effects on gut functionality and on the oxidative status of fish lipids, a fact that could result in beneficial consequences on other parameters linked to the health status of aquacultured fish. The favourable effects have been particularly marked on fish fed with diets supplemented with hydrolysed microalgae, and thus, the inclusion of these dietary supplements at low inclusion level seems promising for feeding *S. aurata* fry and juveniles. However, future studies aimed at ascertaining the intrinsic mechanisms of their effects, as well as on the feasibility of its commercial use at large scale in aquafeeds are required.

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V. CONCLUSIONS

Results from the present work lead to draw the following conclusions:

CHAPTER 1.

1. The nutritional characterization shows high protein content as well as balanced amino acid profile in all the microalgae and cyanobacteria species evaluated.

2. The evaluation of the *in vitro* protein hydrolysis reveals differences in the microalgae susceptibility to the hydrolysis by gilthead seabream digestive enzymes, and therefore in their protein bioavailability. Particularly, *Arthrospira platensis, Nannochloropsis gaditana* and *Chlorella vulgaris* seem to be good candidates feeding gilthead seabream.

CHAPTER 2.

1. The dietary inclusion up to 10% of crude or hydrolysed *Arthrospira platensis* does not improve growth performance, but positive effects on gut functionality, muscle lipid peroxidation and lysozyme activity in liver are observed in fish fed on hydrolysed-microalgae supplemented diets compared to those fed with the raw biomass.

2. The results confirm that *Arthrospira platensis* hydrolysate can be included as a functional additive in starter feeds for gilthead seabream, based on the beneficial effects observed in gut functionality, which are of interest during the weaning period.

CHAPTER 3.

1. The use of *Arthrospira* sp. hydrolysate at low dietary inclusion level does not modify the growth performance in gilthead seabream juveniles, but

promotes a noticeable positive effect on digestive functionality and the improvement of the skin colour and oxidative status in fish.

2. The incorporation of *Arthrospira* sp. hydrolysate as dietary additive is a useful strategy for feeding *Sparus aurata* juveniles, however future studies for clearing up the intrinsic mechanism of its effect, as well as the feasibility of its commercial use in aquafeeds at large scale are requested.

CHAPTER 4.

1. The inclusion of *Nannochloropsis gaditana* biomass at a low dietary level does not have impact on the growth performance in *Sparus aurata* juveniles, but promotes beneficial effects on digestive functionality, oxidative status of muscle lipids and colour.

2. The results obtained evidence the effectiveness of the cellulase pretreatment for increasing the bioavailability of microalgal metabolites with bioactive potential. However, no conclusive evidence was found regarding the impact of this strategy on most of the physiological parameters studied, except for the enhanced antioxidant effect on the tissue lipids.

OVERALL CONCLUSSION

1. Despite the dietary inclusion of microalgae have no impact on fish growth or nutrient utilization, but positive effects on gut functionality and the oxidative status of fish lipids are confirmed. These effects are particularly marked in fish fed with diets supplemented with the hydrolysed microalgae.

VI. ANNEXES

SCIENTIFIC CONTRIBUTIONS

I. Scientific publications

1. **Galafat A**, Vizcaíno AJ, Sáez MI, Martínez TF, Arizcun M, Chaves-Pozo E & Alarcón FJ (2021) Assessment of dietary inclusion of crude or hydrolysed *Arthrospira platensis* biomass in starter diets for gilthead seabream (*Sparus aurata*). *Aquaculture* 548(2), 737680. DOI: 10.1016/j.aquaculture.2021.737680. IF: 4.09, Q1 (Aquatic Science).

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4. Galafat A, Vizcaíno AJ, Sáez MI, Moya TF & Alarcón FJ (2021) The potential of algae for feeding aquaculture fish. In Potential use of probiotics and micro/macro algae as additives and major ingredients in aquafeeds. Centro Tecnológico del Mar (eds.). p: 65-101. ISBN: 978-84-09-3150-5

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9. Ayala MD, Galián C, Fernández V, Chaves-Pozo E, García D, Sáez MI, **Galafat A**, Alarcón FJ, Martínez TF y Arizcun M (2020) Influence of low dietary inclusion of the microalga *Nannochloropsis gaditana* (Lubian 1982) on performance, fish morphology, and muscle growth in juvenile gilthead seabream (*Sparus aurata*). *Animals* 10(12), 2270. DOI: 10.3390/ani10122270. IF: 2.70, Q1 (Animal Science and Zoology).

II. Conferences

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6. Vizcaíno AJ, Sáez MI, **Galafat A**, Martínez TF, Acién FG, Alarcón FJ (2019) Evaluación de microalgas para su uso en nutrición acuícola. XVII Congreso Nacional de Acuicultura. Cartagena, España.

7. **Galafat A**, Vizcaíno AJ, Sáez MI, Arizcun M, Martínez TF, Alarcón FJ (2019) Inclusión de microalgas en piensos de primera edad para dorada (*Sparus aurata*). Efecto sobre las enzimas digestivas y la mucosa intestinal. XVII Congreso Nacional de Acuicultura. Cartagena, España.

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14. **Galafat A**, Francesca B, Vizcaíno AJ, Oshiro A, Martínez TF, Mancera JM, Alarcón FJ (2017) Incorporación de un hidrolizado de microalga en piensos para juveniles de dorada: efectos sobre el crecimiento y la funcionalidad intestinal. XVI Congreso Nacional de Acuicultura. Zaragoza, España.

15. **Galafat A**, Vizcaíno AJ, Sáez MI, Martínez TF, Alarcón FJ (2017) Evaluación de la hidrólisis *in vitro* de la proteína de *Arthrospira platensis* por las proteasas digestivas de dorada**.** XVI Congreso Nacional de Acuicultura. Zaragoza, España.

16. Vizcaíno AJ, Oshiro A, Sáez MI, **Galafat A**, Martínez TF, Acién FG, Alarcón FJ (2017) Hydrolysate of the microalgae *Arthrospira platensis* in feed for gilthead sea bream (*Sparus aurata*): effect on growth and intestinal functionality. 8th Symposium on microalgae and seaweeds products in plant/soil systems. Mosonmagyarovar, Hungary.

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POTENTIAL USE OF PROBIOTICS AND MICRO/MACRO ALGAE AS ADDITIVES AND MAJOR INGREDIENTS IN AQUAFEEDS

Potential use of probiotics and micro/macro algae as additives and major ingredients in aquafeeds

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CHAPTER 4

The potential of algae for feeding aquaculture fish

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Abstract

Developing sustainable and nutritious aquafeeds still remains as one of the greatest challenges in aquaculture. During the last decades, important efforts have been focused on finding and testing alternative ingredients able to reduce the dependency on regular ingredients. Any alternative feedstuff should not only supply adequate nutritional value, but also be available at industrial scale, as well as economically affordable. In this regard, the use of algae in aquaculture arouses great interest mainly owing to their nutritional composition and the variety of their bioactive compounds, as well as to the potential benefits on different aspects of fish development, such as growth, muscle proximate composition, and digestive functionality, as pointed out by numerous scientific publications. However, there are several technical gaps that need to be addressed before the aquafeed industry can incorporate algae-based ingredients or additives into commercial formulas at large scale, such as those related to safety and regulatory aspects on feed application, production costs, variability in nutrient composition and digestive bioavailability, or the presence of anti-nutritional factors, among others. Therefore, this chapter summarizes the algal nutritional composition as well as relevant publications on the use of algae as dietary ingredient to replace fishmeal and fish oil or as functional additive in practical diets for farmed fish. In addition, major challenges for the use of algae in commercial aquafeeds, and future perspectives are also discussed.

Keywords: Aquafeeds, alternative ingredients, bioactive compounds, marine fish, microalgae, nutrients, fish nutrition, seaweeds.

1. The increasing demand for aquafeeds

According to the latest world FAO statistics, farming of aquatic animals achieved another all-time record in 2018, with 82.1 million tonnes, and it is expected to reach 109 million tonnes in 2030 (FAO, 2020). Within this production, fed aquaculture has outdistanced non-fed aquaculture, and nowadays almost 50% of all the aquaculture production is steadily dependent on commercial aquafeeds. Figure 1 shows a projection of the aquafeed production until 2025. The average growth has been of 10% per year, and the production of feeds is expected to increase up to 87 million tonnes by 2025, although this figure is quite low compared to the global feed production for terrestrial animals, which is expected to reach more than 1 billion tonnes. In spite of this fact, aquafeed production consumes 70% of fishmeal, and over 73% of fish oil marketed in the world.

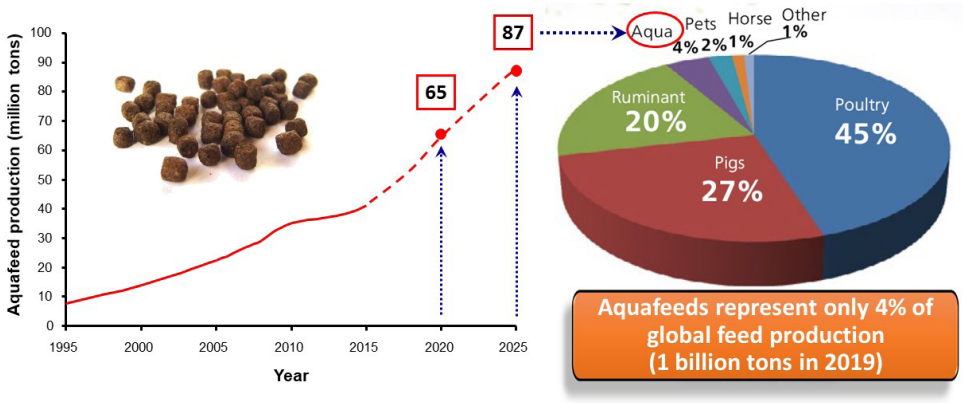

Figure 1. Estimated aquafeed production for 2025.

Indeed, aquaculture industry still heavily relies on fishmeal and fish oil as regular feed ingredients (Yadav *et al*., 2020). Both feedstuffs are considered the most nutritious, digestible, and palatable ingredients for aquafeed production, as well as the major source of essential amino acids, phospholipids, and omega-3 fatty acids, not least docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids (Yarnold *et al*., 2019). Both raw materials come by from wild-harvested fish populations, and so changes in the catches of the target fish species can affect their production. According to FAO (2020), the amount of wild fish catches used for producing fishmeal and fish oil in 2018 (18 million tonnes) was significantly lower than that in 1994 (30 million tonnes). This reduction in the supply of those feedstuffs together with a raising demand driven by a fast-growing aquaculture industry have led fishmeal prices to increase by almost three-fold in the last decade (fishmeal and fish oil current costs are around 1,120-

1,730 and 2,000-2,200 USD per tonne, respectively-HAMMERSMITH Marketing Ltd, September 2020). In this scenario, although the inclusion rates of fishmeal and fish oil in aquafeeds have been progressively reduced, the thriving production of all farmed species has generated a growing demand for such ingredients, mainly attributable to fish aquaculture (Naylor *et al*., 2009). Therefore, finding and testing alternative protein and lipid sources with potential for developing sustainable and nutritious aquafeeds, continue to be a challenge for today's aquaculture (Yarnold *et al*., 2019), and this is the ultimate reason why considerable research effort is being made on this topic.

In this regard, any satisfactory alternative feed ingredient must supply a nutritional value comparable to that of regular ingredients, and should also be palatable, available on a large scale, as well as economically viable (Vizcaíno *et al*., 2014). Conventional land-based crops, especially grains and pulses and their derivatives, are feasible alternatives owing to their low cost, and to the fact that they have proved successful in the elaboration of aquafeeds when used for partial replacement of fishmeal and fish oil. Thus, plant-based ingredients offer positive effects on fish growth, although there are some disadvantages that hinder their inclusion in aquafeeds, especially at high inclusion level. From a nutritional point of view, terrestrial plant feedstuffs are characterized by low digestibility and nutrient bioavailability, and low palatability (Daniel, 2018). In addition, these ingredients usually have imbalanced nutrient profiles, with deficiencies in some essential amino acids, such as lysine, methionine, threonine, and tryptophan, and they can contain anti-nutritive factors that may affect negatively both physiology and growth performance of farmed fish. Other alternatives such as plant oils (e.g. rapeseed oil, cottonseed oil, soybean oil, sunflower seed oil) are rich in n-6 fatty acids but poor in n-3 long chain-PUFA, in such a way that their excessive use may alter the fatty acid profile of fish fillets (Shah *et al.*, 2018). Moreover, intestinal inflammatory phenomena have been described in juvenile salmon fed on 20% soybean meal after 21 days (Booman *et al.*, 2018). In this case, even if fish growth wasn`t affected, enteritis alters the intestinal mucosa integrity and increases the risk of disease.

The above mentioned are the reasons why numerous studies have been carried out to find other alternative ingredients without these limitations. In this point, it is very important to consider that replacing fishmeal is not just substituting the protein of fishmeal, given that fishmeal contains many other important nutrients (nucleotides, n-3 fatty acids, minerals, bioactive compounds, etc.). Fish have requirements for nutrients and not for ingredients. Likewise, fish oil is also more than a source of lipids; it is a source of n-3 fatty acids, but also of cholesterol, vitamins, carotenoids, and other factors. For those reasons, finding alternatives to fishmeal and fish oil is

more than finding cheaper sources of protein or lipid, it is also replacing all the rest of essential nutrients that fish require, including essential amino acids, nucleotides, fatty acids, minerals, vitamins and pigments.

In this regard, algae are interesting alternative ingredients for aquafeeds (Fig. 2). The chemical composition of some algal species has drawn the attention of researchers as an important resource, not only as dietary protein/lipid source, but also as potential additives for providing bioactive and functional compounds to aquafeeds (Shah *et al*., 2018; Vizcaíno *et al*., 2019a).

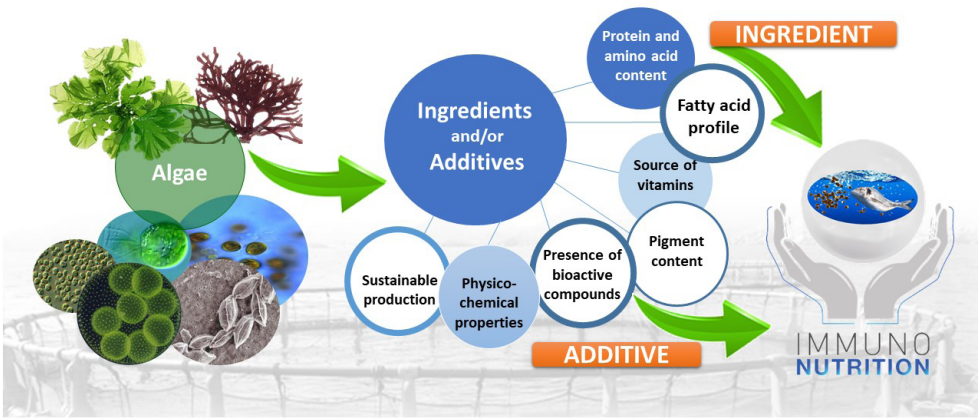

Figure 2. Interest of algae as dietary ingredients/additives for aquafeed manufacture.

2. Relevance of algae in aquafeeds

Since the 1980s, algae play a key role in aquaculture nutrition, either for direct or indirect consumption (Brown *et al*., 1997). However, it has been in the last 25 years when the interest in this resource as potential alternative to fishmeal and fish oil has been progressively increased owing to their nutritional value, rapid growth and its antioxidant value (Roy and Pal, 2015; Vizcaíno *et al*., 2019b).

From a nutritional point of view, microalgae can be used as a natural source of protein, lipids, vitamins, carotenoids, and energy (Shah *et al*., 2018), whereas macroalgae are more appreciated as sources of bioactive compounds, such as pigments, polysaccharides, polyphenols, and vitamins rather than as protein or lipid sources (Moutinho *et al*., 2018). However, the nutritional value of a given algae strain depends of several factors, such as cell size and shape, digestibility, presence of anti-nutritive factors, production of toxic substances, and the specific nutritional requirements of the target animal species of microalgae-supplemented diets (Brown *et al*., 1997).

The chemical composition of algae has been extensively documented in previous studies (Table 1). Overall, the protein content of microalgae ranged from 30 to 55% (on dry matter basis, DM) (López *et al.*, 2010), though there are some genera, such as *Anabaena* sp., *Chlorella* sp., or *Arthrospira* sp. (Cyanobacteria) with higher values (Venkataraman and Becker, 1985). In general, microalgae protein shows a

Table 1. Proximate composition (% dry weight, DM) of several algae species. (CHO: carbohydrates)

	Protein	Lipid	CHO	Source
Marine microalgae				
Anabaena sp.	60.9	14.1	-	Vizcaíno *et al.* (2021)
Dunaliella sp.	52.3	20.3	-	Vizcaíno *et al.* (2021)
Nannochloropsis gaditana	44.9	27.0	-	Vizcaíno *et al.* (2021)
N. gaditana	33.2	27.9	15.9	Di Lena *et al.* (2020)
Pavlova sp.	24–29	9–14	6–9	Becker (1994)
Porphyridium sp.	20.1	4.8	-	Vizcaíno *et al.* (2021)
Schizochytrium sp.	12.5	40.2	38.9	Shields and Lupatsch (2012)
Tetraselmis chuii	46.5	12.3	25	Tibbetts *et al.* (2015)
Tetraselmis sp.	27.2	14.0	45.4	Shields and Lupatsch (2012)
Tetraselmis suecica	26.0	14.7	24.1	Di Lena *et al.* (2020)
T. suecica	36.0	12.9	-	Vizcaíno *et al.* (2021)
Tysochrysis lutea	43.6	17.8	-	Vizcaíno *et al.* (2021)
Freshwater microalgae				
Aphanizomenon flos-aquae	62.0	3.0	23.0	Becker (2007)
Arthrospira maxima	60-71	6-7	13-16	Becker (2007)
Arthrospira platensis	50-65	4-9	8-14	Becker (2007)
A. platensis	46.8	1.4	3.3	Molino *et al.* (2018)
A. platensis	36.8	7.2	-	Vizcaíno *et al.* (2021)
Chlorella ovalis				Slocombe *et al.* (2013)
Chlorella pyrenoidosa	57.0	2.0	26.0	Becker (2007)
Chlorella sp.	43.2	6.5	-	Vizcaíno *et al.* (2021)
Scenedesmus almeriensis	42.8	9.6	-	Vizcaíno *et al.* (2021)
S. almeriensis	49.4	12.0	24.6	Sánchez *et al.* (2008)
Scenedesmus obliquus	50–56	12–14	10–52	Becker (2007)
Spirogyra sp.	25.3	9.3		Vizcaíno *et al.* (2021)
Macroalgae				
Gracilaria cornea	13.5	0.8	39.8	Vizcaíno *et al.* (2016a)
Gracilaria lameneiformis	19.2	0.5	61.3	Xu *et al.* (2011)
Laminaria digitata	15.9	0.5	-	Marsham *et al.* (2007)
Macrocystis pyrifera	5.3-6.1	0.7	-	Cruz-Suárez *et al.* (2009)
Ulva fasciata	8.8-12.3	3.6-5.1	-	McDermid and Stuercke (2003)
Ulva ohnoi	19.2	3.2	29.9	Vizcaíno *et al.* (2019a)
Ulva rigida	14.9	1.2	50.4	Vizcaíno *et al.* (2016a)
Reference ingredient				
Fishmeal	65.0	12.7	-	Vizcaíno *et al.* (2021)
Soybean meal	50.1	2.7	-	Vizcaíno *et al.* (2021)

balanced amino acid profile, similar to that observed in other regular ingredients commonly used in aquafeeds (Becker, 2007). Microalgae contain a lipid content ranging from 2 to 50% (DM), although some genera exceed 80%. Usual values are in the region of 20-50% (Chisti, 2007). The carbohydrate content varies from 5 to 35% and plays an important role in microalgae digestibility (Percival and Turvey, 1974). Regarding macroalgae, the protein content in brown seaweeds ranges from 3 to 15%, and from 14 to 47% (DM) in the case of green and red seaweeds (Arasaki and Arasaki, 1983). The total lipid content is relatively low (0.2-4% DM), while the total amount of carbohydrates ranges from 1.8 to 66% (DM) including simple sugars, soluble carbohydrates, pectin, alginic acid, carrageenan and agar, among others (Wan *et al*., 2019).

2.1. Algal protein

Protein content is the main factor that determines the value of a given alga strain for feeding purposes in aquaculture nutrition (Spolaore *et al*., 2006). Microalgal protein from different species shows similar amino acid profiles, which are characterized by a high content in essential amino acids, as exemplified by the comprehensive study of 40 species of microalgae carried out by Brown *et al*. (1997). This study found that all species showed similar amino acid composition, comparable to that of other regular ingredients. For instance, *Arthrospira* and *Chlorella* biomasses have a protein content above 50%, whose quality is comparable to those of yeast and soybean meal (Kovač *et al*., 2013).

Overall, leucine and arginine are the predominant amino acids in microalgal protein. The content of both amino acids ranges from 5 to 9 g per 100 g protein, while histidine and methionine are typically the most limiting amino acids, with a content around 1.5 to 2 g per 100 g protein. However, microalgae strains like *Dunaliella*, *Scenedesmus* or *Arthrospira* show an amino acid content similar to that of fishmeal (Table 2). Methionine is usually the most limiting amino acid in the ingredients used for aquafeed manufacture, especially when terrestrial plant protein sources are used to replace regular ingredients like fishmeal (Mai *et al*. 2006a,b; Tibbetts *et al*., 2015). Therefore, and based on the dietary amino acid requirements of aquaculture fish (Wilson, 2003), algal protein could be able to provide most of the essential amino acids needed for an adequate growth of the animals.

Similarly, macroalgae protein can be considered relatively well balanced in terms of essential amino acids (Wan *et al*., 2019). Generally speaking, many species contain most of the essential and nonessential amino acids (Gressler *et al*., 2010). Although some commercially important species, like the red seaweed *Palmaria palmata*, lack

some amino acids (e.g., cysteine), they are rich in aspartic acid and glycine, with a content of total essential amino acids comparable to soybean protein (Galland-Irmouli *et al*., 1999).

Most published values on the protein content of algae are based on estimations of crude protein, which quantifies other nitrogenous constituents of algae, such as glucosamides, amines, nucleic acids, and cell wall components, in addition to protein. This leads to overestimation of the true protein content (Becker, 2007). For instance, non-protein nitrogen can reach 11.5% in *Arthrospira*. Even with this overestimate, the nutritional value of algae is high, with average quality being similar to, and even higher than, conventional plant protein sources.

Table 2. Amino acid profile (g/100g protein) of several algae species.

	Val	Met	Ile	Leu	Thr	Phe	His	Lys	Arg	Source
Marine microalgae										
Dunaliella sp.	6.0	2.5	4.5	9.3	5.0	6.0	2.5	6.2	6.6	(2)
Nannochloropsis gaditana	4.9	1.2	3.9	7.3	4.6	4.7	1.6	4.3	5.2	(1)
Tetraselmis suecica	4.7	1.5	4.5	6.8	4.1	4.8	1.6	4.1	5.5	(1)
Tisochrysis lutea	5.0	1.3	4.1	6.9	4.6	5.9	1.8	3.8	6.6	(1)
Freshwater microalgae										
Arthrospira platensis	4.3	2.1	3.6	7.0	4.0	3.9	1.3	4.6	5.1	(1)
Aphanizomenon sp.										(3)
Chlorella sp.	3.0	0.6	1.8	5.0	2.4	3.3	0.4	2.7	2.3	(1)
Scenedesmus almeriensis	5.0	1.3	4.3	6.9	4.1	4.5	1.7	4.8	5.8	(1)
Scenedesmus sp.	6.2	2.4	4.4	9.2	5.6	5.6	2.6	6.6	6.4	(2)
Macroalgae										
Ulva lactuca	6.2	1.6	3.7	6.7	4.7	4.0	1.8	4.2	3.6	(4)
Ulva rigida	5.6	1.5	3.1	5.2	5.0	3.3	1.4	3.7	4.6	(4)
Reference ingredient										
Fishmeal	5.4	3.0	4.7	7.7	4.7	4.1	2.4	7.9	5.7	(5)
Soybean meal	4.8	1.4	4.5	7.7	3.9	5.0	2.7	6.4	7.3	(5)

(1) Vizcaíno et al. (2021); (2) Kent et al. (2015); (3) Kristaki et al. (2011); (4) Shuuluka et al. (2013); (5) Cho & Kind (2010).

2.2. Algal lipid and fatty acid profile

The fatty acid content is another factor that determines the nutritional value of algae (Shah *et al*., 2018). There are numerous scientific publications reporting polyunsaturated fatty acid (PUFA) content of algae, especially microalgae, species used in aquaculture (Dunstan *et al*., 1992; Volkman *et al*., 1989).

In general, many of the microalgae and seaweed species possess a high proportion of PUFA, especially n-3 fatty acids such as docosahexaenoic acid (22:6n-3; DHA), α-linolenic acid (18:3n-3; ALA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; AA) (Brown *et al*., 1997; Wan *et al*., 2019) (Table 3). As shown in Table 1, macroalgae present lower lipid contents compared to those observed in some microalgae species, however, seaweeds may improve the fatty acid profile of feeds (Wan *et al*., 2019; Sáez *et al*., 2020).

Table 3. Polyunsaturated fatty acid content (% of total fatty acids) in several algae species.

	18:2n6	18:3n3	20:4n6	20:5n3	22:6n3	Source
Marine microalgae						
Dunaliella sp.	6.3	15.6		0.2	0.2	(1)
Nannochloropsis sp.	3.5		4.6	30.1		(2)
Schizochytrium sp.	1-2	>1	1	1-16	18-44	(3)
Tetraselmis sp.	9.66	16.17	0.99	4.70		(4)
Tetraselmis sp.	4-7	5-22	<1-4	2-8	<1	(3)
Tetraselmis suecica	6.9	14.9	2	6.2		(2)
Tisochyrsis lutea	7.8	16.3	0.6	0.9	12.0	(2)
Freshwater microalgae						
Scenedesmus sp.	4.67	20.79				(4)
Arthrospira sp.	7.74			5.52		(5)
Chlorella sp.	17.54	20.02				(4)
Scenedesmus almeriensis	6.32	27.9				(6)
Scenedesmus sp.	1-6	>1-3				(3)
Macroalgae						
Ulva lactuca	9.5	0.1	1.8	1.6	0.2	(7)
Ulva ohnoi	2.55	9.70		0.31		(8)
Ulva rigida	14.26	5.18	0.47	0.44	0.52	(9)

(1) Mourente *et al*. (1990); (2) Servel *et al*. (1994); (3) Tibbetts *et al*. (2018); (4) Pratoomyot *et al*. (2005); (5) Sahu *et al*. (2013); (6) Vizcaíno *et al*. (2019b); (7) Cardoso *et al*. (2017); (8) Sáez *et al*. (2020); (9) Ivanova *et al*. (2013).

In general, long-chain n-3 PUFAs are mostly present in marine microalgae strains. Fatty acids like ARA, EPA and DHA can be directly produced by several microalgae species like *Porphyridium*, *Nannochloropsis* and *Schizochytrium* sp. Indeed, the last strain can be used as source of PUFA owing to the high DHA content (up to 49% total lipids) (Ren *et al*., 2010). On the other hand, green microalgae (Chlorophyta) are deficient in long chain PUFA, but contain other fatty acids like linoleic and linolenic acids that are essential for many freshwater fish species. Therefore, the fatty acid content makes algae (especially microalgae) a valuable novel ingredient for replacing fish oil, given that they can mimic the average fatty acid profile found

in forage fish. However, the use of a single strain does not allow to create a "*fish-free*" fish oil alternative. At least two marine microalgae strains should be blended for achieving that purpose.

2.3. Algal carbohydrates

The polysaccharide composition of microalgae varies notably among species (Brown, 2002). In general, glucose is the predominant sugar in some microalgae commonly evaluated for aquaculture purposes, like *Tisochrysis* sp. and *Chlorella* sp. (28-86% total carbohydrates). Also, mannose, fucose, galactose and xylose, among others, are present in different proportions. Most of these sugars are components of the microalgae cell wall, and play a key role as a protective barrier that, in turn, reduces the bioavailability of intracellular nutrients. Extracellular polysaccharides may interfere with nutrient absorption, or conversely, be useful binding agents when it comes to forming feed pellets.

On the other hand, carbohydrates make up one of the largest fractions of seaweeds composition reaching values from 2% to 66%. Polysaccharides, such as cellulose, hemicellulose and lignin, are present in seaweeds, which can have different influence in both feed overall quality (as gelling and stabilising agents), and in animals, as a source of non-starch polysaccharides. While certain carbohydrate fractions as ulvan or β-glucan can have functional effects on fish, other complex carbohydrates as non-starch polysaccharides can induce negative effects on nutrient absorption, growth performance and gut morphology. The latter can be of great interest in the case of farmed carnivorous fish, where carbohydrates are poorly digested and metabolised as energy source (Wan *et al*., 2019). The efficiency of fish to digest the cell wall depends on, the carbohydrate composition, and how carbohydrate fractions are linked to each other, and also on the existence of the appropriate digestive enzymes in the different fish species. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases than carnivorous fish lack.

2.4. Algal pigments

Broadly speaking, algae have an adequate content in high-value carotenoids, such as β-carotene and astaxanthin, (Fig. 3), which are commonly used in aquaculture mainly for their colouring and antioxidant properties, improving the quality and commercial value of farmed fish (Yarnold *et al*., 2019). β-carotene is one of the most demanded pigment with a wide variety of market applications: i) pro-vitamin A (retinol) in food and animal feed, ii) as food colouring agent, additive to cosmetics and multivitamin preparations, and iii) as a food additive under the antioxidant category. This pigment can be naturally produced by the microalgae genus *Dunaliella* that

may synthesize and accumulate up to 16% of its dry weight in the form of cellular β-carotene (Lers *et al*., 1990). Astaxanthin is another pigment that can be synthetized by *Haematococcus* microalgae, *Chlorella zofingiensis* and *Chlorococcum* sp. (Del Campo *et al*., 2004). This compound presents such a antioxidant activity that is has been proposed as "super-vitamin E" (Nakagawa *et al*., 2011). As a natural pigment, astaxanthin is commonly used as a pigmentation source in the aquaculture industry (Canales-Gómez *et al*., 2010). Out of total carotenoid (astaxanthin, cantaxanthin), the astaxanthin was determined as the most important carotenoid in salmon and rainbow trout (Shah *et al*., 2016; Tolasa *et al*., 2005). Astaxanthin cannot be synthesized *the novo* by salmonids, and therefore carotenoid pigments must be supplied in aquafeeds.

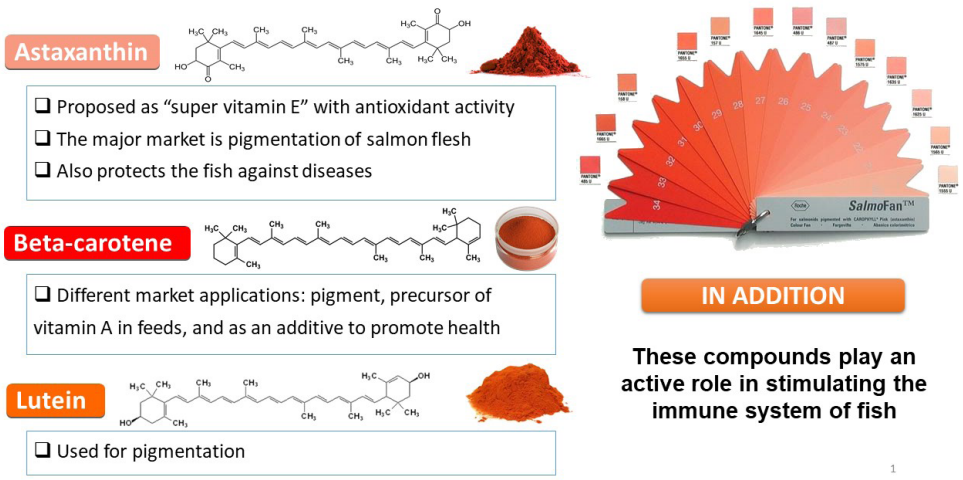

Figure 3. The interest of algae as source of pigments for aquafeeds.

Recently, Sales *et al*. (2020) analyzed the composition of the carotenoid fraction obtained from the microalgae *Nannochloropsis gaditana* (Table 4), and they found that violaxanthin, β-carotene, and neoxanthin were the most abundant carotenoids in extracts, and vaucheroxanthin ester and canthaxanthin, the less abundant. Those authors affirmed that lipid extract from the microalgae *N. gaditana* can be used as dietary additive for feeding juvenile gilthead seabream. The use of extracted compounds instead of the whole biomass can be effective for enhancing the bioavailability of these compounds. In fact, extracted pigments from *Arthrospira platensis*, *Haematococcus pluvialis* and *Chlorella* sp. have been successfully incorporated as supplements for feeding *Lates calcarifer* larvae (Gora *et al*. 2019).

Table 4. Carotenoid content (mg/kg) of the non-saponifiable lipid extract obtained from *Nannochloropsis gaditana* (Data from Sales *et al*., 2020). Values are mean ± SD of triplicate determination.

Carotenoid	Non-saponifiable lipid extract
Neoxanthin	754.97 ± 149.19
Violaxanthin	2137.29 ± 254.97
Antheroxanthin	417.51 ± 70.36
Vaucheroxanthin	78.81 ± 10.6
Zeaxanthin	58.32 ± 8.14
Vaucheroxanthin ester	13.47 ± 2.38
Canthaxanthin	14.09 ± 2.24
β-carotene	925.51 ± 145.4
Total	**4399.97 ± 643.27**

3. The use of algae as ingredient in aquafeeds

The nutritional profile and the variety of nutraceutical compounds in algae justify their potential use as major ingredients, and also as additives in aquafeeds. Indeed, there is abundant scientific literature related to the use of algae-supplemented diets for feeding fish. Thus, studies focussed on the use of microalgae as potential sources of protein, lipid, and functional additives for aquafeeds have been increasing exponentially in the last decade (Tables 5 and 6).

According to previous studies, low level of fishmeal replacement (around 0.5-10%) has positive effects of fish. For instance, the inclusion up to 7.5% *Arthrospira* sp. in diets for rainbow trout (*Oncorhynchus mykiss*) did not negatively affect growth and nutrient utilization. Weight gain and carotenoid concentration in skin and fillets both increased in fish fed on 7.5% microalgae-supplemented diet (Teimouri *et al*., 2013). Similarly, *Scenedesmus* sp. and *Desmodesmus* sp. at 5 or 10% replacement of fishmeal in practical diets for Atlantic salmon (*Salmo salar*) have been successfully evaluated without causing negative effects on growth, nutrient utilization, and body proximate composition of fish (Kiron *et al*., 2016), and even an increase in total n-3 and PUFA content in fish was reported (Gong *et al*., 2019). The inclusion up to 15% of *Nannochloropsis gaditana*, *Tisochrysis lutea* and *Scenedesmus almeriensis* were successfully used in diets for Senegalese sole (*Solea senegalensis*) (Vizcaíno *et al*., 2019b). Vizcaíno *et al.* (2014) also pointed out that the dietary inclusion up to 38% *S. almeriensis* in diets for gilthead seabream (*S. aurata*) juveniles caused positive effect on gut functionality.

On the other hand, microalgae could be a key ingredient for designing a fish oil replacement that contains essential fatty acids, such as EPA and ARA. Encouraging

Table 5. Recent studies on applications of microalgae as dietary ingredient or additive in aquafeeds.

Microalgae	Fish species	Use	Effect on fish	S
Arthrospira sp.	*Pelteobagrus fulvidraco*	4% AI	(↔) growth performance, (↑) antioxidant and immune response	(1)
Arthrospira sp.	*Oncorhynchus mikiss*	7.5% FMR	(↔) fish growth, (↑) carotenoid concentrations in skin and fillet	(2)
Arthrospira sp.	*Sparus aurata*	4% AI	(↑) activity digestive enzymes, (↑) intestinal mucosa structure, and (↓) oxidation of muscle lipids	(3)
Chlorella vulgaris	*Paralichthys olivaceus*	15% FMR	(↑) growth performance, (↑) antioxidant enzyme activity and lipid metabolism	(4)
Desmodesmus sp.	*Salmo salar*	10% FMR	(↔) growth rate, condition factor, protein efficiency ratio and body proximate composition	(5)
Isochrysis sp.	*Dicentrarchus labrax*	36% FOR	(↔) feed intake, growth performance (↑) greenish pigmentation of the skin	(6)
Nannochloropsis & Isochrysis sp.	*Gadus morhua*	15% FMR	(↑) feed intake and growth	(7)
Nannochloropsis & Schizochytrium sp.	*P. olivaceus*	100% FOR	(↔) growth performance and nutrient utilization	(8)
Nannochloropsis sp.	*D. labrax*	15% FMR	(↔) growth performance, proximate composition and intestinal integrity	(9)
Pavlova viridis & Nannochloropsis sp.	*D. labrax*	100% FOR	(↔) growth performance and nutrient utilization	(10)
Phaeodactylum tricornutum	*S. salar*	6% FMR	(↔) growth, nutrient digestibility and feed utilization	(11)
Scenedesmus almeriensis	*S. aurata*	38% FMR	(↔) growth and nutrient utilization. (↑) intestinal enzyme activities and absorptive surface	(12)
Scenedesmus sp.	*S. salar*	10% FMR	(↔) feed intake, growth, chemical composition. (↑) n-3 PUFA content	(13)
Schizochytrium sp.	*S. salar*	5% FMR	(↔) health and fillet nutritional quality	(14)
Schizochytrium sp.	*Oreochromis niloticus*	100% FOR	(↑) growth performance, nutrient utilization, and n-3 FUFA in fillet	(15)
Spirulina maxima	*O. niloticus*	30% FMR	(↔) growth	(16)
Spirulina sp.	*Puntius gelius*	20% FMR	(↑) growth and feed conversion ratio	(17)
T. galbana, S. almeriensis & N. gaditana	*Solea senegalensis*	15% FMR	(↔) growth performance and nutrient utilization. (↑)intestinal absorptive capacity	(18)
Tetraselmis suecica	*D. labrax*	20% FMR	(↔) growth performance	(19)

Use: FMR: fishmeal replacement: **FOR**: fish oil replacement; **AI**: algae inclusion.
Effect: (↓) reduced; **(↑)** increased/improved; **(↔)** no effect.
S: [1] Liu *et al*. (2020); [2] Teimuri *et al*. (2013); [3] Galafat *et al*. (2020); [4] Rahimnejad *et al*. (2017); [5] Kiron *et al*. (2016); [6] Tibaldi *et al*. (2015); [7] Walker and Berlinsk (2011); [8] Quiao *et al*. (2014); [9] Valente *et al*. (2019); [10] Haas *et al*. (2016); [11] Sørensen *et al*. (2016); [12] Vizcaíno *et al*. (2014); [13] Gong *et al*. (2019); [14] Kousoulaki *et al*. (2015); [15] Sarker *et al*. (2016); [16] Rincón *et al*. (2012); [17] Hajiahmadian *et al*. (2012); [18] Vizcaíno *et al*. (2018); [19] Tulli *et al*. (2012)

results were recently obtained in marine fish species by Tibaldi *et al*. (2015). These authors used dried *T. lutea* biomass to replace up to 36% fish lipid in a diet with low level of fish oil, and they did not find adverse effects on growth performance of European seabass (*Dicentrarchus labrax*). Similarly, the heterotrophic microalgae *Schizochytrium* sp. has been successfully used on different fish species. The high DHA content of this microalga has allowed the total replacement of fish oil in diets

Table 6. Recent studies on applications of seaweeds as dietary ingredient/additive in aquafeeds.

Seaweed	Fish	Use	Effects on fish	S
Palmaria palmata	*Salmo salar*	15% AI	(↔) growth performance and feed utilization	(1)
Porphyra dioica & *Ulva* sp.	*Oreochromis niloticus*	10% AI	(↔) growth performance or body composition	(2)
Sargassum horneri	*Scophthalmus maximus*	10% AI	(↔) growth performance. (↑) non-specific immune response and resistance to pathogenic bacteria	(3)
Ulva lactuca	*Solea senegalensis*	10% AI	(↔) growth performance and feed utilization	(4)
U. ohnoi	*S. senegalensis*	5% AI	(↓) growth and pancreatic protease activity. (↑) absorptive surface of the intestinal mucosa	(5)
U. rigida & *Gracilaria cornea*	*Sparus aurata*	15% AI	(↔) growth and fish chemical composition. (↔) intestinal structure	(6)

Use: AI: algae inclusion.
Effect: (↓) reduced; **(↑)** increased/improved; **(↔)** no effect.
S: [1] Wan *et al*. (2016); [2] Silva *et al*. (2015); [3] Wang *et al*. (2019); [4] Moutinho *et al* (2018); [5] Vizcaíno *et al* (2019a); [6] Vizcaíno *et al*. (2016a)

for Nile tilapia (*Oreochromis niloticus*) (Sarker *et al*., 2016). The results obtained revealed positive effects on growth and nutrient utilization, as well as an increase on n-3 long chain PUFA accretion in fillets.

Seaweeds have also been evaluated in recent years as a novel and sustainable resource for aquafeed manufacturing (Vizcaíno *et al*., 2016a, 2019a). In spite of the fact that their protein content is low compared to other ingredients used in aquafeeds, seaweeds are also rich in biologically active compounds, such as polysaccharides, pigments, polyphenols, and vitamins, which might exert certain beneficial effects on farmed fish (Wan *et al*., 2019). Overall, there are numerous studies pointing to promising results in terms of growth, survival, and nutrient utilization in different farmed fish fed on seaweeds up to 10-15% inclusion level. Some species of the genus *Ulva* have been successfully evaluated as a dietary ingredient for gilthead seabream (Vizcaíno *et al*., 2016a), Senegalese sole (Moutinho *et al*., 2018) or Nile tilapia (Silva *et al*., 2015) without negative effects on growth and nutrient utilization. Similarly, the inclusion up to 10% of *Sargassum horneri* had no adverse effects on growth performance of juvenile turbot (*Scophthalmus maximus*). The inclusion of *S. horneri* also enhanced the activity of non-specific immune enzymes and the resistance against pathogenic bacteria (Wang *et al*., 2019).

In summary, several beneficial effects of algae have been reported in numerous studies, such as: i) improved growth, feed utilisation, and survival rate, ii) increased

lipid metabolism, iii) antioxidant properties, iv) enhanced body composition, and flesh quality, v) enriched skin and flesh pigmentation, vi) improved integrity of intestinal mucosa, vii) enhanced activity of digestive enzymes, viii) reinforced immune system, and, ix) invigorated resistance to stress, and against pathogens.

Given the nutritional composition and the reported effect on fish, it seems that algae are interesting alternative ingredients/additives for aquafeeds. Herbivorous and omnivorous species tolerate well high inclusion levels of algae compared to carnivorous fish species. However, the results reported suggest that the optimum dietary algae inclusion level should vary depending on the algae and on the farmed fish species considered. In this regard, the effect of algae seems to be dose-dependent, and species-specific, and consequently, it is difficult to establish a general rule about the use of algae in aquafeeds. Therefore, specific research should be carried out on each specific case, and more research is needed to evaluate the potential of algae as protein sources, and pinpointing factors affecting their effectiveness should be undertaken.

4. Current challenges in the use of microalgae in aquafeeds

4.1. Safety and regulatory aspects of algae in aquafeeds

The use of algae in foods and feeds is increasingly relevant as the components of microalgae have the potential to be competitive with the same components of other origins. For instance, microalgae used commonly for the production of food supplements, such us species of the genus *Isochrysis*, *Chaetoceros*, *Tetraselmis*, *Pavlova*, *Skeletonema*, *Dunaliella*, *Nannochloropsis*, *Phaedactylum*, *Chlorella*, do not produce toxins. But, it should be considered that, even within a given species, differences exist between toxic and non-toxic strains. For this reason, it is very important to know their safety aspects at species level. The competitiveness of algae-based products is based not only on technical and economical aspects, but also on the regulations ruling their use (Enzing *et al*., 2014). The safety aspects of algae used in aquafeeds have been analysed by the European Food Safety Authority (EFSA). The placing in the European Union market of algae or its components for food and feed purposes is regulated by three legal dispositions: i) EC regulation 178/2002, on food safety; ii) EC regulation 258/97, on novel foods and novel food ingredients; and iii) EC regulation 1924/2006, on nutrition and health claims for foods. Given that several compounds derived from algae are used as feed additives, the Regulation (EC) 1333/2008 also applies. In the past, prior to placing in the market, food and feed business operators were obliged to apply for authorization for the use of proteins derived from algae in feed under Directive 82/471/EEC. Assessment of

safety and nutritional value should be done according to the guidelines in Directive 83/228/EEC using the "*Guidance for the assessment of biomasses for use in animal nutrition*" published by the European Food Safety Authority (EFSA, 2011).

Algae belong to the group "*algae and prokaryotes organism*" in the Annex I of Regulation EC 752/2014. Algae or their products used as feed should fulfil all the legal maximum levels mentioned in Directive 2002/32/EC. The regulation EU 68/2013 established the "*Catalogue of Feed Materials*" and includes algae within the group 7 of the list of feed materials, specifically in "*Other plant, algae, and products derived thereof*" (Table 7). Within the European Union, the EFSA requires the safety assessment of any new compound intended for use in food and feed before it is authorized to be placed on the market.

Table 7. Information in the group "Other plants, algae and products derived thereof" (source: Commission Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials).

Number	Name [1]	Description	Compulsory declarations
7.1.1	**Algae**	Algae, live or processed, including fresh, chilled or frozen algae. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.2	**Dried algae**	Product obtained by drying algae. This product may have been washed to reduce the iodine content. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.3	**Algae meal**	Product of algae oil manufacture, obtained by extraction of algae. May contain up to 0.1% of antifoaming agents	Crude protein Crude fat Crude ash
7.1.4	**Algal oil**	Product of the oil manufacture from algae obtained by extraction. May contain up to 0.1% of antifoaming agents	Crude fat Moisture if > 1%
7.1.5	**Algae extract**	Watery or alcoholic extract of algae that principally contains carbohydrates. May contain up to 0.1% of antifoaming agents	
7.2.6	**Seaweed meal**	Product obtained by drying and crushing macro-algae, in particular brown seaweed. This product may have been washed to reduce the iodine content. May contain up to 0.1% of antifoaming agents	Crude ash

[1] The name shall be supplemented by the species.

4.2. Price

The use of algae proteins instead of fishmeal in aquafeeds would allow for a decoupling of aquaculture production from wild fisheries. Until now, fishmeal and fish oil are substantially cheaper than microalgae, which prevents microalgae to enter

the aquafeed market. The lack of alternatives to microalgae for feeding fish larvae and juveniles assures a market for microalgae in fish hatcheries. The use of algae as feed ingredient in aquafeeds looks promising, as microalgae have a favourable protein and lipid composition. They can be used in feeds at inclusion levels of about 10%, and have additionally positive effects on the health of animals. The replacement of soybean meal in compound feed production represents a potentially large market for algae (Pereira *et al*., 2020). The outlook for microalgae to be used as feed additives is promising, due to the many positive effects on animal health described. Furthermore, the aim in certain countries to reduce the use of antibiotics is a driver to the use of other health-improving feed additives, being algal and their derivatives potential candidates for this purpose.

A critical factor that will determine the commercial viability of algae is their competitiveness compared to other raw sources currently on the market. For example, a major application for the production of microalgae is lipid extraction for obtaining biofuel. However, although algal biomass is a greener, environmentally friendly alternative, biofuel it is not yet competitive compared to fossil fuels (Cuellar-Bermudez *et al*., 2015). The same situation applies in the case of algae for commercial aquafeeds. The competitiveness of microalgae could be further increased by taking a holistic view, maximising the extraction of all available high-value components by cascading biorefinery. Similarly, seaweeds are ideally suited for cascading biorefinery, because they contain many high-value components, together with bulky low-value components that are considered raw materials for the bio-based industry, as with an economic value (Cian *et al*., 2012). Currently, the relatively high cost of microalgae compared to regular ingredients (Fig. 4) limits their use in high-value fish production (Yarnold *et al*., 2019).

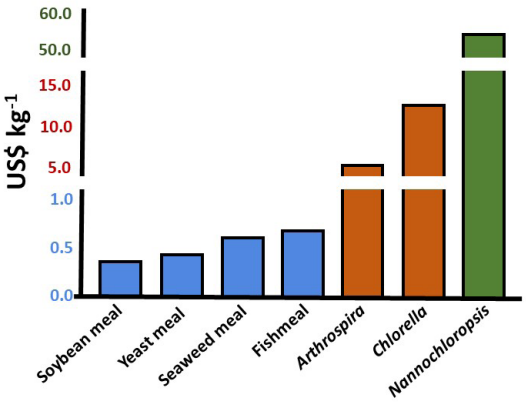

Figure 4. Price comparison of fishmeal alternatives as formulated-feed ingredients. Image modified from Yarnol *et al.* (2019).

It has been estimated that algal meal prices of 0.66 and 2.65 € per kg would be needed to replace fishmeal and soybean meal, respectively, in diets for tilapia (a relatively low-value fish). The study of Vizcaíno *et al*. (2014) pointed out that culture technologies should reduce substantially the cost of microalgae production, and even considering a cost of 5.5 € per kg (Norsker *et al*., 2011), the large scale utilization of microalgae in aquafeeds remains a constrain for the aquaculture industry. Given that algae are expensive to produce, especially microalgae, their use as bulk ingredients for aquafeed formulae is likely to require improved production efficiency and further cost reduction by using biorefinery approaches. However, their main advantage from an economical standpoint their richness in fatty acids, pigments, vitamins, minerals, and bioactive compounds, which make them excellent high-value additives and supplements to blend into a wide range of aquafeeds, even when using at low dietary inclusion level.

An example of cascading biorefinery is the SABANA project (grant # 727874 from the European Union's Horizon 2020 Research and Innovation program), which aims at developing a large-scale integrated microalgae-based biorefinery for the production of valuable products for the aquaculture (Fig. 5).

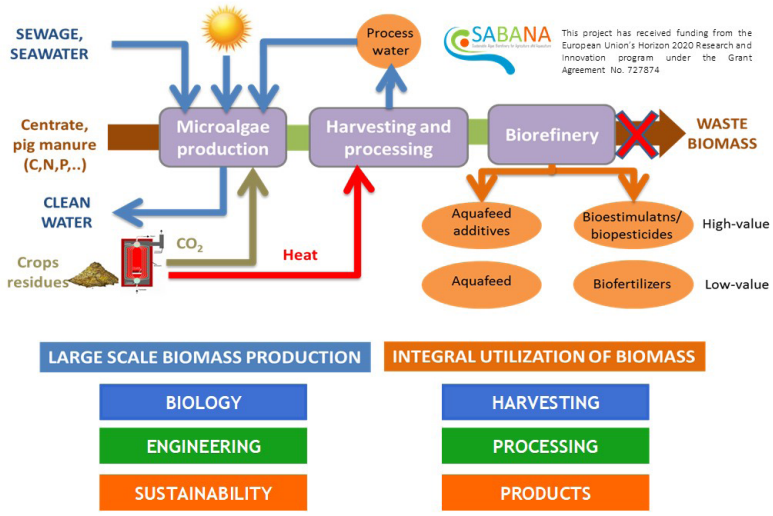

Figure 5. Detail of the block diagram for producing and processing the microalgal biomass in the Project SABANA.

In this project, natural seawater and sunlight are used to growth microalgae. Instead of chemical fertilizers, wastewaters are used as nutrient source for producing large amounts of biomass that are being processed for obtaining valuable products

for aquafeeds. The project is divided in two major tasks; i) one is related to the improvement of technology for large scale biomass production (including biological, engineering and sustainability aspects), and ii) the second one focuses on the development of methods for integral utilization of the biomass (including harvesting, processing, and evaluation of pre-commercial products). The objective of this project is to achieve a zero-waste process in a demonstration facility of 5 hectares located in the University of Almeria, with capacity to produce 300 tonnes of algal biomass per year with an economic cost of around 1 € per kg dry weight.

4.3. Variability in nutrient composition

An additional challenge, particularly relevant in algae production, is the variability in their chemical composition, which is highly dependent on the species strain, the growth medium, the harvesting period, and the production method, among other factors. For instance, the protein content can vary by season, temperature, and location in which the algae are cultured and/or harvested (Joubert and Fleurence, 2008). The relative composition of specific proteins can also differ, changing the concentrations of amino acids consequently. For example, annual monitoring of *P. palmata* harvested on the Atlantic coast showed that protein levels were highest in winter and spring months, varying from 9 to 25%, and peaking in May (Galland-Irmouli *et al*., 1999). In microalgae, Adams *et al*. (2013) described that short-term effect of nitrogen limitation generally includes an increase in lipid and carbohydrate contents, and a decrease in the growth rate and the content of crude protein, although the extent of this response varies markedly between species. In the light of the data, the development of protocols for optimizing the biochemical composition of algae should expect to play an important role in the future production processes.

4.4. Presence of anti-nutritional factors

The presence of anti-nutritive factors is one of the most important issues derived from using novel dietary ingredients in aquafeeds. These compounds can exert negative effects on the absorption of nutrients and micronutrients, hampering the normal functioning of certain organs, so they are one of the main drawbacks limiting their practical use in formulated feeds (Vizcaíno *et al*., 2020).

Anti-nutritive factors comprise a wide variety of compounds, such as protease inhibitors, phytohemagglutinin, lectins, phytic acid, saponins, phytoestrogens or antivitamins (Prabhu *et al*., 2017). In general, these substances have been related to plant-derived feedstuffs, although recent studies have also documented their presence in some algae species (Oliveira *et al*., 2009; Mæhre, 2015; Vizcaíno *et al*., 2020). Overall, the ability of microalgae to inhibit fish digestive proteases seems

to be low, with inhibition values lower than 20% against control assays (Vizcaíno *et al*., 2021). However, other studies pointed to the existence of protease inhibitors in some macroalgae species such as *Ulva rigida*, *Ulva ohnoi*, *Gracilaria cornea* and *Sargassum sp*., which may exert not only reduced proteolysis within digestive tract, but also increased pancreatic secretion as an attempt to overcome their anti-nutritional effects (Sáez *et al*., 2013; Diken *et al*., 2016; Vizcaíno *et al*., 2019a; 2020).

Sáez *et al.* (2013) evaluated the effect of the inclusion of *Gracilaria corne*a and *Ulva rigida* as dietary ingredients on the intestinal proteolytic activity of juvenile gilthead seabream. The results obtained evidenced that digestive proteases were affected by algae-supplemented diets, as fish displayed different alkaline protease enzyme activity levels after a 70-day feeding trial. In particular, the proteolytic activities in fish fed *Ulva* supplemented-diets were significantly lower than those of fish fed on control diet. The presence of protease inhibitors in macroalgae may contribute to the progressive decrease in the proteolytic activity in fish fed diet with increasing levels of *Ulva*. However, the decline in the level of alkaline protease activity was not accompanied by a decrease in fish growth and feed utilization, since all fish grew similarly. The existence of a compensation mechanism against dietary protease inhibitors in juvenile gilthead seabream has been proved by Santigosa *et al*. (2010), who found similar results when fish were fed on diets with soybean trypsin inhibitor.

Vizcaíno *et al*. (2020) also assessed the existence in *Ulva ohnoi* of substances able to inhibit the digestive proteases of gilthead seabream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*) and seabass (*Dicentrarchus labrax*) (Fig. 6). In that study, inhibition plots and zymograms were obtained in order to illustrate the response of fish proteases after incubation with crude or thermal treated *U. ohnoi.* Digestive proteases of all marine fish tested showed susceptibility to *Ulva* protease inhibitors, although considerably high concentration of *Ulva* was needed to cause high inhibition values. According to the estimation of Vizcaíno *et al*., the amount of *Ulva* required to reach 50% inhibition of digestive proteases would represent a dietary inclusion of approximately 40% to 53%, being these values quite far from those used in the formulation of commercial aquafeeds. Moreover, the effect of thermal treatment on the capacity of *Ulva* to inhibit trypsin activity evidenced that such inhibitors are susceptible to temperature. A treatment of 80 °C for 15 min reduced the inhibitory capacity by 50%, and above 75% as prolonged times were applied. Deactivation of anti-nutritive factors is also an important issue to be considered in raw material processing during aquafeed manufacture, and in the case of *U. onhoi* the heat treatment seems to be enough for inactivating these compounds.

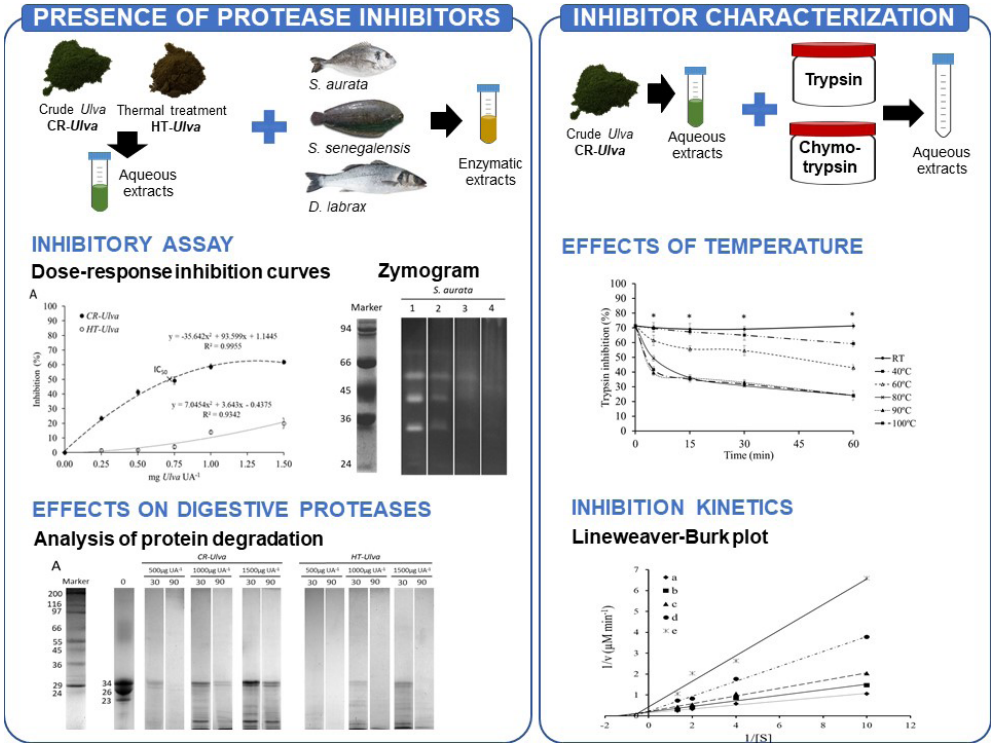

Figure 6. A summary of the results achieved in the study by Vizcaíno *et al.* (2020).

4.5. Algae digestibility

Not only the nutrient composition of algae determines their interest as ingredients in aquafeeds, but also factors such as feed attractiveness (e.g., smell, taste), accessibility (e.g., cell/pellet size, buoyancy), and nutrient availability should be considered. The high cell-wall recalcitrance of most algae is detrimental to digestibility and assimilation of intracellular nutrients, especially for carnivorous fish with a short digestion phase (e.g., seabass, turbot). The efficiency of marine animals to digest the cell walls depends on the carbohydrate composition, on how they are linked to each other, as well as on the existence of adequate fish digestive carbohydrases. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases, but carnivorous fish do not, and this fact should be taken into consideration when formulating aquafeeds (Shi *et al*., 2017). Consequently, it may be reasonable to think that any strategy aimed at improving the bioavailability of the inner compounds might be of great interest for using algae in aquafeeds. Several procedures have been evaluated with the aim of releasing inner components of algae

(Tibbetts *et al*., 2017; Teuling *et al*., 2019) but when they come to large-scale cell lysis, the enzymatic hydrolysis is likely one of the most promising strategies, not least owing to its economic viability. By following this procedure, even low inclusion level of enzyme-hydrolysed algae might well improve the physiological aspects in fish in a manner similar to including higher amounts of raw algae in aquafeeds (Tchorbanov and Bozhkova, 1988; Galafat *et al*., 2020). Alternatively, fermentation can also increase protein digestibility due to the degradation of insoluble polysaccharides, such as xylan. This last has been reported after fermentation of *Palmaria palmata* biomass using the fungal mould *Trichoderma pseudokoningii*, which was found to decrease the xylan content (Marrion *et al*., 2003)

Little research has been carried out to assess the digestibility of algal protein by fish digestive enzymes (Tibbetts *et al*., 2015; 2016; Vizcaíno *et al*., 2019b). Previous studies provided useful species-specific information about the manner that digestive enzymes of farmed fish hydrolyse algal proteins. The *in vitro* study of Vizcaíno *et al.* (2019b) reported that microalgae show *in vitro* protein degradation values around 50%, which are similar to those described in other raw materials commonly used in aquafeeds, such as soybean protein concentrate or fishmeal (Fig. 7).

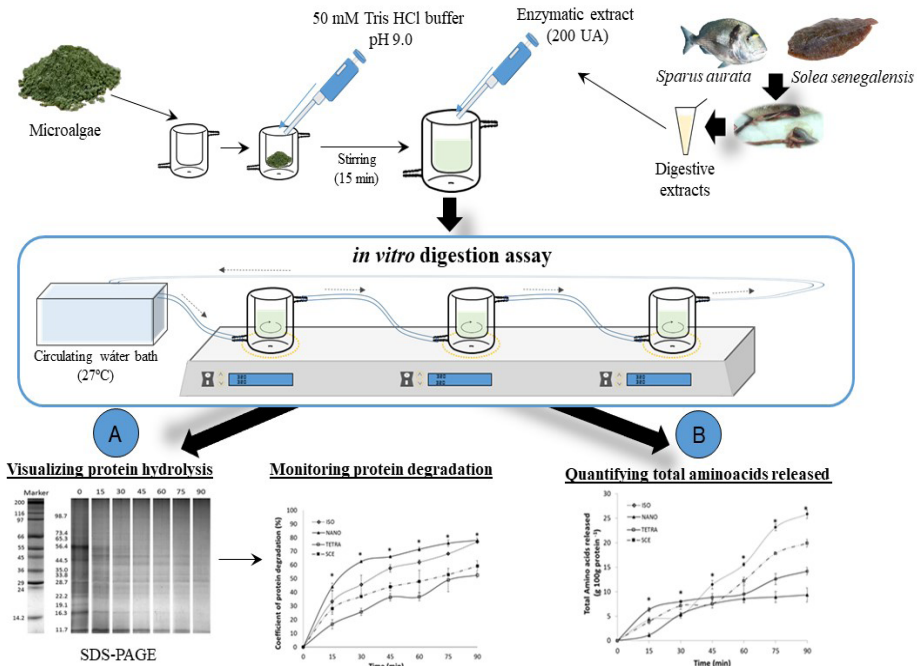

Figure 7. A summary of the *in vitro* study carried out for assessing the hydrolysis of algal protein by digestive proteases of juvenile gilthead seabream (Vizcaíno *et al*., 2019).

Some microalgae species like *Tysocrysis* sp. or *Dunaliella* sp. may reach even high proteolysis by *S. aurata* digestive proteases (>75%). This high *in vitro* protein hydrolysis is related to the fact that these microalgae have no distinct cell wall, and consequently, it is expected that cells could be easily hydrolysed by fish digestive enzymes (Vizcaíno *et al*., 2019b). On the contrary, in other species like *Nannochloropsis* sp., *Chlorella* sp. or *Scenedesmus* sp., protein is less susceptible to the action of fish proteases, and consequently, proteolysis values are lower than 50% (Vizcaíno *et al*., 2021). These microalgae possess a thick cell wall containing cellulose, hemicellulose, pectin and glycoprotein, which determines a low bioavailability of intracellular components (Bernaerts *et al*., 2018). In spite of the presence of intestinal amylase activity in some marine fish, like gilthead seabream, the lack of digestive cellulases prevent the breakdown of the algal cell wall. The effective breakdown of algae cellulosic cell wall is a key factor for improving nutrient bioavailability in monogastric animals, not least in fish. Consequently, it would be advisable to include a previous step for cell wall disruption and/or hydrolysis before using algal biomass in aquafeed manufacturing.

5. Evaluation of algae in aquafeeds

5.1. Effect on growth and nutrient utilization

Several studies have reported that the dietary inclusion of microalgae for feeding farmed fish has no negative effects on growth performance and nutrient utilization (Shah *et al*., 2018; Roohani *et al*., 2019). Nevertheless, adverse effects on fish growth have also been reported (Walker and Berlinsky, 2011; Gong *et al*., 2019). Differences in response seems to be influenced by several factors, such as fish and algae species, inclusion level, and nutritional composition of algae (Shah *et al*., 2018). Several microalgae species, such as *Tisochrysis lutea*, *Tetraselmis suecica*, *Nannochloropsis gaditana*, *Arthrospira platensis* and *Scenedesmus almeriensis*, have been successfully tested as dietary ingredients for different farmed fish species (Fig. 8). The dietary inclusion of *T. suecica* (5%) improved growth performance of gilthead seabream fry (Vizcaíno *et al*., 2016b). Similarly, Vizcaíno *et al.* (2018) confirmed that microalgae inclusion up to 15% did not cause negative effects on growth performance of Senegalese sole juveniles. The study of Perera *et al.* (2020) evaluated two commercial compounds extracted from microalgae, i) LB-GREENboost (LBGb), and ii) LB-GUThealth (LBGh) developed by LifeBioencapsulation S.L. (Almería, Spain) included at 0.5% and at 1% in feeds. In this case, all fish groups grew allometrically from 12-13 g to 37-39 g with an overall weigh gain (WG) of ~200 % and specific growth rates (SGR) of 1.26-1.30%.

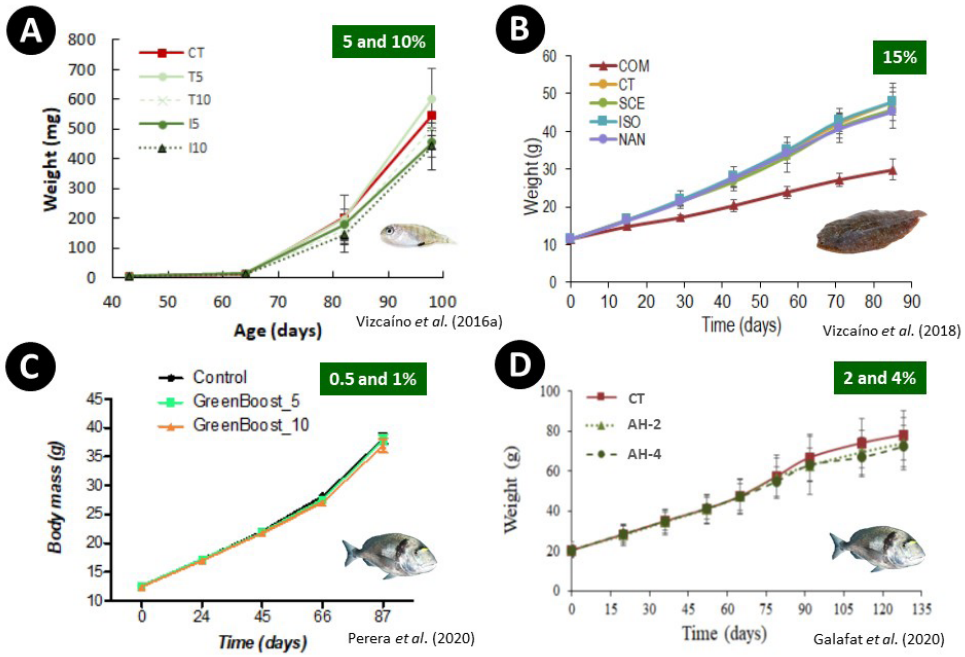

Figure 8. Different case studies for assessing the effect of microalgae-supplemented diets on fish growth. **A**) Gilthead seabream fry fed control (CT), and *Tetraselmis suecica* (T5 and T10) and *Isochrysis galbana* (I5 and I10) at 5 and 10% dietary inclusion. **B**) Senegalese sole juveniles fed commercial (COM), control (CT), and *Scenedesmus almeriensis* (SCE), *Isochrysis galbana* (ISO) and *Nannochloropsis gaditana* (NAN) at 15%. **C**) Gilthead seabream juveniles fed control, and the microalgal additive GreenBoost at 0.5 and 1%. **D**) Gilthead seabream juveniles fed control (CT), and *Arthrospira* sp. protein hydrolysate at 2 and 4% (AH-2 and AH-4).

Another recent study evaluated the inclusion *Arthrospira* protein hydrolysate up to 4% as a novel strategy aimed at increasing the nutritional and functional properties of the crude raw biomass, reporting the lack of negative effects on growth performance in gilthead seabream juveniles (Galafat *et al*., 2020). In the above-mentioned studies, the inclusion of microalgae did not affect feed intake, although altered feeding behaviour and decreased feed consumption has been reported owing to high inclusion level (Dallaire *et al*., 2007). On the other hand, it is important to keep in mind that despite microalgae do not increase growth performance of fish, they have a significant effect on nutrient utilization, reducing feed conversion ratio (FCR) (Perera *et al.,* 2020).

Similarly, the effects of macroalgae on fish seem to be species-specific, and they depend on the proportion of biomass used. It has been reported that low dietary level of macroalgae leads to positive effects on growth performance and nutrient utilization

of farmed fish (Vizcaíno *et al*., 2016a; 2019a). On the contrary, Valente *et al*. (2006) reported that the incorporation of 10% *Gracilaria cornea* affected negatively growth performance in *Dicentrarchus labrax*. These detrimental effects have been attributed to the existence of anti-nutritive factors, as described Vizcaíno *et al*. (2020), which might affect the bioavailability and/or digestibility of nutrients. In this sense, Vizcaíno *et al*. (2019a) found that FCR was increased as a result of including 5% *Ulva onhoi* in experimental diets for Senegalese sole juveniles. This fact might be a consequence of the high content in soluble and insoluble polysaccharides that can provoke a rapid transit of feed through the fish digestive tract, this increasing FCR and even impairing the specific growth rate (Vizcaíno *et al*., 2016a). Nevertheless, Moutinho *et al*. (2018) reported that the dietary inclusion of 10% *U. lactuca* for 5 months (from 23 g up to 60 g body weight) did not cause any detectable impact on growth performance and feed utilization in juvenile Senegalese sole. The disparate response of fish after *Ulva* dietary administration described in the literature might be related to differences in factors such as fish physiological maturity, the strain of *Ulva*, the length of the feeding trial, and even the dietary inclusion level of the seaweed.

5.2. Effect on muscle proximate composition

Overall, the use of microalgae in aquafeeds has yielded controversial effects on the chemical composition of fish. Thus, whereas Vizcaíno *et al*. (2014) reported that the inclusion of *Scenedesmus almeriensis* of up to 39% in diets for gilthead seabream juveniles did not affect fish body composition, other studies showed that microalgae modify the protein and lipid content in liver and muscle (Vizcaíno *et al*., 2016b; Galafat *et al*., 2020). Specifically, the last study found that dietary microalgae decreases muscle lipid content in gilthead seabream fry and juveniles. Similar findings were reported by Roohani *et al.* (2019), who described that *Spirulina platensis* increased protein and decreased fat content in *Salmo trutta* juveniles. These authors pointed out that several algal compounds, especially vitamins, minerals, essential amino acids and fatty acids, may activate fish metabolism, and particularly the use of lipid as energy source, which leads to reduced tissue storage. In the case of macroalgae, Sáez *et al*. (2020) also described a reduction in muscle lipid content in Senegalese sole fed with diets supplemented with 5% *Ulva onhoi,* and Ergün *et al*. (2009) described that fish fed *Ulva*-enriched diets showed increased muscle protein content. Previous studies attributed the effects on lipid metabolism and muscle fat deposition to the high content in vitamin C of *Ulva* (Ortiz *et al*., 2006). From the above-mentioned studies, it is clear that algae supplementation is an interesting strategy aimed at modulating the composition of fish muscle, which might be highly desirable in terms of quality of aquaculture products.

Another advantage of algae for feeding fish is that animals generally reflect in muscle the dietary fatty acid profiles. This way, inclusion of algal biomass with the appropriate fatty acid profile can lead to significant increase in n-3-PUFAs content in the fillet. Regarding the modulation of fatty acid content, García-Márquez *et al*. (2020) described in Nile tilapia that a short feeding pulse with *Scenedesmus almeriensis*-enriched diets reduced the levels of saturated fatty acid in muscle, while increased levels of ARA, EPA and DHA in muscle. In this case, the partial replacement of fish derivatives by 25% with *S. almeriensis,* and its administration over a short period of time (30 days) represents an opportunity for producers to further improve the nutritional value of tilapia fillets, this leading to higher market value of fish products. In Senegalese sole, also 5% dietary supplementation with *Ulva* reduced muscle total lipid content and favoured muscle selective retention of n-3 PUFA, not least eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Sáez *et al*., 2020). A similar effect was observed by Vizcaíno *et al*. (2016b) in gilthead seabream fry fed low dietary level of microalgae, where the inclusion of *Tetraselmis suecica* significantly increased the proportion of 18:3n-3 and the addition of *Tisochrysis galbana* increased DHA content in fish tissues. Moreover, the use of algae oils containing either EPA and DHA was effective in the total replacement of fish oil in practical (15% fishmeal) diets for marine fish, and ensured high nutritional quality of the fish fillet, increasing DHA content (Carvalho *et al*., 2020). The results of the previous studies clearly indicate that algae and their derivatives are useful for providing n-3 PUFA in aquafeeds, and can contribute to reduce the use of fish oil worldwide.

5.3. Effects on gut functionality

One of the most important aspects related to the dietary inclusion of algae is their effects on the functionality and integrity of fish gut, given that growth is directly linked to nutrient digestion and absorption processes (Vizcaíno *et al*., 2019a). One of the key roles of the intestinal epithelium is to complete the final stages of the digestion, as well as to absorb dietary nutrients. A healthy gut helps the better absorption and bioavailability of nutrients from feed while acts as a physical barrier for avoiding the diffusion of pathogens and toxins from the lumen to the mucosal tissues. A single layer of epithelial cells separates the intestinal lumen from the underlying sterile tissue, and any alteration in the barrier integrity strongly activates immune cells and cause chronic inflammation of the intestinal tissues. This is why the integrity of the intestinal mucosa is a key factor in fish nutrition.

In this regard, various studies performed by Vizcaíno *et al*. (2014, 2016a, b, 2018, 2019a) demonstrated that the dietary inclusion of algae induces noticeable changes in several enzymes involved in the digestive and absorptive processes. Pancreatic

and intestinal brush border enzymes are correlated with the nutritional status of fish (Alarcón *et al*., 1998). Hence, their activities are used as indicators of the digestive and absorptive capacity of fish. With the latter in mind, the digestive enzyme activities quantified in those studies were differentiated in two groups; i) on the one hand, total alkaline protease activity, trypsin, chymotrypsin activities are used as indicators of the digestive capacity of fish to hydrolyse dietary protein, and, ii) on the other, leucine aminopeptidase and alkaline phosphatase activities are used as indicators of the intestinal absorptive capacity. Results obtained in gilthead seabream and Senegalese sole neither showed negative effects on the activity of enzymes from the pancreatic secretion nor on brush border enzymes; on the contrary, even increased activity of the enzymes associated to the brush border membrane were observed owing to the dietary inclusion of some microalgae (*Scenedesmus almeriensis*, *Tisochrysis lutea* or *Tetraselmis suecica*).

These enzymes play a key role in the final digestion stages of dietary protein, allowing amino acid absorption or transport by enterocytes. Particularly, alkaline phosphatase is a dominant enzyme in the intestinal mucosa, and it is commonly used either as an indicator of the intestinal integrity, or as a general marker of nutrient absorption. For this reason, the increase in these activities can be associated with an amelioration in the overall efficiency of digestive and absorptive processes. Regarding the use of aquafeeds supplemented with seaweeds, Vizcaíno *et al.* (2016a, 2019a) described contrasting results; whereas *Ulva lactuca* caused a decrease in the intestinal proteolytic activity in gilthead seabream and Senegalese sole juveniles, the use of *Ulva onhoi* increased alkaline phosphatase activity in Senegalese sole. An explanation of this differential effect could be attributable to the different dietary level used in these feeding trials.

In this way, the higher dietary level, the lower digestive proteolytic activity. In the case of *Ulva* sp., Vizcaíno *et al*. (2019b) suggested its use as an additive for improving the intestinal epithelium of Senegalese sole, but only for a short period of time in order to avoid undesirable effects on digestive proteolytic enzymes. A healthy gastrointestinal tract is also crucial for optimal growth performance. According to Sweetman *et al*. (2008), the study of the intestinal mucosa can be used as a valuable tool to know how diet or other factors, such as infectious diseases or anti-nutritional compounds, can influence its structure and morphology.

One of the major limitations for using proteins from plant origin in aquafeeds is their impact on the digestive system, including the reduced height of villi and enterocytes, low brush border integrity and supranuclear vacuolization in enterocytes, presence of leucocytes in *lamina propria* and submucosa as well as the presence of different

inflammatory symptoms, among other events (Cerezuela *et al*., 2012). Light and electron microscopy are useful tools for assessing how algae-supplemented diets might affect the integrity of the intestinal mucosa of fish. Both, light microscopy, and transmission and scanning electron microscopy (TEM and SEM) images obtained from intestinal section of fish fed algae-supplemented diets have been used for assessing the integrity of the apical brush border. This way, while TEM images offer information of the length, diameter and absorption surface of microvilli, SEM images enable to measure the enterocyte apical area (Fig. 9). The studies carried out on marine fish species (gilthead seabream and Senegalese sole) at different developmental stages (fry and juvenile) revealed that the inclusion of algae provoked positive changes in microvilli length, enterocyte apical area, and increased absorption surface in enterocytes (Vizcaíno *et al*., 2014, 2016b, 2019a). It is a general rule in these studies that those changes revealed an overall increased absorptive capacity in the intestines, as well as a reinforced intestinal mucosa as physical barrier in fish fed microalgae-supplemented diets.

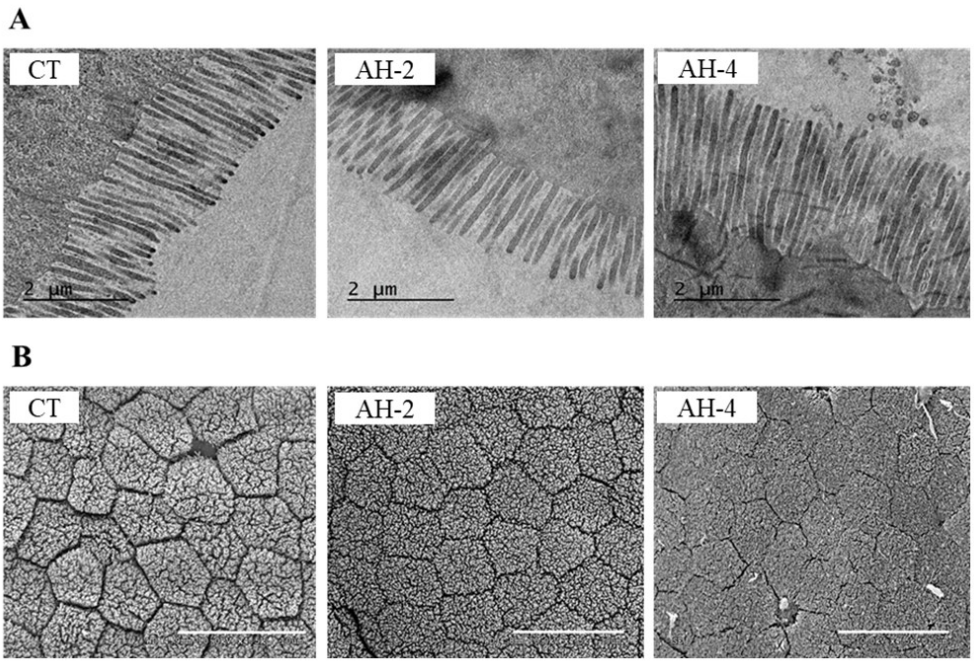

Figure 9. TEM (A) and SEM (B) micrographs from the anterior intestine of juvenile gilthead seabream fed the experimental diets. (TEM bar: 2 µm; SEM bar: 10 µm). CT: control, and AH-2: 2% *Arthrospira* sp. hydrolysate, AH-4: 4% *Arthrospira* sp. hydrolysate diets. Image taken from Galafat *et al*. (2020).

6. Conclusions

Increased demand for aquaculture fish equates to higher requirements for aquafeeds. Feed producers are continuously seeking for sustainable novel ingredients to provide fish with adequate nutrition. Algae as feed ingredients and/or additives can offer the desired nutritional profile in aquafeed formulation. In spite of the fact that algae production has improved significantly in the recent years, production systems do not fulfil yet the desired biomass productivity to gather commercial benefits. For this reason, production costs must be reduced significantly in order to achieve a massive use of algae as regular ingredients in fish commercial formulas.

The algal species used for aquafeed manufacture can vary according to the target species and the type of nutrient required (protein, lipid, DHA, EPA). For instance, some species of algae (*Spirulina*, *Chlorella* and *Nannochloropsis*) are so rich in protein content that almost half (40 to 65%) of its biomass is protein. Algae can accumulate high quantities of lipid when grown under stress, and some species have a nutritionally valuable fatty acid profile, which includes polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Algae also accumulate higher amounts of carotenoids as an adaptation to the changing environment. Carbohydrates constitute from 2 to 66% in dry weight in algae, and they vary according to their growth conditions and the age of the cultures. However, a concern with using algae for feeding purposes is the presence of a thick cell wall rich in polysaccharides of varied nature in many species, which is resistant to enzyme hydrolysis. Hence, this cell wall is difficult for certain fish species to break down and digest. Aquafeed producers can cope with this inconvenience by implementing procedures to process the biomass aimed at releasing the internal nutrients, so that they are readily available for the fish to be digested and absorbed.

Each algae species has its own nutrient profile. However, this nutritional content may vary depending on temperature, incident light quality and intensity, medium conditions, growth phase, photoperiod and harvesting methods. Assuming that enough quantities of algae biomass were available at a suitable price, algal producers and aquafeed manufacturers would still need to take into account the eventual variations in proximate composition existing among different strains and growing conditions. Further effort is needed to ensure a consistent chemical composition of algal biomasses so that manufacturers can readily incorporate this novel source instead of other regular ingredients in commercial aquafeeds.

Numerous studies evidenced beneficial effects of using algae in aquafeeds, specifically focussing on growth, feed utilisation, and survival rate. Algae are also useful for modulating the fat content and fatty acid profile composition, the skin and

flesh pigmentation, and the quality of fish flesh. There are also evidences that dietary inclusion of algae improves integrity of intestinal mucosa, and might enhance activity of some digestive enzymes. However, results reported in the literature indicate that the optimum dietary algae inclusion level should vary depending on the algae and on the farmed fish species considered. Overall, herbivorous and omnivorous fish tolerate high inclusion levels of algae compared to carnivorous species. The effect of algae seems to be dose-dependent, and species-specific, and consequently, it is difficult to establish a general rule about the use of algae in aquafeeds. Therefore, specific research should be carried out on each specific algae strain and fish species.

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Assessment of dietary inclusion of crude or hydrolysed *Arthrospira platensis* biomass in starter diets for gilthead seabream (*Sparus aurata*)

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ABSTRACT

This work evaluates the effects of the dietary inclusion of crude or hydrolysed *Arthrospira platensis* (Cyanobacteria) biomass on growth, muscle composition, digestive functionality and immune activities in gilthead seabream (*Sparus aurata*) fry (20.32 mg mean body weight). A 40-day feeding trial was conducted, aimed at assessing four experimental diets that included 5 or 10% (w/w) *A. platensis*, either crude or hydrolysed, plus a microalgae-free diet as control batch. Overall, none of the dietary treatments caused negative impacts on fish growth, body composition, muscle fatty acid profile, or innate immune response. Thus, the dietary inclusion of both crude and hydrolysed *A. platensis* reduced significantly the oxidation of muscle lipids, especially when using hydrolysed biomass, regardless of the dietary inclusion level. In relation to digestive enzymes, significantly higher levels of trypsin, chymotrypsin and leucine aminopeptidase activities were measured in fish fed on *A. platensis*-supplemented diets compared to control fish. In addition, within each inclusion level (5 or 10% w/w), those animals fed with diets that included the hydrolysed biomass yielded consistently higher digestive enzyme activities than those receiving the crude biomass. Microalgae dietary inclusion also induced favourable changes in fish gut morphology, according to the increase in microvilli length and diameter observed. This fact might well have contributed to reinforce the role of the intestinal mucosa as a protective barrier against microorganisms, as well as to enhance the absorptive capacity of the intestinal mucosa. Finally, 10% inclusion of microalgae hydrolysate enhanced lysozyme activity in liver, this fact suggesting improved protection against infectious diseases. In conclusion, the positive effects observed in fish fed with the diets including *A. platensis* up to 10% (not least the hydrolysed biomass) with regard to the different parameters assessed (digestive enzyme activities, intestinal epithelium ultrastructure, muscle lipid oxidation, and lysozyme activity) suggest the benefits of including this product in starter feeds for gilthead seabream fry.

1. Introduction

Early stages in the life cycle of marine fish are critical for the subsequent developmental changes taking place, in which fish undergo drastic morphological and physiological changes that determine further viability (Torres et al., 2020). Besides organ differentiation and morphogenesis, the larval stage is characterized by the highest growth rate throughout the productive cycle of commercial fish (Savoie et al., 2011). Consequently, considerable research effort has been made to develop inert microdiets that must fulfill certain premises for adequate larval development. Besides being tasty and economical, the most important of such requirements is to provide the necessary protein, amino acids, and fatty acids (Conceição et al., 2007; Vizcaíno et al., 2016; Khoa et al., 2019). These requirements have been traditionally met by the use of fishmeal and fish oil as the main protein and fatty acid sources, although plant protein ingredients, such as soybean meal, have also been included in feedstuffs (Ayala et al., 2020). However, in recent years, microalgae species and specific strains of cyanobacteria have emerged as a raw material of extraordinary interest in aquaculture (Shah et al., 2018; Han et al., 2019). Owing to their chemical composition, species of the genus *Arthrospira* are considered excellent candidates that have been successfully used as ingredients in feeds for several fish

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species (Mahmoud et al., 2018; Liu et al., 2019; Niccolai et al., 2019; Rajasekar et al., 2019). Despite this, the relatively high cost of microalgae, compared to conventional ingredients, limits their use in commercial aquafeeds (Yarnold et al., 2019). Even though cheaper than other microalgae species commercially available, the current price of freeze-dried *A. platensis* (around 15 USD kg^{-1}) is, by far, above the average price of fishmeal (in the region of 1.6–2.0 USD kg^{-1}), and therefore it is not yet feasible to replace fishmeal as the main ingredient in aquaculture feeds. Hence, the interest of microalgae as potential functional additive in aquafeeds, instead of a major dietary ingredient, is increasing considerably (Galafat et al., 2020).

On the other hand, microalgae inclusion in aquafeeds becomes more difficult in the case of early life stages because fish larvae cannot handle dietary components like juveniles. The scarce enzyme activity during these stages hinders digestion processes (Khoa et al., 2019). Namely the limited luminal protease activity affects directly protein digestion, a factor that has been pointed out as a major limitation to the utilization of diets by fish larvae (Cahu and Infante, 2001; Cai et al., 2015). In this regard, this work raises the question of whether protein hydrolysates obtained from microalgae could be used as dietary ingredient in weaning diets for marine fish larvae. Earlier, Galafat et al. (2020) corroborated the potentially positive physiological effects of *Arthrospira* sp. protein hydrolysates as bioactive additive in diets for juvenile gilthead seabream (*S. aurata*). According to this work, an enzymatic pretreatment could release low molecular weight bioactive peptides and free amino acids that could be easily absorbed by enterocytes, this leading to earlier maturation of digestive organs, as well as to improved nutrient digestibility and acceptability by the animal (Srichanun et al., 2014). Beside this, a range of molecules that are known to be bioactive or so-called "nutraceutical" effects. These bioactive compounds might trigger host immune reactions in response to pathogen surface molecules, thus improving anti-inflammatory and antimicrobial abilities of the host (Novak and Vetvicka, 2009), apparently by raising both the non-specific and the specific immunity of fish (Sahoo and Mukherjee, 2001). This fact might well be critical in early life stages in which the specific immunity is not well established, and the immune response of fish depends on non-specific activities (Uribe et al., 2011). It has been recently described an immunity communication between gut and liver in which more specific immune proteins and transcript were detected in gut and more non-specific immune molecules in liver (Wu et al., 2016). The potential role of microalgae enzymatic hydrolysates in aquafeeds on the abovementioned physiological phenomena at early stages of fish development remains unexplored. Therefore, this research is aimed at assessing the effects of low dietary inclusion levels of crude and hydrolysed *Arthrospira platensis* on fish growth performance, proximate composition, fatty acid composition, muscle lipid oxidation, digestive functionality, and innate immune activities in gilthead seabream fry.

2. Material and methods

2.1. Microalgae biomass

Crude biomass of *A. platensis* (crude protein: 65% dry weight, DW; crude lipid: 5%, DW) was provided by Biorizon Biotech (Almería, Spain). The term microalgae is used to refer to prokaryotic species such as cyanobacteria *A. platensis* (Cyanophyceae) with microscopic size. Cyanobacterium *Spirulina platensis* and *A. platensis* are considered as synonyms to each other as indicated by Guiry and Guiry (2018). The microalgal protein hydrolysate was produced from the crude raw biomass after performing an enzymatic hydrolysis following the method described by Saadaoui et al. (2019), as modified by Galafat et al. (2020). Briefly, a sludge containing 150 g L^{-1} microalgae biomass was incubated with 0.2% *w*/w mixture of commercial proteases (Alcalase 2.4 L® and Flavourzyme 1000 L® from Novozymes A/S, Bagsvaerd, Denmark) under controlled conditions (pH 8.0 and 50 °C under continuous stirring) during 4 h. Immediately after the hydrolysis, the reaction mixture was heated at 80 °C for 15 min in order to inactivate commercial proteolytic enzymes. The hydrolysate was then kept at 4 °C until use.

2.2. Experimental diets

Five iso-nitrogenous (59%, DW) and isolipidic (18.0%, DW) experimental diets were formulated; two of them contained 5 and 10% w/w microalgae raw biomass (designated as C-5 and C-10, respectively); two other experimental groups included 5 and 10% w/w hydrolysed biomass (designated as H-5 and H-10, respectively), and a fifth diet, microalgae-free, was used as the control batch (CT). The formulation and chemical composition of the experimental diets are shown in Tables 1, 2 and 3. The experimental diets were designed and manufactured by CEIMAR-University of Almería (Service of Experimental Diets) (Almeria, Spain) using standard aquafeed processing procedures. Particle size of the microdiets elaborated ranged from 0.3 to 0.8 mm.

2.3. Fish and experimental design

Larval rearing took place at the Planta de Cultivos Marinos facility of the Instituto Español de Oceanografía (IEO, Puerto de Mazarrón, Murcia, Spain). Previously, fertilized eggs of gilthead seabream (*S. aurata*) were stocked in a 500 L cylindrical-conical incubator with gentle

Table 1
Ingredients and proximate composition (g kg^{-1} DW) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
Ingredients					
Fishmeal LT94[1]	685.0	641.0	597.0	641.0	597.0
A. platensis meal[2]		50.0	100.0		
A. platensis hydrolysate[3]				50.0	100.0
Attractant premix[4]	80.0	80.0	80.0	80.0	80.0
Wheat gluten[5]	50.0	50.0	50.0	50.0	50.0
Soybean protein concentrate[6]	20.0	20.0	20.0	20.0	20.0
Fish oil	53.0	55.0	58.0	55.0	58.0
Soybean lecithin[7]	40.0	40.0	40.0	40.0	40.0
Choline chloride	2.0	2.0	2.0	2.0	2.0
Wheat meal[8]	17.0	9.0		9.0	
Betaine	2.0	2.0	2.0	2.0	2.0
Vitamins and minerals premix[9]	30.0	30.0	30.0	30.0	30.0
Vitamin C	1.0	1.0	1.0	1.0	1.0
Binder (alginate)	20.0	20.0	20.0	20.0	20.0
Proximate composition (%, DW)					
Crude protein	58.9	59.1	59.3	59.3	59.4
Crude lipid	18.1	18.0	17.9	18.4	18.3
Ash	16.0	16.0	15.7	15.8	16.1
Crude fiber	2.1	1.9	2.3	2.0	2.2
NfE[10]	4.9	5.0	4.8	4.5	4.0

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. [1](protein: 69.4%; lipid: 12.3%), Norsildemel (Bergen, Norway); [2](protein: 60.5%; lipid: 5.6%); [3]Liquid product containing 150 g microalgae meal L^{-1}; [4](50% squid meal, 25% shrimp meal, 25% krill meal); [5](protein: 76.0%; lipid: 1.9%); [6](protein: 50.0%; lipid: 1.0%); [7]Lecico P700 IP (Lecico GmbH, Germany) [8](protein: 12.0%; lipid: 2.0%); [9]Vitamin & Mineral Premix: Vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain); [10]NfE: Nitrogen free extract calculated as 100 - (% crude protein + % ether extract + % ash + % crude fiber). DW: dry weight.

Table 2
Fatty acid composition (% DW of relevant fatty acids) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
14:0	3.0	3.0	3.1	2.8	2.9
16:0	16.0	16.8	16.5	16.8	16.4
18:0	2.8	2.7	2.7	2.7	2.8
18:1n9	19.0	19.2	19.3	19.5	19.3
18:2n6	12.6	12.9	12.7	13.4	13.0
18:3n3	2.0	2.0	1.9	1.8	1.9
20:4n6, ARA	0.9	0.9	0.9	0.9	0.9
20:5n3, EPA	7.5	7.3	7.2	6.8	7.1
22:6n3, DHA	10.8	10.6	10.4	10.3	10.5
SFA	21.9	22.5	22.2	22.3	22.1
HUFA	21.9	21.4	21.6	20.9	21.4
n3	24.5	23.8	23.8	23.0	23.7
n6	13.4	13.8	14.1	14.3	13.9
n9	5.9	5.7	5.8	5.4	5.6

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. DW: dry weight.

Table 3
Aminoacids profile of *A. platensis* biomass and the experimental diets (g 100 g^{-1} DW).

	A. platensis	CT	C-5	C-10	H-5	H-10
NEAA						
Ala	5.0	3.6	3.2	3.7	3.3	3.6
Asp	6.5	5.3	4.9	4.7	4.9	4.8
Cys	0.3	0.3	0.2	0.3	0.2	0.3
Glu	8.9	8.9	7.9	8.8	8.0	8.5
Gly	3.3	3.6	3.2	3.5	3.2	3.4
Pro	4.9	5.1	4.5	4.7	4.5	4.8
Ser	3.4	2.6	2.4	2.6	2.4	2.5
Tyr	3.2	2.0	1.9	1.9	1.9	2.0
EAA						
Arg	4.1	3.4	3.2	3.5	3.2	3.4
His	1.2	1.3	1.3	1.2	1.2	1.3
Ile	3.1	2.1	2.0	2.4	2.0	2.3
Leu	5.1	4.0	3.8	4.1	3.8	4.1
Lys	4.2	5.6	5.2	5.4	5.1	5.4
Met	0.9	1.6	1.4	1.5	1.4	1.5
Phe	3.6	2.6	2.4	2.5	2.4	2.6
Thr	3.3	2.4	2.1	2.3	2.2	2.2
Val	3.4	2.5	2.3	2.8	2.4	2.7
EAA/NEAA	0.9	0.8	0.8	0.9	0.8	0.9

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. EAA: essential aminoacids, NEAA: non-essential aminoacids. DW: dry weight.

aeration until hatching. The incubator was supplied with ultraviolet-irradiated seawater at 19 ± 0.5 °C, same as spawning water temperature. Then, hatched larvae were transferred to a 5000-L tank where they began to be fed with rotifers (*Brachionus plicatilis*) once the mouth opening occurred (5 days post-hatching, dph) until 27 dph, at a density of 20 rotifers mL^{-1}. From 20 to 27 dph, Artemia nauplii at a concentration of 1–3 nauplii mL^{-1} were introduced in the tank, and Artemia metanauplii from 26 dph until the weaning period was completed (50 dph). From 40 to 50 dph, larvae were progressively weaned through a co-feeding regime based on Artemia metanauplii and the inert commercial diet (Gemma Wean 0.2, Skretting). The amount of Artemia was progressively reduced from 3 to 0.5 Artemia mL^{-1}, whereas inert feed supply increased. After weaning, gilthead seabream larvae were transferred to 170 L tanks (510 larvae $tank^{-1}$; 3 larvae L^{-1}) in an open circulation system five days before the beginning of the feeding trial. UV sterilized seawater (38‰ salinity; 20–23 °C) was provided into the tank system at an exchange rate of 150 L h^{-1}. Supplemental aeration was provided in order to maintain dissolved oxygen above 6.5 mg L^{-1}, and photoperiod was fixed on a 14-h light/10-h dark cycle (450 lx). Ammonia (<0.1 mg L^{-1}), nitrite (<0.2 mg L^{-1}), and nitrate (<50 mg L^{-1}) were determined once weekly.

From 55 dph onwards, larvae (~ 24 mg, average initial body weight) were exclusively fed with the experimental diets to apparent visual satiation (*ad libitum*) six times daily. The different dietary treatments (CT, C-5, C-10, H-5 and H-10) were randomly assigned to the experimental tanks. Each dietary treatment was tested in triplicate (5 feeding treatments × 3 tanks per treatment) for 40 days. Feeding rate and feed size were equal in all tanks, and those were adjusted according to fish age, larval weight, and water temperature, following the recommendations for gilthead seabream fry provided by Skretting España (Burgos, Spain).

2.4. Fish sampling

At the beginning, larvae (55 dph) were individually weighed and measured after 12-h fasting. During the feeding trial, fasted fish from each tank were randomly sampled at 75 and 95 dph. In each sampling point, 100 fish per tank were weighed, measured and sacrificed by anaesthetic overdose (50 ppm clove oil) according to the requirements of the Council Directive 2010/63/UE. One pool of complete intestines (15 animals) from fish previously fasted for 6-h was prepared from each tank. The biological material was then processed to obtain crude extracts prior to determine digestive enzyme activities. In brief, intestines were manually homogenized in distilled water at 4 °C to a final concentration of 0.5 g mL^{-1}. Supernatants were obtained after centrifugation (16,000 ×*g* for 12 min at 4 °C) and stored at −20 °C until further analysis. For proximate composition analysis, one hundred of 95 dph-fry carcasses (without viscera) per tank were freeze-dried. In addition, muscle samples (1 g) of a pool of ten fish per tank were used for lipid oxidation determinations, and the excess sample was freeze-dried for fatty acid composition. The intestines of three specimens from each tank were collected at 95 dph for examination by transmission (TEM) and scanning (SEM) electron microscopy. Finally, also at 95 dph, the liver of three fish from each tank were extracted, weighed, and individually kept at −80 °C until processing for the analysis of immune-related parameters.

2.5. Growth performance, nutrient utilization and somatic indices

Growth performance was assessed by different parameters according to the following formulae: daily gain (DG, g day^{-1}) = (Wf − Wi) / days; specific growth rate (SGR, %/d) = (Ln (Wf) − Ln (Wi) / days) × 100; condition factor (K) = (Wf / SL^3) × 100, where Wf was the final weight (g), Wi was the initial weight (g) and SL was the standard length. Somatic indices were also calculated: Hepatosomatic Index (HSI, %) = (liver weight (g) /whole body weight (g)) × 100¸ and Viscerosomatic Index (VSI, %) = (visceral weight (g) /whole body weight (g)) × 100.

2.6. Proximal composition and fatty acid profile

Chemical analysis of feeds and carcasses was carried out following the methods by AOAC (2000) for dry matter and ash, whereas crude protein (N × 6.25) was determined by using elemental analysis (C:H:N) with a Fisons EA 1108 analyser (Fisons Instruments, Beverly, MA, USA). Total lipid content was analysed following the procedures described by Folch (1957). Fatty acid profile of feeds and muscle samples was determined following the method of Rodríguez-Ruiz et al. (1998), using a gas chromatograph. For amino acid analysis, microalgal biomass and experimental diets were hydrolysed (20 mg in 1 mL HCl 6 M) at 110 °C during 24 h under an inert atmosphere (N_2). After that, 50 μL of the hydrolysate were mixed with 50 μL of 6 M NaOH. Then 100 μL of internal standard (2.5 mM norleucine) and 800 μL sodium citrate loading buffer (pH 2.2) were added and mixed by vortex for 5 s, and then filtered (0.2 μm). A sample (20 μL) of this mixture was analysed with an amino acid

analyser Biochrom 30+ amino acid analyser (Biochrom LTD Cambridge, UK) according to the manufacturer's protocol.

2.7. Muscle lipid oxidation

Lipid oxidation in muscle samples was estimated by measuring thiobarbituric acid-reactive substances (TBARS) following the method of Buege and Aust (1978). Samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO_4, 0.1% (*v*/v) Triton X-100 solution. The mixture was centrifuged (10,000 ×*g*, 20 min, 4 °C). Supernatants were mixed in a 1:5 ratio (v/v) with 2-thiobarbituric acid (TBA) reagent (0.375% *w/v* TBA, 15% w/v TCA, 0.01% w/v 2,6- di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl), heated for 15 min and centrifuged (3600 ×*g*, 10 min, 4 °C). The absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

2.8. Digestive enzyme activities

Total alkaline protease activity was determined according to the method described by Alarcón et al. (1998) using buffered 5 g L^{-1} casein (50 mM Tris-HCl, pH 9.0) as substrate. The amount of enzyme releasing 1 μg tyrosine per minute was defined as one unit of activity, considering an extinction coefficient for tyrosine of 0.008 μg^{-1} cm^{-1} mL^{-1}, measured spectrophotometrically at 280 nm. Trypsin and chymotrypsin activities were measured spectrophotometrically at 405 nm using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-nitroanilide) as substrate, according to Erlanger et al. (1961), and 0.2 mM SAPNA (N-succinyl-(Ala) 2-Pro-Phe-P-nitroanilide), following the method described by Del Mar et al. (1979), respectively. Leucine aminopeptidase activity was quantified using 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer (pH 8.8) as substrate according to Pfleiderer (1970). Alkaline phosphatase activity was determined using buffered *p*-nitrophenyl phosphate (pH 9.5) as substrate, following the method described by Bergmeyer (1974). For trypsin, chymotrypsin and leucine aminopeptidase activities, the amount of enzyme that released 1 μmol of p-nitroanilide (pNA) per minute was defined as one unit of activity, considering as extinction coefficient 8800 M cm^{-1}, measured spectrophotometrically at 405 nm. For alkaline phosphatase, one unit of activity was defined as the amount of enzyme that released 1 μg of nitrophenyl per min (extinction coefficient 17,800 M cm^{-1} at 405 nm).

2.9. Ultrastructural analysis of intestinal mucosa

Intestine samples from the anterior region were collected for evaluation by electron microscopy. Samples for scanning electron microscopy (SEM) were previously washed with 1% *S*-carboxymethyl-L-cysteine (Sigma) for 20 s in order to remove the epithelial mucus, and fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h. Then, samples were washed and dehydrated in graded ethanol. Critical point drying of samples (CDP 030 Critical point dryer, Leica Microsystems, Madrid, Spain) was carried out by using absolute ethanol as intermediate fluid and CO_2 as transition fluid. After that, dried samples were mounted on supports and fixed with graphite (PELCO® Colloidal Graphite, Ted Pella INC., Ca, USA) and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). All samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). Samples for transmission electron microscopy (TEM) were fixed in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5 (4 h, 4 °C). Then, intestine sections were washed with PBS for 20 min, subjected to a post-fixation step with 20 g L^{-1} osmium tetroxide, and dehydrated by consecutive immersion (20 min each) in ethanol solution of gradients from 50% to 100% (v/v). Samples were embedded for two hours in 1:1 Epon resin: absolute ethanol mixture under continuous shaking, and then included in pure Epon resin during 24 h, and polymerized at 60 °C. Finally, the ultrathin cuts were placed on 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C transmission electron microscopy (Carl Zeiss, Barcelona, Spain) at 100 Kv. Visualization fields were recorded at ×16,000 magnification.

SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software. Microvilli length (ML) and microvilli diameter (MD) as well as the number of microvilli within 1 μm distance (Vizcaíno et al., 2014) were determined in TEM micrographs. SEM images were used to obtain several measurements of enterocyte apical area (EA). Finally, data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno et al. (2014).

2.10. Antimicrobial activities in liver homogenates

Liver samples, stored at −80 °C, were homogenized (1 mg of tissue mL^{-1}) in phosphate buffer saline (PBS, pH 7,4) and protease, antiprotease, peroxidase, lysozyme and bactericidal activities were analysed in extracts. Protease activity was determined by measuring the hydrolysis of azocasein, according to the protocol by Charney and Tomarelly (1947) modified as described in Chaves-Pozo et al. (2019). Results were expressed as relative values, considering 100% protease activity that observed for a protease standard solution (10 μL of 2 mg mL^{-1} proteinase K (AppliChem) in PBS) under the specified assay conditions. Results were then expressed as % μg^{-1} of tissue. The antiprotease activity was determined by the ability to inhibit proteinase K activity using a modification of the protocol described in Ellis (1990) as described elsewhere (Chaves-Pozo et al., 2019). The percentage of inhibition of proteinase K activity for each sample was calculated as 100 − (% of protease activity). Results were expressed as % μg^{-1} of tissue. Peroxidase activity was measured in extracts according to Quade and Roth (1997) and Chaves-Pozo et al. (2019). One unit was defined as the amount of activity producing an absorbance change of 1, and the activity was expressed as units (U) μg^{-1} of tissue. The lysozyme was measured using a modification of the turbidimetric method described by Parry Jr et al. (1965), using 0.3 mg mL^{-1} freeze-dried *Micrococcus lysodeikticus* (Sigma M-3770) in phosphate citrate buffer (0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2) as substrate. The reduction in absorbance at 450 nm was measured immediately every 30 s during 15 min at 22 °C in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. Results were expressed as units (U) μg^{-1} of tissue. Bactericidal activity was determined by evaluating their effects on the bacterial growth of *Vibrio harveyi*, as explained elsewhere (Sunyer and Tort, 1995; Chaves-Pozo et al., 2019). Results were corrected with the absorbance measured in each sample at the initial time point and expressed as % μg^{-1} of tissue.

2.11. Statistical analysis

Data were presented as mean ± SD. The effects of the different dietary treatments were determined by one-way ANOVA considering a significance level of 95% to indicate statistical difference ($P < 0.05$), followed by a generalized lineal statistical model (GLM analysis). Significant differences were determined using Tukey's multiple comparison test. Data with nonparametric distribution were analysed using Kruskal-Wallis one-way analysis. Also, discriminant analysis (DA) was conducted in digestive enzyme activities and morphometrical analysis of TEM and SEM images. The estimation of the validity of the discriminant function was based on the significance of Wilk's Lambda and the percentage of correct assignment to a certain diet. All statistical analyses were performed with Statgraphics Plus 4.0 (Rockville, Maryland, USA) software.

3. Results

3.1. Growth performance and proximate composition

The evolution of fish growth during the feeding trial is shown in Fig. 1. Dietary inclusion of *A. platensis* biomass, either crude or hydrolysed, did not affect fish growth or nutrient utilization. In fact, final body weight (FBW), daily gain (DG), specific growth rate (SGR) and Fulton's body condition factor (K) were similar in all the experimental groups (Table 4). Regarding somatic indices, hepatosomatic index (HSI) tended to decrease in fish fed on hydrolysed *Arthrospira*-supplemented diets, although significant differences with respect to CT group were only observed for H-10 lot ($P < 0.05$). Viscerosomatic index (VSI) remained unaffected.

The proximate composition of eviscerated fish is shown in Table 5. The inclusion of *Arthrospira* biomass, crude or hydrolysed, did not modify protein, lipid, or ash contents of gilthead seabream fry ($P < 0.05$).

3.2. Fatty acid profile

Muscle fatty acid composition of gilthead seabream fry is shown in Table 6. All the experimental groups showed similar saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid contents ($P > 0.05$). PUFAs were the predominant lipid fraction (32.68–33.42%), followed by MUFAs (30.77–31.18%) and SFAs (22.97–24.90%). Considered individually, muscle content of myristic acid (C14:0) and palmitic acid (C16:0) was significantly lower in fish fed on H-10 and H-5 diets, respectively, compared to the rest of dietary treatments evaluated. However, fish fed with crude *A. platensis*-supplemented diets (C-5 and C-10) showed the highest values of palmitic acid. Regarding polyunsaturated fatty acids, the inclusion of microalgae hydrolysates (H-5 and H-10 diets) yielded a significant decrease in eicosapentaenoic acid (EPA, 20:5n3) content in comparison with CT group ($P < 0.05$), whereas docosahexaenoic acid (DHA, 22:6n3) tended to increase in these experimental groups, although no significant differences were observed. As a result, fish fed on diets containing microalgae hydrolysates (H-5 and H-10) showed lower EPA/DHA ratios ($P < 0.05$).

3.3. Muscle lipid oxidation (TBARS)

Results of muscle TBARS are shown in Fig. 2. Overall, fish fed on microalgae-supplemented diets showed a significant decrease in TBARS values compared to CT group ($P < 0.05$), especially in C-10, H-5 and H-10 experimental groups.

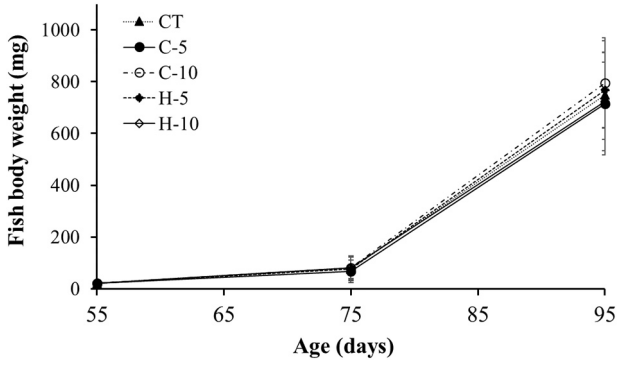

Fig. 1. The final body weight of gilthead seabream fry fed with experimental diets for 40 days. Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

3.4. Digestive functionality

Intestinal enzyme activities were measured at two time points throughout the feeding trial (Fig. 3). In general, the results obtained revealed that the dietary inclusion of crude and hydrolysed *A. platensis* had no adverse impact on the digestive enzymes evaluated, regardless of the dietary inclusion considered. Overall, the activity level of the different digestive enzymes evaluated increased significantly with larval age regardless of dietary treatment used ($P < 0.001$) (Table 7). At 75 dph, an increase of trypsin and chymotrypsin activity levels was found in groups fed with crude and hydrolysed microalgae compared to control (CT) ($P > 0.001$). Moreover, results showed that the use of the hydrolysates (H-5 and H-10) increased the chymotrypsin and total alkaline protease activity levels compared to fish fed with crude microalgal biomass (chymotrypsin, $P = 0.001$; total alkaline protease, $P = 0.012$). Regarding brush border enzymes, the inclusion of the microalgae biomass increased significantly both leucine aminopeptidase ($P = 0.015$) and alkaline phosphatase ($P = 0.004$) activities. Fish fed on C-5 and H-10 diets showed higher leucine aminopeptidase activity than CT group. For alkaline phosphatase, all the experimental groups fed on *A. platensis* supplemented diets, especially H-10 group, displayed higher activity than fish receiving CT diet. At the end of the feeding trial (95 dph) only chymotrypsin, and leucine aminopeptidase activities increased owing to the inclusion of the microalga. C-10 and H-10 showed higher chymotrypsin activity levels than those observed in the control fish, whereas the increase of leucine aminopeptidase activity ($P < 0.05$) was observed in most groups feed on microalgae-supplemented diets, except C-10 group.

On the other hand, TEM and SEM images showed an intestinal mucosa with normal appearance in all the fish fed with the experimental diets. In general, a regular and densely packed microvilli on the enterocyte surface was observed in all the specimens without any sign of structural damage or microvilli alterations (Fig. 4A and B).

Morphometric analysis of TEM and SEM images revealed that the dietary inclusion of both crude and hydrolysed *A. platensis* caused a significant effect on microvilli length, microvilli diameter, enterocyte apical area and absorption surface ($P < 0.001$) (Table 8). Overall, fish fed on microalgae-supplemented diets showed significantly increased microvilli length and microvilli diameter compared to fish of CT group, not least owing to the use of the microalgal hydrolysate ($P < 0.001$), regardless the dietary inclusion level. In addition, H-10 fed fish showed the highest values of enterocyte apical area (EA) and enterocyte absorption surface (TAS).

Values of digestive enzyme activities and ultrastructural analysis of intestinal mucosa were analysed by discriminant analysis (DA) (Fig. 5, Table 9). Grouping was recognized on the basis of the diet ingested by fish. DA confirmed that function 1 could discriminate clearly diet H-10, which was plotted much further to the right than the other dietary treatments. The second function provided certain discrimination between the CT diet and the microalgae-supplemented diets, but not nearly as clear as that provided by the first function.

3.5. Immune system activities

The immune activities analysed in liver extracts did not show significant differences between treatments ($P > 0.05$) (Table 10). These results indicate that the microalgae-supplemented diets did not cause any negative impact on the innate immune activities in the liver of gilthead seabream fry. On the contrary, protease activity tended to increase in fish fed on C-5 and H-10 diets, and lysozyme and bactericidal activities tended to increase in H-10 diet, although these changes were only significant for lysozyme activity (H-10 group), likely owing to the great variability observed between specimens.

Table 4
Growth performance and nutrient utilization parameters of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*p-value*
Growth performance						
Final body weight (FBW, mg)	749.78 ± 126.28	714.23 ± 196.81	795.19 ± 173.62	766.79 ± 190.87	723.78 ± 190.95	*0.276*
Fulton's condition factor (K)	1.41 ± 0.12	1.36 ± 0.08	1.37 ± 0.03	1.48 ± 0.07	1.34 ± 0.04	*0.300*
Daily gain (DG, mg day^{-1})	19.93 ± 2.94	16.10 ± 4.58	17.98 ± 4.04	17.31 ± 4.44	16.32 ± 0.44	*0.277*
Specific Growth Rate (SGR, %/d)	8.17 ± 0.40	8.01 ± 0.65	8.29 ± 0.53	8.19 ± 0.59	8.05 ± 0.62	*0.215*
Somatic index						
Hepatosomatic Index (HSI, %)	2.42 ± 0.42 b	2.29 ± 0.61 b	2.10 ± 0.63 b	1.95 ± 0.65 ab	1.77 ± 0.36 a	*<0.001*
Viscerosomatic Index (VSI, %)	6.30 ± 1.20	6.39 ± 1.12	6.23 ± 1.06	5.82 ± 1.00	6.22 ± 1.27	*0.296*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate tanks ($n = 3$). Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

Table 5
Carcass proximate composition (% of DW) of gilthead seabream at the end of the feeding trial.

	Protein	Lipid	Ash
CT	66.17 ± 0.16	15.24 ± 0.27	3.76 ± 0.02
C-5	65.81 ± 1.54	15.46 ± 0.28	3.65 ± 0.08
C-10	67.17 ± 0.04	15.23 ± 0.19	3.67 ± 0.21
H-5	65.08 ± 0.12	15.43 ± 0.37	3.63 ± 0.05
H-10	67.39 ± 0.02	15.53 ± 0.48	3.59 ± 0.04
P-value	*0.088*	*0.780*	*0.219*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. DW: dry weight.

4. Discussion

The dietary inclusion of microalgae hydrolysates emerges as a novel strategy in aquaculture nutrition. The enzymatic hydrolysis of microalgae biomass may increase extraction yields of high-value products or improve the bioavailability of intracellular components (Galafat et al., 2020; Siddik et al., 2021a, 2021b), and therefore an improvement on fish growth parameters derived from its dietary inclusion could be expected. However, the results obtained in this work revealed that the dietary inclusion of crude and hydrolysed *A. platensis* biomass had no significant effect on body growth parameters after a 40-day feeding trial. These results are in agreement with those reported by Galafat et al. (2020), who pointed out similar effects in gilthead seabream juveniles fed on *Arthrospira* sp. protein hydrolysate up to 4% inclusion level. Similarly, Ayala et al. (2020) did not observe differences in growth rates and feed intake in gilthead seabream juveniles attributable to the dietary inclusion of *Nannochloropsis gaditana* either raw or hydrolysed. On the contrary, other authors reported that low dietary inclusion of different microalgae species, such as *Tetraselmis suecica, Phaeodactylum* sp., *Nannochloropsis* sp., or *Chlorella* sp., in microdiets led to improved growth performance in gilthead seabream and Senegalese sole fry (Vizcaíno et al., 2016; Peixoto et al., 2021). These disparate results confirm that microalgae have a species-specific and dose-dependent effect on growth performance and nutrient utilization of farmed fish.

Regarding somatic indices, the dietary inclusion of hydrolysed *A. platensis* decreased hepatosomatic index (HSI), especially with the highest inclusion level. This reduction of HSI has been previously documented in several studies (Vizcaíno et al., 2016; Tulli et al., 2012), and seems to be related to the existence of a direct relationship with lipid metabolism. Thus, some microalgae compounds are apparently able to modulate to a certain extent lipid metabolism by increasing the efficiency of lipid mobilization from liver to muscle, especially in phases of rapid growth (Knutsen et al., 2019). However, the results obtained in this work indicate that the dietary inclusion of crude or hydrolysed *A. platensis* did not modify gilthead seabream fry body composition, in line with earlier reports on juveniles of the same (Galafat et al., 2020), and other farmed fish species (Sørensen et al., 2017; Valente et al., 2019). The influence of diets on fish body composition can also be

Table 6
Muscle fatty acid composition (% fatty acids) of gilthead seabream fry fed with the experimental diets for 40 days.

	CT	C-5	C-10	H-5	H-10	*P-value*
14:0	2.40 ± 0.07 b	2.39 ± 0.04 b	2.40 ± 0.07 b	2.33 ± 0.09 ab	2.14 ± 0.01 a	*0.029*
16:0	16.86 ± 0.12 b	17.25 ± 0.05 c	17.59 ± 0.26 c	16.27 ± 0.14 a	16.71 ± 0.01 b	*0.002*
18:0	4.99 ± 0.02	4.89 ± 0.14	4.90 ± 0.06	4.37 ± 0.02	4.57 ± 0.02	*0.594*
16:1n7	4.59 ± 0.08	4.75 ± 0.18	4.78 ± 0.11	4.89 ± 0.04	4.87 ± 0.02	*0.153*
18:1n9	17.82 ± 0.07	17.97 ± 0.16	18.24 ± 0.32	12.21 ± 0.12	18.16 ± 0.15	*0.254*
18:1n7	3.26 ± 0.03	3.29 ± 0.03	3.25 ± 0.07	3.26 ± 0.01	3.24 ± 0.01	*0.729*
18:2n6	10.81 ± 0.01 a	11.32 ± 0.17 b	11.70 ± 0.22 b	11.63 ± 0.06 b	11.72 ± 0.03 b	*0.003*
18:3n3	1.44 ± 0.01	1.50 ± 0.02	1.53 ± 0.06	1.52 ± 0.02	1.45 ± 0.02	*0.087*
20:1n9	5.10 ± 0.06	4.88 ± 0.07	4.81 ± 0.31	4.82 ± 0.06	4.57 ± 0.19	*0.180*
20:4n6, ARA	0.46 ± 0.01	0.39 ± 0.01	0.42 ± 0.03	0.36 ± 0.02	0.38 ± 0.05	*0.069*
20:5n3, EPA	5.13 ± 0.13 c	5.22 ± 0.06 c	4.99 ± 0.01 c	3.81 ± 0.03 b	3.44 ± 0.04 a	*<0.001*
22:5n3	1.72 ± 0.04 b	1.70 ± 0.04 ab	1.66 ± 0.01 ab	1.60 ± 0.00 a	1.62 ± 0.01 a	*0.020*
22:6n3, DHA	12.61 ± 0.34 ab	12.11 ± 0.39 a	11.94 ± 0.22 a	12.45 ± 0.12 ab	13.21 ± 0.01 b	*0.026*
SFA	24.25 ± 0.17	24.53 ± 0.15	24.90 ± 0.39	22.97 ± 0.25	23.41 ± 0.04	*0.489*
MUFA	30.77 ± 0.07	30.89 ± 0.44	31.08 ± 0.81	31.18 ± 0.16	30.85 ± 0.01	*0.845*
PUFA	33.35 ± 0.51	33.41 ± 0.43	33.42 ± 0.01	32.68 ± 0.06	33.07 ± 0.12	*0.605*
Other FA	9.94 ± 0.29 ab	9.42 ± 0.08 ab	8.64 ± 0.92 a	11.43 ± 0.35 b	10.83 ± 0.17 b	*0.010*
n3	20.90 ± 0.51 b	20.53 ± 0.46 ab	20.12 ± 0.18 ab	19.39 ± 0.17 a	19.71 ± 0.06 ab	*0.029*
n6	11.27 ± 0.00 a	11.71 ± 0.18 ab	12.11 ± 0.25 b	11.99 ± 0.07 b	12.11 ± 0.02 b	*0.007*
n9	22.92 ± 0.12	22.85 ± 0.23	23.04 ± 0.63	23.04 ± 0.18	22.73 ± 0.05	*0.519*
n3/n6	1.85 ± 0.05	1.75 ± 0.07	1.66 ± 0.05	1.62 ± 0.02	1.63 ± 0.01	*0.595*
EPA/DHA	0.41 ± 0.00 c	0.43 ± 0.01 d	0.42 ± 0.01 cd	0.31 ± 0.00 b	0.26 ± 0.00 a	*<0.001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of triplicate determination. Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

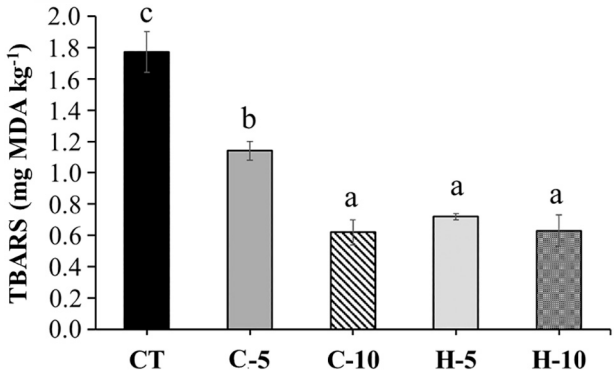

Fig. 2. Muscle thiobarbituric acid-reactive substances (TBARS) content of gilthead seabream fry after 40-day feeding trial (mean ± standard deviation, $n = 12$). Values with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

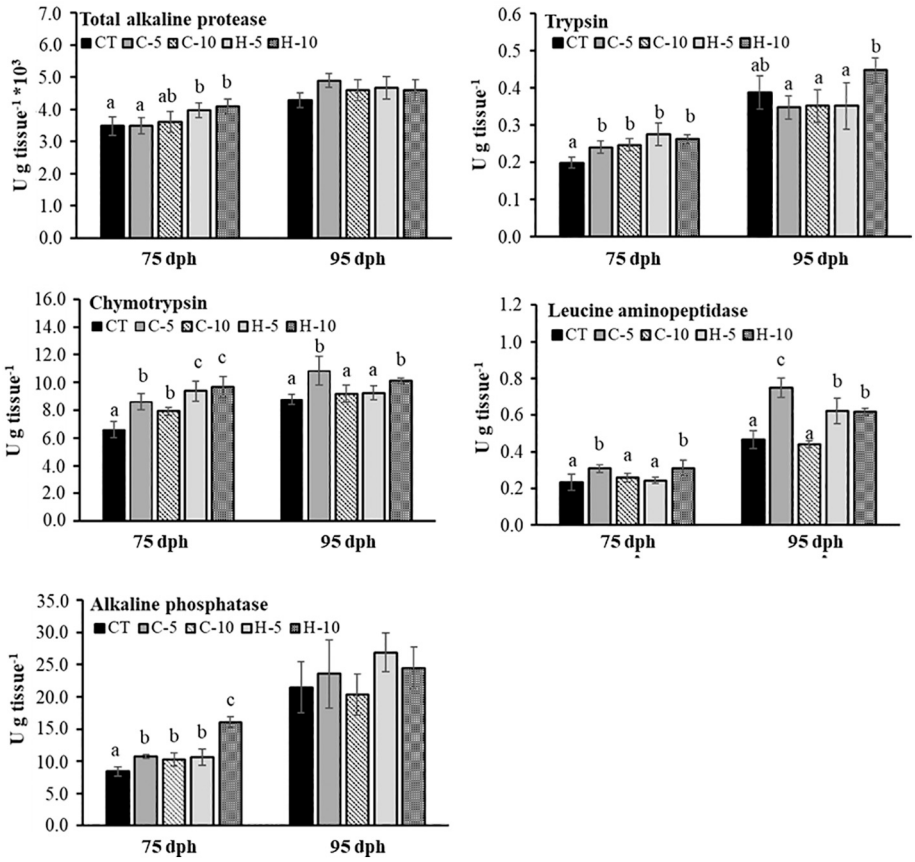

Fig. 3. Enzyme activities measured in intestinal extracts of *S. aurata* fry fed with the experimental diets for 40 days. Values are mean ± SD ($n = 9$, nine pools of intestines per dietary treatment). Values in the same day with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$). CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Table 7
Evaluation of different factors on digestive enzyme activities in the intestinal extracts of gilthead seabream at the end of the feeding trial.

	Age	Microalgae	Inclusion level	Hydrolysis treatment
Total alkaline protease	<0.001	0.038	0.847	0.224
Trypsin	<0.001	0.419	0.301	0.146
Chymotrypsin	<0.001	<0.001	0.606	0.048
Leucine aminopeptidase	<0.001	0.017	0.262	0.781
Alkaline phosphatase	<0.001	0.095	0.595	0.114

Data are mean ± SD ($n = 9$). Factors are: i) Age of fish larvae; ii) Microalgae (with or without microalgae inclusion), iii) Inclusion level (microalgae dietary inclusion level), and iv) Hydrolysis treatment (crude biomass *vs* hydrolysed biomass).

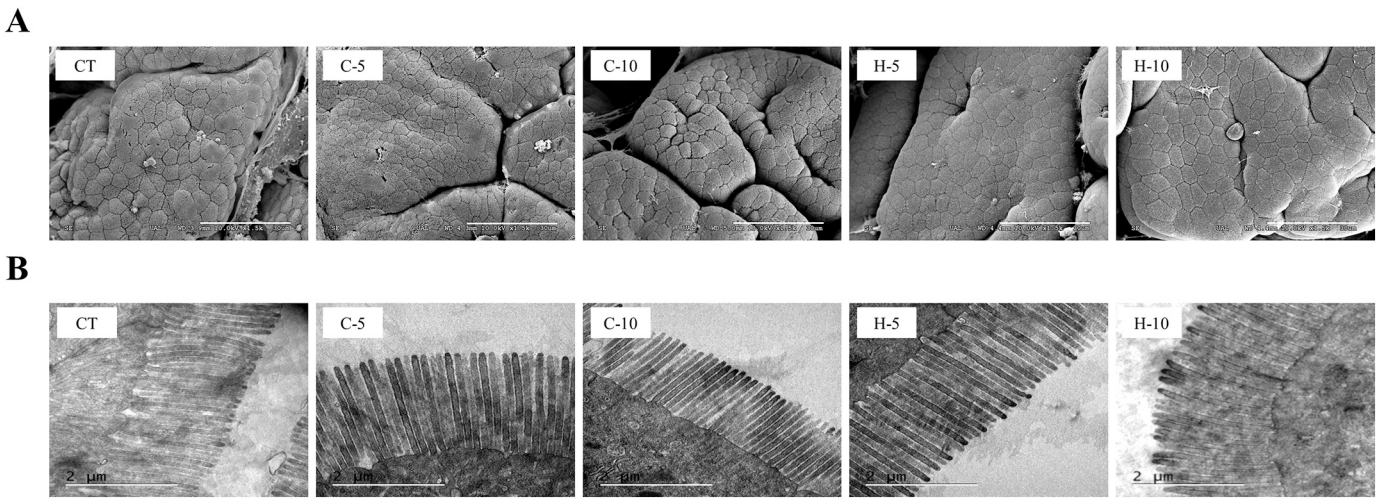

Fig. 4. Comparative SEM (A) and TEM (B) micrographs from the anterior intestinal regions of *S. aurata* fry at the end of the feeding trial. CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. (SEM bar: 30 µm; TEM bar: 2 µm).

Table 8
Microvillar morphology of the anterior intestine of gilthead seabream fry fed with the experimental diets for 40 days.

	ML (µm)	MD (µm)	EA (µm^2)	TAS (µm^2)
CT	1.84 ± 0.16 a	0.12 ± 0.01 a	27.04 ± 3.21 b	1012.01 ± 157.51 a
C-5	2.06 ± 0.13 b	0.14 ± 0.01 c	22.56 ± 3.70 a	1089.77 ± 194.24 a
C-10	1.88 ± 0.19 a	0.14 ± 0.01 c	22.65 ± 3.64 a	1021.84 ± 148.08 a
H-5	2.09 ± 0.13 b	0.15 ± 0.01 d	22.92 ± 3.39 a	1079.58 ± 158.57 a
H-10	2.89 ± 0.43 c	0.13 ± 0.01 b	29.84 ± 2.94 c	1930.06 ± 311.07 b
P-value	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. ML: microvilli length; MD: microvilli diameter; EA: enterocyte apical area; TAS: enterocyte absorption surface. Values are mean ± SD of 60 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

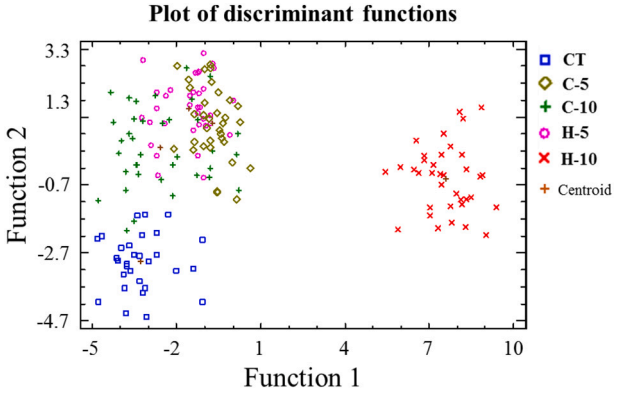

Fig. 5. Plot of the first two discriminant functions established by the discriminant analysis (DA) of digestive functionality analysis of *S. aurata* fry fed with the experimental diets.

Table 9
Summary of the results in the discriminant analysis (DA) of the different dietary treatments considering digestive enzyme activities and ultrastructural analysis of the intestinal mucosa as predictor variables.

Discriminant function	Eigenvalue	Relative percentage	Canonical correlation	Wilk's λ	χ^2	Degree of freedom	*P*-value
1	8.31057	81.45	0.94477	0.0358087	760.8052	16	<0.001
2	1.83474	17.98	0.80451	0.3334	250.9873	9	<0.001

Table 10
Antimicrobial activities in liver extracts of gilthead seabream fry fed with the experimental diets for 40 days.

	Antiprotease (% g tissue^{-1})	Protease (% μg tissue^{-1})	Peroxidase (U μg tissue^{-1})	Lysozyme (U μg tissue^{-1})	Bactericidal activity (% μg tissue^{-1})
CT	0.09 ± 0.04	0.02 ± 0.01	0.68 ± 0.06	0.05 ± 0.01 a	0.32 ± 0.12
C-5	0.09 ± 0.04	0.46 ± 0.24	0.86 ± 0.10	0.06 ± 0.01 a	0.48 ± 0.16
C-10	0.07 ± 0.02	0.03 ± 0.01	0.74 ± 0.07	0.05 ± 0.01 a	0.42 ± 0.09
H-5	0.08 ± 0.01	0.01 ± 0.00	0.67 ± 0.07	0.04 ± 0.00 a	0.37 ± 0.07
H-10	0.07 ± 0.03	0.34 ± 0.18	0.84 ± 0.11	0.16 ± 0.06 b	0.72 ± 0.21
P-value	*0.985*	*0.198*	*0.433*	*0.019*	*0.156*

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± SD of 9 measures obtained from three specimens per replicate tank. Values in the same column with different lowercase letter indicate significant differences among dietary treatments ($P < 0.05$).

assessed by means of the muscle fatty acid profile (Fernandes et al., 2012). According to previous studies (Tibaldi et al., 2015; Cardinaletti et al., 2018; He et al., 2018), certain degree selective retention of specific fatty acids in the muscle of fish fed with microalgae-supplemented diets has been observed, especially EPA and DHA. Thus, muscle EPA contents were lower in fish fed on microalgae hydrolysate diets (H-5 and H-10) than those measured in the respective experimental diets, a fact that seems to be related to the modulating effect on lipid metabolism previously mentioned and with a higher catabolic use of this specific fatty acid (Tocher, 2003). Both EPA and DHA play a key role in fish development, and their dietary deficiency is related to adverse effects on fish growth and survival (Glencross, 2009; Tocher, 2010). In this work, EPA/DHA ratios in all the experimental diets ranged from 0.66 to 0.69, similar to the optimum (0.67) for gilthead seabream (Rodriguez et al., 1997), although this factor did not affect growth performance and nutrient utilization at the end of the feeding trial, as it has been observed in previous works (Vizcaíno et al., 2016; Atalah et al., 2007).

On the other hand, a significant reduction in MDA levels was observed in muscle of fry fed the microalgae-supplemented diets. This phenomenon might well be directly related to enhanced scavenging activity against reactive oxygen species, resulting in lower muscle lipid oxidation (Teimouri et al., 2019). Microalgae contain a wide range of bioactive compounds like astaxanthin and xanthophylls able to prevent oxidative stress, as previously observed in fish fed on diets supplemented not only with *Arthrospira* sp. (Abdelkhalek et al., 2015; Teimouri et al., 2019; Galafat et al., 2020), but also with other microalgae species (Qiao et al., 2019; Batista et al., 2020). It is worth mentioning that low dietary inclusion of *Arthrospira* hydrolysate (H-5) caused a notable reduction in muscle MDA level; in fact, similar to that observed in fish fed with 10% crude microalgal biomass. This could be explained by the fact that enzymatic hydrolysis might have facilitated the release of antioxidant compounds that otherwise are less available within the intact cells (Liu et al., 2019; Afify et al., 2018).

In addition to the effects on growth and fish chemical composition, the nutritional value of first-feeding diets, combined with other biotic and abiotic factors, play a key role on the ontogeny of the digestive tract in marine fish larvae (Yúfera et al., 2000; Zambonino-Infante and Cahu, 2001). Indeed, imbalanced diets can lead to a disruption in maturation processes, such as a reduction in pancreatic secretory capacity (Zambonino-Infante et al., 2008). The dietary inclusion of microalgae and cyanobacteria has been previously evaluated in different farmed fish species, and roughly, no detrimental effects have been described on proteolytic enzyme activity (Vizcaíno et al., 2016; Gong et al., 2019; Galafat et al., 2020). In this work, partial replacement of fish meal with crude and hydrolysed *A. platensis* did not impair the pancreatic secretion of proteolytic enzymes, quite the contrary, given that fish fed on microalgae-supplemented diets showed higher trypsin and chymotrypsin activity levels than fish fed a fishmeal:fish oil (FM:FO) diet, especially in the case of fish fed with the microalgae hydrolysate. Both enzymes play a key role in early life stages, since digestion relies mainly on these pancreatic enzymes until a gastric digestion was attained (Hamre et al., 2013; Khoa et al., 2019). These enzymes are modulated by dietary protein content, so that the increase in activity observed could be related to an increase in the availability of peptides and amino acids, which may stimulate the pancreatic secretion (Zambonino-Infante et al., 2008; Hamre et al., 2013). Indeed, Gisbert et al. (2018) evidenced differences in chymotrypsin activity in fish related to differences in the distribution of peptide molecular weight among hydrolysates obtained from fish, yeast and pig blood. Regarding brush border enzymes, our results revealed a lack of negative effects on leucine aminopeptidase and alkaline phosphatase activities in fish fed on *A. platensis* supplemented feeds. Fish from these experimental groups, especially H-5 and H-10, showed significantly increased activities for both enzymes, which seems to be related to adequate maturation of the intestine (Zambonino-Infante and Cahu, 2001).

Despite this, the improvement in fish digestive enzyme activities did not translate into increased growth performance. This finding might be explained by two different hypotheses; on the one hand, the increase in enzyme activity levels could be associated with a compensation mechanism against dietary changes (Santigosa et al., 2008); and on the other, it could be related to a saturation of the peptide transport system in the intestinal brush-border membrane. In fact, the use of protein hydrolysates may induce an overload of short peptides, and/or impaired utilization of the small peptides and free amino acids, which are used for energy production rather than for growth (Yúfera et al., 2018).

In addition to the effects on the digestive enzyme activities, inadequate dietary and environmental conditions may interfere with the complex regulation mechanisms involved in epithelial development, disrupting epithelial integrity (Rønnestad et al., 2013). According to previous studies, the dietary inclusion of different species of microalgae exerts positive effects on the apical brush border integrity, such as increased microvilli length, enterocyte apical area, and increased absorption surface in enterocytes (Galafat et al., 2020; Vizcaíno et al., 2016; Araújo et al., 2016). In line with these studies, the results obtained revealed that the inclusion of *A. platensis* biomass caused favourable effects on gut morphology of gilthead seabream fry, especially in the case of the hydrolysed microalgae. Hence, changes observed in microvilli length and microvilli diameter might improve the contribution of the intestinal mucosa as a natural barrier, whereas increased total absorption surface observed in fish fed on 10% hydrolysed biomass might enhance the absorptive capacity of the intestinal mucosa (Vizcaíno et al., 2014).

Regarding the innate immune system, it has been reported that the dietary inclusion of crude microalgae could modulate slightly protease activity, peroxidase and antibacterial activities, but not lysozyme activity (Chaves-Pozo et al., 2014), while stimulated cellular immune activities were reported in some cases (Cerezuela et al., 2012). In this study, no detrimental effects on the immune status of gilthead seabream larvae could be attributed to *A. platensis*-supplemented diets, but on the contrary, fish fed on 10% hydrolysed microalgae showed increased lysozyme activity levels, this fact suggesting better defence against infectious diseases. However, the discrepancies observed among different studies assessing the impact of microalgae on the activity of the innate immune system suggest both a species-specific and dose-specific effect on the results reported.

In conclusion, the results obtained in this study indicate that

A. platensis hydrolysate can be used as dietary ingredient in starter feeds for gilthead seabream fry. The use of enzymatically hydrolysed microalgae up to 10% inclusion level did not improve body growth parameters, but yielded positive effects on gut functionality, muscle lipid peroxidation, and lysozyme activity in liver, which seems to be associated with an increase in the nutritional and functional properties of this biomass compared with the raw microalgae. These positive effects confirm that this supplement can be included as a functional ingredient in starter feeds for gilthead seabream.

Authors' contributions

Alarcón, F.J. and Martínez T.F. conceived and designed the experiments and prepared the aquafeeds. Galafat, A., Vizcaíno, A.J., and Sáez, M.I., performed the fish sampling. Arizcun, M. and Chaves-Pozo, E., participated in sampling, fish maintenance and immune activities analysis. Galafat, A., Vizcaíno, A.J., Sáez, M.I., Martínez, T.F., Arizcun M., and Chaves-Pozo E., performed analytical analysis and discussed the data. Galafat, A., Vizcaíno, A.J., and Alarcón, F.J., performed the data analysis and drafted the manuscript. All authors critically revised and approved the manuscript.

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Statement of informed consent, human/animal rights

The authors state that no conflicts, informed consent, human or animal rights are applicable. All studies involving fish were conducted in accordance with the requirements of the Directive 2010/63/EU, and the Spanish legislation (Real Decreto 53/2013), regarding the ethical rules applicable in research involving laboratory animals. Thereby, all the procedures were authorized by the Bioethics and Animal Welfare Committee of the Instituto Español de Oceanografía (REGA code ES300261040017).

Author agreement statement

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Evaluation of *Arthrospira* sp. enzyme hydrolysate as dietary additive in gilthead seabream (*Sparus aurata*) juveniles

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Abstract

The aim of this work was to evaluate the effects of the dietary inclusion of *Arthrospira* sp. enzyme hydrolysate on gilthead seabream (*Sparus aurata)* juveniles in a 128-day feeding trial. Algal hydrolysate was tested at low inclusion level, namely, 2 and 4%, against a control diet without *Arthrospira* sp. supplementation. At the end of the feeding trial, fish body weight was recorded for growth evaluation. No significant differences were found among the experimental groups regarding growth performance or nutrient utilization, despite the fact that those animals fed with diets enriched with *Arthrospira* displayed higher trypsin, chymotrypsin, and leucine aminopeptidase enzyme activities, compared to fish fed with control diet. The ultrastructural study of the intestinal mucosa also revealed increased microvilli length and absorptive capacity in fish fed with *Arthrospira* sp. diets, especially at 4% inclusion level. *Arthrospira* supplementation was also responsible for lower lipid oxidation in muscle tissue, as well as for remarkable colour differences in skin, compared to control animals.

These results suggest, overall, that low dietary supplementation with this microalgal hydrolysate might improve not only the intestinal ultrastructure and functionality but also muscle pigmentation and antioxidant capacity of juvenile gilthead seabream.

Keywords Growth performance · Intestine ultrastructure · Functional additive · Microalgae hydrolysate

Introduction

The interest in microalgae has increased strongly in the last years, given that they have valuable potential for reducing the dependence on unsustainable conventional raw ingredients in aquafeeds (Shah et al. 2018). The use of microalgae in aquaculture can be approached from two perspectives: on one hand, taking into account their nutritive value as protein and lipid sources, and on the other, considering that microalgae also have plenty of substances with potential bioactive effects. Abundant literature on the first consideration is available, but it is likely that the main constraint for extensive utilization of microalgae consists of the fact that any satisfactory alternative feed ingredient must be able to supply comparable nutritional value at competitive cost. To date, this is far from being achieved, given that any large-scale practical utilization of microalgae relies on a significant reduction in production costs. With regard to the second aspect mentioned above, growing interest is currently being paid to the fact that microalgae can accumulate useful metabolites, normally at relatively low concentration, with potentially bioactive effects. Thereby, the interest in microalgae as potential nutraceutical additive in aquafeeds is increasing considerably (Chakraborty and Hancz 2011; Cardinaletti et al. 2018; Shah et al. 2018).

Numerous studies have reported that microalgae can be used as dietary ingredient or additive in aquafeeds without exerting negative impacts on fish growth and nutrient utilization (De Cruz et al. 2018; Perez-Velazquez et al. 2018) and even yielding valuable effects on lipid metabolism (Robin and Vincent 2003), fish gut functionality (Vizcaíno et al. 2016, 2018), and oxidative status and lipid utilization in different fish species (Kiron 2012; Teimouri et al. 2013; Roy and Pal 2015; Amer 2016). In addition, positive effects have been reported in rainbow trout

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(*Oncorhynchus mykiss*) related to pigmentation attributes (Teimouri et al. 2013) and lipid peroxidation (Teimouri et al. 2016, 2019).

The genus *Arthrospira* (filamentous Cyanobacteria) is known for its high protein content, up to 70% on dry matter basis (Santigosa et al. 2011; Macias-Sancho et al. 2014; Ansarifard et al. 2018), with amino acid profiles comparable to those found in some reference feed proteins (Becker 2007). *Arthrospira* is also rich in polyunsaturated fatty acids (PUFAs), mainly gamma-linolenic acid (18:3n6) (Ronda et al. 2012), as well as in vitamins (A and B_{12}), minerals, and pigments with acknowledged antioxidant activity, such as carotenoids (Pugh et al. 2001; El-Sheekh et al. 2014; Adel et al. 2016; Velasquez et al. 2016), phycobilins, and phycocyanins (Mahmoud et al. 2018; Takyar et al. 2019). Thus, the dietary inclusion of *Arthrospira* has been evaluated in different fish species (Hussein et al. 2013; Kim et al. 2013; Teimouri et al. 2013; Velasquez et al. 2016; De Cruz et al. 2018; Perez-Velazquez et al. 2018), these studies reporting, overall, a lack of negative effects on growth performance or nutrient utilization but even favourable impacts on fish physiology. However, the potential effects of *Arthrospira* on fish growth and objective quality parameters of Mediterranean fish species remain virtually unknown.

However, microalgae also display certain disadvantages from a nutritional point of view, such as the structure and composition of their cell wall, which is a protective barrier that reduces the bioavailability of the intracellular nutrients (Wu et al. 2017). The efficiency of marine animals to digest the cell walls depends on the carbohydrate composition, on how they are linked to each other, and on the existence of suitable digestive enzymes. Overall, herbivorous and omnivorous species possess a wide range of carbohydrases, but carnivorous fish do not, and this fact should be taken into consideration when it comes to formulating aquafeeds. Consequently, it may be reasonable to think that any strategy aimed at improving the bioavailability of the inner compounds, not only of *Arthrospira* but of any other species, might enable to include microalgae at low inclusion level in aquafeeds. Several procedures have been evaluated with the aim of releasing inner components of microalgae (Tibbetts et al. 2017; Agboola et al. 2019; Teuling et al. 2019), but when it comes to large-scale cell lysis, enzymatic hydrolysis is one of the most promising strategies, not least owing to its economic viability. By following this procedure, even low inclusion level of enzyme-hydrolysed microalgae in aquafeeds might well improve the physiological aspects in fish in a manner similar to including higher amounts of raw microalgae (Tchorbanov and Bozhkova 1988). To our knowledge, despite the potential of this procedure to increase nutrient bioavailability and functional properties, the use of microalgae enzymatic hydrolysates in aquafeeds remains unexplored. Thus, the production of microalgal hydrolysates is a promising strategy that deserves further research efforts.

Protein hydrolysates are believed to be more effective than intact protein or free amino acids from a nutritional point of view. The enzymatic hydrolysis of proteins results in the formation of a mixture of free amino acids, di-, tri-, and oligopeptides, and enhances the occurrence of polar groups and the solubility of hydrolysate compounds. The dietary use of protein hydrolysates of different origins in some species of farmed fish has proved several positive bioactive effects, such as antioxidant, antimicrobial, or anti-inflammatory, and beneficial effects on the functionality of the intestinal mucosa (Leduc et al. 2018; Zamora-Sillero et al. 2018). In the case of algae protein, enzymatic hydrolysis could release low molecular weight bioactive peptides and free amino acids, which might enable not only increased bioavailability but also lead to potential positive physiological effects (Morris et al. 2007; Chalamaiah et al. 2012; Montone et al. 2018; Wang et al. 2018).

In this piece of research, we hypothesise that *Arthrospira* sp. enzyme hydrolysate might improve some parameters related to growth performance, muscle lipid oxidation, skin pigmentation, and digestive functionality of juvenile gilthead seabream when added a low dietary inclusion level. The overall objective of this study is focused specifically on the assessment of the potential effects of low level of microalgae protein hydrolysate as functional additive in practical diets for juvenile fish of this Mediterranean species.

Materials and methods

Arthrospira sp. hydrolysate

Arthrospira sp. hydrolysate was produced starting from a sludge containing up to 150 g L^{-1} of microalgae biomass after performing an enzymatic hydrolysis with a mixture of commercial proteases under controlled conditions (pH 8.0 and 50 °C under continuous stirring) for 4 h providing 0.2% w/w proteases (Alcalase 2.4 L and Flavourzyme 1000 L from Novozymes A/S, Denmark), following a modification of the method described by Saadaoui et al. (2019). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson Unit (AU) per gramme. One AU is the amount of enzyme, which, under standard conditions, digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one mequivalent of tyrosine released per minute. Flavourzyme 1000 L is a protease complex of *Aspergillus oryzae* that contains both endo- and exoprotease activities. It has an activity of 1.0 leucine aminopeptidase (LAPU) unit g^{-1}. One LAPU is the amount of enzyme that hydrolyses 1 μmol of leucine-p-

nitroanilide per minute. Immediately after the hydrolysis, the reaction mixture was heated at 80 °C for 15 min in order to inactivate the proteolytic enzymes. Total free amino acids were quantified spectrophotometrically at 340 nm using L-leucine as standard (Church et al. 1983). In brief, triplicate samples of 50 μL were withdrawn from microalgal protein hydrolysate, and 50 μL of 20% trichloroacetic acid (TCA) were added with the purpose of stopping the enzyme reaction. Afterwards, protein precipitates were discarded by centrifugation (12,000 rpm, 15 min at 4 °C), and the supernatants were stored at − 20 °C until further analysis. Finally, SDS-PAGE (Laemmli 1970) for crude *Arthrospira* sp. meal and its protein hydrolysate was performed in order to identify the protein fractions and their molecular weight.

Experimental diets

Three isonitrogenous (450 g crude protein kg^{-1}) and isolipidic (170 g crude lipid kg^{-1}) experimental feeds were formulated, control without microalgae (CT), plus AH-2 and AH-4, containing 2% and 4% (DM basis) *Arthrospira* sp. hydrolysate, respectively. The formulation and chemical composition of the experimental diets are shown in Table 1. Before adding fish oil and diluted choline chloride, feed ingredients were finely ground and mixed in a vertical helix mixer (Sammic 13 M-11, 5-L capacity, Sammic SA, Spain) for 20 min. Then the algae hydrolysate was added, and water content was adjusted to provide 400 mL per kg of ingredient mixture to obtain a homogenous dough. The dough was passed through a single screw laboratory extruder (Miltenz 51SP, JS Conwell Ltd., New Zealand) in order to obtain 2- and 3-mm pellets. The feeds were dried in a 12-m^3 drying chamber with forced-air circulation (Airfrio, Spain) at 30 °C for 24 h and stored at − 20 °C until use. An attractant premix was added (50 g kg^{-1}) to improve feed palatability (according to Barroso et al. 2013). The experimental diets were produced by LifeBioencapsulation SL (Spin-off, Universidad de Almería, Spain).

Feeding trial and sampling

Feeding trial was carried out at the *Servicios Centrales de Investigación en Cultivos Marinos* (SCI-CM, CASEM, Universidad de Cádiz, Puerto Real, Spain). All experimental procedures complied with the Guidelines of the European Union (Directive 2010/63/UE) regarding the use of laboratory animals. The competent Ethical Committee approved the experimental procedures involving the use of fish (Junta de Andalucía, reference number 06/02/2020/011). A total of 180 gilthead seabream juveniles (20 g average body weight) were selected and randomly distributed in 9 tanks (triplicate tanks per dietary treatment) of 75-L capacity (400 g average biomass per tank). Fish were fed with a commercial diet (Skretting España, 45% crude protein, 19% crude lipid) during a 15-day acclimation period. Afterwards, experimental diets were offered twice per day (9:00 and 17:00) at 2% of the biomass, until triplication of the initial body weight. The 128-day feeding trial was carried out in a flow-through filtered (1 μm) seawater system sterilized by UV, under constant temperature (19.0 ± 1.1 °C), salinity (35 ± 1 ‰), and natural

Table 1 Ingredient composition and proximate composition (g kg^{-1} on dry matter basis) of the experimental diets

	CT	AH-2	AH-4
Ingredients			
Fishmeal[1]	374.2	358.4	340.6
Arthrospira sp. hydrolysate (g dry matter)[2]		20	40
Attractant premix[3]	50	50	50
Wheat gluten[4]	95	95	95
Soybean meal[5]	165	165	165
Fish oil	72.8	73.9	74,9
Soybean oil	28	28	28
Wheat flour[6]	170	164.7	161.5
Betaine	5	5	5
Vitamins and minerals premix[7]	20	20	20
Binder (guar gum)[8]	20	20	20
Proximate composition			
Crude protein	449.9	450.3	449.5
Crude lipid	169.7	170.1	170.3
Ash	70.8	69.2	67.2
Crude fibre	34.7	34.6	34.5
NfE[9]	274.8	275.9	278.5

CT control diet, *AH-2* 2% *Arthrospira* hydrolysate-supplemented diet, *AH-4* 4% *Arthrospira* hydrolysate-supplemented diet

[1] Protein, 69.4%; lipid, 12.3%; Norsildemel (Bergen, Norway)

[2] Liquid product containing 150 g microalgae meal L^{-1}

[3] 50% squid meal, 25% shrimp meal, 25% krill meal

[4] Protein, 76.0%; lipid, 1.9%

[5] Protein, 50.0%; lipid, 1.0%

[6] Protein, 12.0%; lipid, 2.0%

[7] Vitamin and mineral premix: vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K_3 (menadione sodium bisulphite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B_2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (LifeBioencapsulation premix)

[8] EPSA (Sevilla, Spain)

[9] *NfE* nitrogen-free extract calculated as 100 − (% crude protein + % ether extract + % ash + % crude fibre)

photoperiod (light:dark, LD; from 11:13 h in February to 13:11 h in May). The water in all the tanks was oxygen-saturated (> 90% O_2 saturation) with air stones. Water ammonia (< 0.1 mg L^{-1}), nitrite (< 0.2 mg L^{-1}), and nitrate (< 50 mg L^{-1}) were determined with commercial kits (SERA GmbH, Heinsberg, Germany).

Fish were individually weighed every 2 weeks after a 24-h fasting period in order to determine growth and feed utilization parameters. At the end of the feeding trial, 16 fish per tank were killed by anaesthetic overdose (200 mg L^{-1} clove oil) followed by spine severing. Sampled fish were dissected, and the digestive tract and dorsal muscle were removed. Dorsal muscle samples were carefully washed, dried, and packaged in transparent sterile polyethylene bags and stored in a cold room (4 ± 1 °C). Muscle samples were withdrawn from each dietary treatment at day 1 and 8 of cold storage. Colour parameters were determined on the right side of the anterior dorsal skin of fish, and then a portion of muscle tissue (5 g) was used for lipid oxidation determination (TBARS). The rest of individual muscle samples were freeze-dried and stored at − 20 °C for further analysis of muscle proximate composition. For digestive enzyme activity determinations, intestines from nine fish per tank were randomly pooled to obtain three enzymatic extracts from each experimental tank. Intestine samples were homogenized in distilled water at 4 °C (0.5 g mL^{-1}). Supernatants were obtained after centrifugation (11,200 × *g*, 12 min, 4 °C) and stored in aliquots at − 20 °C until further use. Total soluble protein was determined using bovine serum albumin as standard (according to Bradford 1976). Finally, the intestines of three specimens from each tank were collected for examination by transmission (TEM) and scanning (SEM) electron microscopy.

Growth performance, nutrient utilization, and somatic indices

Growth performance was assessed by different parameters according to the following formulae: daily gain (DG, g day^{-1}) = ($W_f - W_i$) / days; specific growth rate (SGR, %) = (Ln W_f − Ln W_i) / days × 100; condition factor (K) = (W_f / SL^3) × 100, where W_f was the final weight (g), W_i was the initial weight (g), and *SL* was the standard length.

Nutrient utilization indices were estimated as follows: feed conversion ratio (FCR) = total feed intake on dry basis (g) / weight gain (g) and protein efficiency ratio (PER) = WG / total protein ingested (g), where *WG* was the weight gain (g).

Proximate analysis

Proximate analysis of feeds and muscle samples were carried according to AOAC (2000) for dry matter and ash, whereas crude protein (N × 6.25) was determined by using elemental analysis (C:H:N) (Fisons EA 1108 analyzer, Fisons Instruments, USA). Total lipid content was analysed following the procedure described by Folch (1957).

Skin colour determinations

Instrumental colour was measured in triplicate on the right side of the dorsal fish skin by L*, a*, and b* system (CIE 1978), using a Minolta chroma meter CR-400 (Minolta, Japan). The lightness (L*, on a 0–100 point scale from black to white), redness (a*, estimates the position between red, positive values, and green, negative values), and yellowness (b*, estimates the position between yellow, positive values, and blue, negative values) were recorded.

Muscle lipid oxidation

Lipid oxidation in muscle samples was estimated by thiobarbituric acid-reactive substances (TBARS) at 1 and 8 dpm (days *post-mortem*). TBARS were measured in muscle samples according to the method of Buege and Aust (1978). Samples (1 g) were homogenized in 4 mL 50 mM NaH_2PO4, 0.1% (v/v) Triton X-100 solution. The mixture was centrifuged (10,000 × *g*, 20 min, 4 °C). Supernatants were mixed in a 1:5 ratio (v/v) with 2-tiobarbituric acid (TBA) reagent (0.375% w/*v* TBA, 15% w/v TCA, 0.01% w/v 2,6-di-tert-butyl-4-methylphenol (BHT) and 0.25 N HCl). The mixture was heated for 15 min and centrifuged (3600 × *g*, 10 min, 4 °C), and the absorbance of supernatants was measured at 535 nm. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg of muscle after comparing with a MDA standard.

Digestive enzyme activities

Total alkaline protease activity in the digestive extracts was spectrophotometrically determined using 5 g L^{-1} casein in 50 mM Tris-HCl (pH 9.0) as substrate, according to Alarcón et al. (1998). One unit of total protease activity was defined as the amount of enzyme that released 1 μg of tyrosine per minute in the reaction mixture, considering an extinction coefficient for tyrosine of 0.008 μg^{-1} mL^{-1} cm^{-1}, measured at 280 nm. Trypsin and chymotrypsin activities were determined by using 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4-p-nitroanilide) as substrate, according to Erlanger et al. (1961), and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-p-nitroanilide) according to DelMar et al. (1979); both substrates dissolved in 50 mM Tris-HCl and 10 mM $CaCl_2$ buffer (pH 8.5). Leucine aminopeptidase activity was quantified according to Pfleiderer (1970), using 2 mM L-leucine-p-nitroanilide (LpNa) in 100 mM Tris-HCl buffer, pH 8.8, as substrate. For trypsin, chymotrypsin, and leucine aminopeptidase activities, one unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol of p-nitroanilide

(pNA) per minute, measured spectrophotometrically at 405 nm, considering an extinction coefficient of 8800 M cm^{-1}. Alkaline phosphatase activity was determined by using p-nitrophenyl phosphate in 1 M diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5, as substrate, following the method described in Bergmeyer (1974). For alkaline phosphatase, one unit of enzyme activity was defined as the amount of enzyme that releases 1 μg of nitrophenyl per min, considering a molar extinction coefficient of 17,800 M cm^{-1} for p-nitrophenol measured at 405 nm.

Ultrastructural study of intestinal mucosa

Intestine samples were collected for electron microscopy evaluation. Samples for scanning electron microscopy (SEM) were previously washed with 1% S-carboxymethyl-L-cysteine (Sigma Chem.) for 20 s, with the aim of removing the epithelial mucus. Afterwards, the samples were fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.2) for 24 h, after which samples were washed and dehydrated in graded ethanol. Then samples were critical point dried with absolute ethanol as intermediate fluid and CO_2 as transition fluid (CDP 030 Critical point dryer, Leica Microsystems, Spain). After drying, specimens were mounted on supports and fixed with graphite (PELCO Colloidal Graphite, Ted Pella INC., USA) and then gold sputter coated (SCD 005 Sputter Coater, Leica Microsystems). Finally, all samples were screened with a scanning electron microscopy (HITACHI model S-3500, Hitachi High-Technologies Corporation, Japan). Samples for transmission electron microscopy (TEM) were fixed (4 h, 4 °C) in 25 g L^{-1} glutaraldehyde and 40 g L^{-1} formaldehyde in phosphate buffer saline (PBS), pH 7.5. Next, intestine sections were washed with PBS for 20 min, and then, a post-fixation step with 20 g L^{-1} osmium tetroxide was carried out. Then, samples were dehydrated by consecutive immersion (20 min each) in ethanol solution of gradients ranging from 50 to 100% (v/v). Next, samples were embedded for 2 h, in 1:1 mixture of Epon resin and 100% (v/v) ethanol under continuous shaking, and then they were included in pure Epon resin and polymerized at 60 °C. Finally, the ultrathin sections were placed on a 700 Å copper mesh and stained with uranyl acetate and lead citrate. The mesh observation was performed with a Zeiss 10C TEM at 100 Kv (Carl Zeiss, Spain). Visualization fields were recorded at ×16,000 magnification. SEM and TEM visualization fields were recorded, and digital images were analysed using UTHSCSA ImageTool software (University of Texas Health Science Center, San Antonio, TX, USA). Microvilli length (ML) and microvilli diameter (MD) and the number of microvilli within 1 μm distance (Vizcaíno et al. 2014) were determined in TEM micrographs. SEM images were used to obtain several measurements of enterocyte apical area (EA). Finally, data obtained from TEM and SEM images were used to estimate the total absorption surface per enterocyte (TAS) according to Vizcaíno et al. (2014).

Statistical analysis

All assays were repeated at least three times with three replicates. Data were expressed as mean ± SE. Comparison of means was carried out by one-way ANOVA with a 5% level of probability ($p < 0.05$) followed by a multiple comparison test. Data in percentage (%) were arcsine (×1/2)-transformed, checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). When the data did not meet the ANOVA assumptions, a Kruskal-Wallis one-way analysis of variance on ranks was used. When the Kruskal-Wallis test showed significance, and Dunn's method of multiple comparisons was used to compare individual medians. All statistical analyses were performed with Statgraphics Plus 4.0 (USA) software.

Results

Characterization of the protein hydrolysate of *Arthrospira* sp.

Figure 1 shows the proteinograms of raw *Arthrospira* sp. meal and its protein hydrolysate. Raw meal showed a complex protein profile made up of several fractions with a wide range from 13 to 86 kDa. However, microalgal protein hydrolysate shows only two protein fractions (57 and 39 kDa) in the range of molecular weight visualized in the proteinogram. In addition, quantification of total free amino acids revealed that *Arthrospira* sp. hydrolysate contained higher level of free amino acids (84.06 ± 3.23 mg leucine equivalents (100 mg)$^{-1}$ protein) than those found in the raw microalgae biomass (31.5 ± 3.09 mg leucine equivalents (100 mg)$^{-1}$ protein).

Growth performance and nutrient utilization

Growth of gilthead seabream juveniles fed experimental diets for 128 days is shown in Fig. 2.

All dietary groups showed similar final body weight, DG, and SGR, without significant differences ($p > 0.05$), although mean values were slightly lower in AH-2 and AH-4 groups. Similarly, no significant differences were observed in FCR and PER mean values (Table 2).

Muscle proximate composition

Muscle chemical composition is shown in Table 3. Protein content increased significantly in fish fed with diets supplemented with *Arthrospira* hydrolysate, especially in AH-2

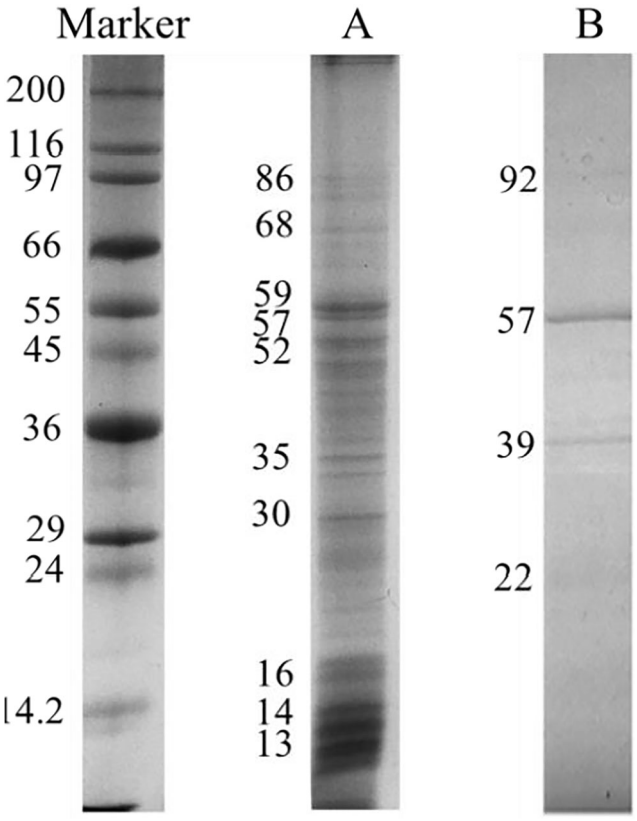

Fig. 1 SDS-PAGE of the raw *Arthrospira* sp. biomass (**a**) and its protein hydrolysate (**b**). Figures at the left of each proteinogram indicate the molecular mass (kDa) of the main protein fractions separated. Marker: 5 μL of wide range molecular weight marker (S-84445, Sigma, St. Louis, USA) ranging from 6.5 (aprotinin, bovine lung) to 200 kDa (myosin, porcine heart)

group, whereas a significant decrease in the lipid content was observed in fish fed with AH-2 and AH-4 diets. Moisture and ash content were similar among dietary treatments.

Instrumental skin colour determinations

Initial L* values were significantly higher in AH-2-fed fish, compared to CT and AH-4 groups (Table 4). After 8 days of cold storage, values remained stable in AH-2 and AH-4, whereas in CT significantly decreased ($p < 0.001$). CT presented a* negative values indicating a skin greenish coloration. However, values for AH-2 and AH-4 were positive which evidenced a slightly red coloration, though they decreased significantly at 8 dpm ($p = 0.040$ and < 0.001). Skin b* values in CT were positive and sharply decreased during storage under refrigeration. Nevertheless, values of AH-2 and AH-4 significantly increased, indicating a yellowish colour of the skin.

Muscle lipid oxidation (TBARS)

Muscle TBARS content in CT group showed significantly higher values (Table 4). Muscle lipid oxidation increased during cold storage ($p = 0.015$ and $p = 0.019$), although TBARS values were significantly lower at any sampling time in specimens fed with *Arthrospira* hydrolysate compared to CT fish ($p = 0.019$ and $p = 0.015$).

Digestive enzyme activities

Trypsin, chymotrypsin, and leucine aminopeptidase activities significantly increased in fish fed with *Arthrospira* hydrolysate-supplemented diets ($p = 0.001$, $p = 0.001$, and $p < 0.001$, respectively) compared to control fish (Table 5). Fish fed with AH-4 showed the highest enzyme activity levels. Total alkaline protease and alkaline phosphatase activities did not differ among experimental groups ($p = 0.160$ and $p = 0.844$).

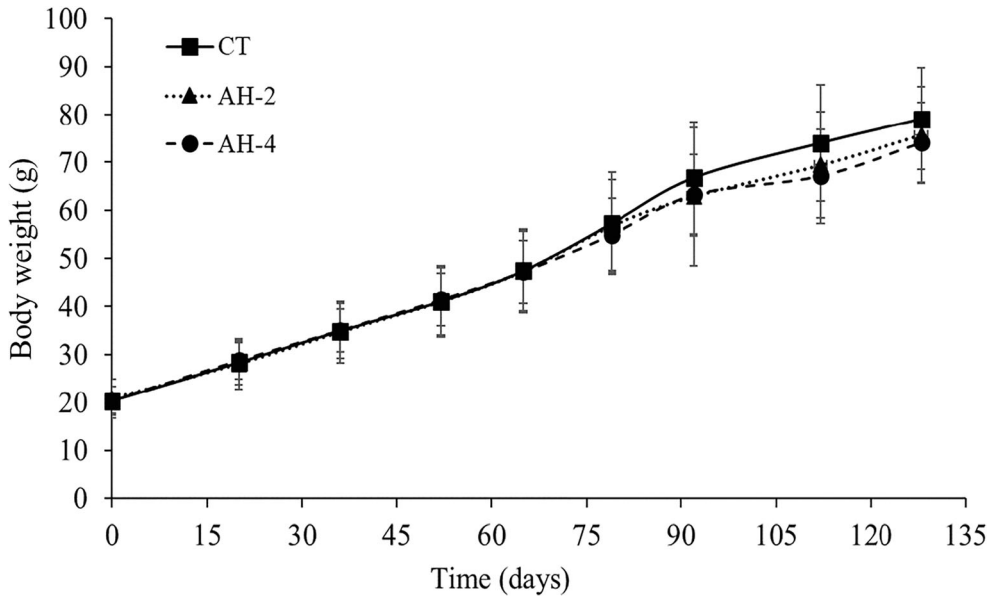

Fig. 2 Time course of changes in body weight of fish fed with the experimental diets. *CT* control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate

Table 2 Growth performance and nutrient utilization parameters of gilthead seabream juveniles fed with the experimental diets during the 128-day feeding trial

	CT	AH-2	AH-4	*p*
Growth and nutrient utilization				
Initial body weight (g)	20.32 ± 0.48	20.80 ± 0.52	20.51 ± 0.36	*0.7701*
Final body weight (g)	79.09 ± 1.75	75.80 ± 1.38	74.06 ± 1.15	*0.0529*
Fulton's condition factor	1.80 ± 0.03	1.81 ± 0.05	1.77 ± 0.04	*0.9404*
Daily gain (DG, g day−1)	0.46 ± 0.06	0.43 ± 0.01	0.42 ± 0.02	*0.5803*
Specific growth rate (SGR)	1.05 ± 0.13	0.99 ± 0.04	0.98 ± 0.05	*0.8948*
Feed conversion ratio (FCR)	0.47 ± 0.03	0.41 ± 0.04	0.42 ± 0.05	*0.5789*
Protein efficiency ratio (PER)	2.15 ± 0.15	2.49 ± 0.24	2.45 ± 0.33	*0.6054*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate tanks

Ultrastructural study of the intestinal mucosa

TEM and SEM observations evidenced that all specimens presented intestinal mucosa without any evidence of abnormality (Fig. 3). Nevertheless, the morphometric analysis of the intestinal microvilli carried out on both TEM and SEM images evidenced a significant increase in microvilli length (ML) and microvilli diameter (MD) in fish fed with AH-4 diet. Enterocyte apical area values were similar in all dietary treatments ($p = 0.211$), but total enterocyte absorption surface (TAS) was significantly higher in fish fed with AH-4 diet compared to CT group ($p < 0.001$) (Table 6).

Discussion

The use of *Arthrospira* hydrolysates in aquafeeds arises as a novel strategy aimed at increasing the nutritional and functional properties of the original raw biomass, by turning the proteins into low molecular weight peptides and free amino acids with higher bioavailability (Chalamaiah et al. 2012). The existence of low molecular mass molecules and free amino acids as a result of the enzymatic hydrolysis has been proposed as an interesting dietary supplement for aquacultured fish (Xu et al. 2016). Indeed, the potential beneficial effects derived from the use of protein hydrolysates in aquafeeds have been proven previously (Bui et al. 2014; Khosravi et al. 2015). However, to our knowledge, studies focused specifically on the assessment of microalgae hydrolysates for this purpose are not available and even less with regard to *Arthrospira* sp.

Table 3 Muscle proximate composition (g kg^{-1} dry weight) and moisture (%) of gilthead seabream at the end of the feeding trial

	CT	AH-2	AH-4	*p*
Total protein	745.31 ± 3.83a	789.38 ± 0.51c	772.19 ± 0.77b	*0.035*
Total lipid	166.65 ± 2.36c	141.72 ± 1.02a	157.61 ± 0.57b	*<0.001*
Ash	54.06 ± 3.32	55.23 ± 1.87	54.82 ± 2.13	*0.114*
Moisture (%)	74.51 ± 0.63	75.67 ± 1.02	74.89 ± 0.71	*0.7302*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determination ($n = 3$). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

The dietary inclusion of *Arthrospira* sp. raw biomass in aquafeeds has been evaluated previously in different fish species with favourable results. Thus, Adel et al. (2016) and Yu et al. (2018) revealed that the incorporation of 10% *Arthrospira* in feeds yielded positive effects on growth performance in great sturgeon and coral trout (*Plectropomus leopardus*), respectively. Similarly, Kim et al. (2013) reported positive effects on fish performance with 5% *Arthrospira* inclusion level in feeds for parrot fish (*Oplegnathus fasciatus*). However, results obtained in our work revealed that the dietary inclusion of *Arthrospira* protein hydrolysate up to 4% did not increase fish performance after a 128-day feeding trial.

Although some studies have reported the effect of *Arthrospira* in muscle composition of fish, disparate results have been reported. Hence, Velasquez et al. (2016) observed no changes in protein, lipid, moisture, or ash contents; on the contrary, our results (Table 3) revealed modifications in muscle protein and lipid contents in fish fed with the diets containing the microalgal protein hydrolysate. Similarly, Roohani et al. (2019) reported that *Arthrospira* (*Spirulina*) *platensis* increased protein and decreased fat content in *Salmo trutta* juveniles. These authors pointed out that the presence of several nutrients (not least vitamins, minerals, essential amino acids, and fatty acids) in microalgae might activate fish metabolism. Chen et al. (2019) found that *Arthrospira* contains substances able to modulate lipid metabolism in rodents, although the chemical nature of those compounds remains to be ascertained. According to Kim et al. (2013), *Arthrospira* might well have certain impact on lipid turnover, mainly through the use of dietary lipids as energy source, this leading to reduced muscle lipid storage. The same effect has been reported previously for other microalgae species (Hussein et al. 2013; El-Sheekh et al. 2014; Vizcaíno et al. 2014, 2016). The dietary inclusion of *Arthrospira* sp. also yields muscle fish with high protein content, an effect that might be highly desirable for final aquaculture products. In agreement, Xu et al. (2018) reported increased muscle protein content as a result of feeding juvenile *Cyprinus carpio* with enzymatic hydrolysates of insect meal. Two hypotheses might explain such increase; on one hand, a raise in the bioavailability and absorption rate of small peptides and free amino acids that enables enhanced body protein synthesis and on the other hand, the increased activity of the digestive enzymes that might promote a more efficient hydrolysis/absorption of nutrients directly involved in the synthesis of tissue protein. Further studies are required in order to verify these hypotheses.

Table 4 Changes in skin colour and muscle TBARS content during cold storage in gilthead seabream fed the experimental diets for 128 days

		CT	AH-2	AH-4	*p*
L*	1	76.4 ± 1.8^{aB}	83.3 ± 1.9^{b}	74.8 ± 1.3^{a}	0.009
	8	59.6 ± 2.6^{aA}	77.6 ± 2.4^{b}	76.5 ± 1.4^{b}	<0.001
	p	*<0.001*	*0.828*	*0.072*	
a*	1	-1.3 ± 0.4^{a}	2.3 ± 0.2^{bB}	3.5 ± 0.3^{cB}	<0.001
	8	-1.3 ± 0.2	1.9 ± 0.2^{A}	1.6 ± 0.3^{A}	0.353
	p	*0.409*	*0.040*	*<0.001*	
b*	1	5.6 ± 0.6^{B}	5.7 ± 0.8^{A}	4.3 ± 1.0^{A}	0.169
	8	2.0 ± 0.4^{aA}	6.7 ± 0.6^{bB}	6.3 ± 0.7^{bB}	<0.001
	p	*0.002*	*0.027*	*0.030*	
TBARS	1	0.40 ± 0.01^{bA}	0.34 ± 0.01aA	0.34 ± 0.02^{aA}	0.019
	8	0.50 ± 0.01^{bB}	0.44 ± 0.01^{aB}	0.42 ± 0.01^{aB}	0.005
	p	*0.012*	*0.015*	*0.019*	

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE ($n = 9$). Superscript lowercase letters indicate differences ($p < 0.05$) attributable to diets; superscripts capital letters indicate differences attributable to storage time

It is a well-known phenomenon that the skin of many species of commercial fish lacks colour and brightness in captivity, a feature directly linked to the consumer's acceptance of fish, which, accordingly, influences their market value. In this regard, different studies have described positive effects of the addition of *Arthrospira* at low inclusion level on the pigmentation attributes in different fish species (Kumprom et al. 2011; Teimouri et al. 2013; Abdulrahman and Ameen 2014; Roohani et al. 2019). Our results indicate that juveniles fed with *Arthrospira*-supplemented diets showed a skin lighter, reddish, and yellowish, and these differences remained stable over 8 days of cold storage. Similar results indicating intensified redness and yellowness in fish skin were found in golden carp (*Carassius auratus*; Kumprom et al. 2011), common carp (*Cyprinus carpio*; Abdulrahman and Ameen 2014), and rainbow trout (Teimouri et al. 2013), fed with *Arthrospira* at a low dietary inclusion level. This improvement in colorimetric parameters could be associated to the fact that most microalgae species are natural sources of pigments (Begum et al. 2016), which might play decisive role on the quality of the final product (Ginés et al. 2004). In this regard, changes in colour parameters observed could likely be attributed to the carotenoid content of *Arthrospira* (Lu et al. 2003; Teimouri et al. 2013), and thus, xanthophylls (mainly lutein and zeaxanthin) could explain the increased yellowness measured in the skin of fillets (Table 4).

Table 5 Digestive enzyme activities (U g^{-1} tissue) measured in intestine of gilthead seabream juveniles fed experimental diets for 128 days

	CT	AH-2	AH-4	*p*
Total alkaline protease	118.4 ± 11.26	95.6 ± 2.77	124.3 ± 14.72	*0.160*
Trypsin	27.8 ± 1.38a	32.7 ± 0.72b	37.9 ± 1.02 c	*0.001*
Chymotrypsin	25.4 ± 1.14a	32.8 ± 1.39b	44.9 ± 3.94c	*0.001*
L-aminopeptidase	0.22 ± 0.02a	0.40 ± 0.01b	0.38 ± 0.01c	*<0.001*
Alkaline phosphatase	57.2 ± 3.77	54.5 ± 3.18	54.9 ± 3.03	*0.844*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE of triplicate determinations per tank ($n = 9$). Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

Muscle lipid oxidation increased during the storage of fish fillets, as evidenced by the significant increase of TBARS. However, values for this parameter in muscle were significantly lower in the specimens fed with AH-2 and AH-4 diets (Table 4). The antioxidant capacity of *Arthrospira* sp. is well-known, owing to the high content in different bioactive substances playing a key role in the inhibition of lipid peroxidation (Deng and Chow 2010; Kim et al. 2013). Beyond their influence on colour parameters, xanthophylls have a potent antioxidant capacity against reactive oxygen species (ROS) (Hallerud 2014) that could explain the reduced lipid peroxidation of muscle lipids found in those animals fed with AH diets. Moreover, it has also been described that *Arthrospira* sp. contains considerable amount of the enzyme superoxide dismutase that might decrease the rate of formation of free radicals, this resulting in lower muscle lipid oxidation at inclusion levels from 10 to 2.5% (Teimouri et al. 2016). Similar results were reported in clownfish (*Oplegnathus fasciatus*) (Kim et al. 2013) and in tilapia (Amer 2016), attributed to the inclusion of *Arthrospira pacifica* and *Arthrospira* sp. as dietary additives.

The activity of digestive enzymes is not only a reliable indicator of the nutritional status of fish (Cahu and Infante 2001; Cara et al. 2007) but also a valuable tool for estimating the digestive and absorptive capacity of animals after a dietary treatment (Alarcón et al. 1998; Messina et al. 2019). The existence of changes in the digestive-absorptive processes influenced by the dietary inclusion of microalgae has been previously assessed in aquacultured fish such as seabream (Vizcaíno et al. 2014, 2016), seabass (Messina et al. 2019), Senegalese sole (Vizcaíno et al. 2018), common carp (Ansarifard et al. 2018), or great sturgeon (Adel et al. 2016). The present study confirmed that dietary inclusion of *Arthrospira* hydrolysates increased the activity of some digestive enzyme activities, despite the low inclusion levels tested. Thus, trypsin and chymotrypsin activities increased significantly in fish fed with *Arthrospira*-supplemented diets, a fact that might have contributed to increasing the availability of substrates for muscle protein accretion. Vizcaíno et al. (2016) reported similar results in *Sparus aurata* fed with microalgae-supplemented diets, which

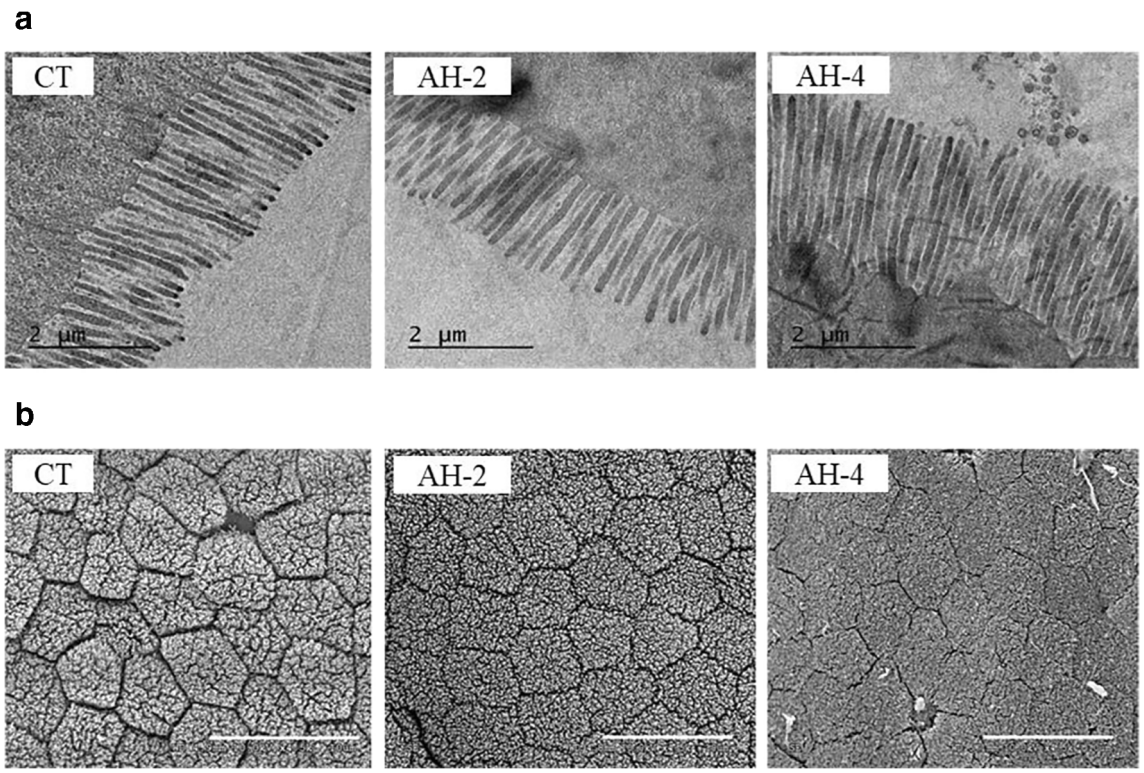

Fig. 3 TEM (**a**) and SEM (**b**) micrographs from the anterior intestine of juvenile gilthead seabream fed with experimental diets (TEM bar, 2 μm; SEM bar, 10 μm). *CT* control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate

might be related to the existence of compensatory mechanisms in fish against dietary changes. In line with the above, it has been reported that digestive protease and amylase activities increased adding 3% dietary supplementation with plant protein hydrolysate in juvenile blunt snout bream *Megalobrama amblycephala* (Yuan et al. 2019). Regarding brush border enzymes, a significant increase in the activity of leucine aminopeptidase was observed in fish fed with the diets containing the microalgal hydrolysate (Table 5). Leucine aminopeptidase and alkaline phosphatase play a crucial role in the final stages of the digestive process, facilitating the absorption and transport of nutrients through the enterocytes (Infante and Cahu 2001). In fact, both enzymes are used as indicators of the intestinal integrity (Wahnon et al. 1992) or as general markers of nutrient absorption (Silva et al. 2010). Previous studies proposed that the higher the activity levels of these enzymes, the better the efficiency of the digestive processes and the intestinal absorptive capacity (Infante and Cahu 2001). However, Messina et al. (2019) reported that alkaline phosphatase activity was not affected when fishmeal was replaced by microalgae, indicating no major functional changes in the gut integrity of European seabass (*Dicentrarchus labrax*).

Table 6 Microvilli morphometric parameters of the anterior intestine of juvenile gilthead seabream fed with the experimental diets for 128 days

	CT	AH-2	AH-4	*p*
ML (μm)	1.83 ± 0.05a	1.70 ± 0.05a	2.62 ± 0.05b	*< 0.001*
MD (μm)	0.13 ± 0.00a	0.13 ± 0.00a	0.14 ± 0.00b	*0.012*
EA (μm^2)	23.36 ± 0.82	21.75 ± 0.51	21.18 ± 1.01	*0.211*
TAS (μm^2)	767.90 ± 20.28a	751.45 ± 20.85a	1347.44 ± 20.95b	*< 0.001*

CT control, *AH-2* 2% *Arthrospira* hydrolysate, *AH-4* 4% *Arthrospira* hydrolysate. Values are mean ± SE ($n = 50$). *ML* microvilli length, *MD* microvilli diameter, *EA* enterocyte apical area, *TAS* total enterocyte absorption surface. Values in the same row with different lowercase letter indicate significant difference ($p < 0.05$)

In addition to the activity of the digestive enzymes, the intestinal mucosa plays a key role in the digestive and absorptive processes (Sweetman et al. 2008), as well as acting as a protective barrier against pathogenic microorganisms (Wilson and Castro 2011). The study of the intestinal mucosa also enables to know the influence of dietary treatments on its structure and morphology. Several studies revealed that the dietary inclusion of plant protein ingredients, algae, or probiotics can lead to morphological changes in the structure of the digestive mucosa, which are linked to important consequences on the digestive physiology and the absorption capacity of the intestinal mucosa. This has been described in different fish species, such as gilthead seabream (Cerezuela et al. 2012; Vizcaíno et al. 2016), rainbow trout (Araújo et al. 2016), goldfish (*Carassius auratus*) (Omnes et al. 2015), or

Senegalese sole (Vizcaíno et al. 2018). Until now, knowledge regarding the effects of protein hydrolysates on the intestinal structure is scarce. In the present study, the ultrastructural analysis revealed a significant effect of *Arthrospira* hydrolysate on the morphology of the intestinal mucosa, especially in those fish fed with 4% inclusion level. In agreement, it has been described that the dietary inclusion of microalgal hydrolysates can reduce mucosal barrier damage, as well as prevents colonic inflammation in mice (Wang et al. 2018). These authors evidenced that the oral administration of microalgae hydrolysates reversed the progression of dextran sulphate sodium-induced colitis and also prevented acute inflammation in that murine model. In agreement, the inclusion of 5% dietary shrimp hydrolysate resulted in larger intestinal villi and also modulated the transcriptomic response of the intestinal mucosa in European seabass (Leduc et al. 2018). Yuan et al. (2019) reported that 3% cottonseed meal protein hydrolysate increased the length of the intestinal microvilli in juvenile blunt snout bream (*Megalobrama amblycephala*). In our study, changes observed on microvilli length and microvilli diameter can be interpreted as an overall increase in enterocyte absorption surface and, consequently, an enhanced intestinal absorption capacity. This increased absorption area might have been responsible for higher amino acid uptake in the anterior intestine, this yielding higher protein accretion in muscle, especially in fish fed with 4% microalgae hydrolysate.

In conclusion, our results show that juvenile gilthead seabream fed with *Arthrospira* hydrolysate increased the activity of key digestive enzymes, improved the intestinal mucosa structure, and reduced the oxidation of muscle lipids. Thus, this supplement (especially when used at 4% inclusion level) could be useful for maintaining the overall condition status in juveniles of this fish species. The incorporation of microalgal hydrolysate as dietary additive seems promising for feeding *S. aurata* juveniles, not least due to the stimulating effect observed on the intestinal mucosa and as a natural alternative for the improvement of the skin colour in cultured fish. However, future studies should be focused on the intrinsic mechanism of their effects, as well as on the feasibility of its commercial use in aquafeeds at large scale.

Authors' contributions A. Galafat and A.J. Vizcaíno performed the fish sampling. I. Jérez-Cepa and J.M. Mancera participated in sampling and fish maintenance. A. Galafat, A.J. Vizcaíno, and M.I Sáez performed analytical analysis. F.J. Alarcón prepared the aquafeeds. A. Galafat, F.J. Alarcón, and A.J Vizcaíno performed the data analysis and drafted the manuscript. F.J. Alarcón and T.F. Martínez designed the work. All authors critically revised and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of informed consent, human/animal rights All studies involving fish were conducted in accordance with the requirements of the Directive 2010/63/EU, and the Spanish legislation (Real Decreto 53/2013), regarding the ethical rules applicable in research involving laboratory animals. Thereby, all the procedures were authorized by the Bioethics and Animal Welfare Committee of UCA (Universidad de Cádiz, Spain).

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