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Partial and total replacement of fishmeal by a vegetable and animal proteins blend and inclusion of *Isochrysis galbana* in diets for gilthead seabream (*Sparus aurata* L.): effects on growth and feed efficiency

TRABAJO FIN DE MÁSTER

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ABSTRACT

A trial was conducted to evaluate the effect of partial and total replacement of fishmeal (FM) by a vegetable and animal proteins blend as well as the inclusion of a microalgae in diets for gilthead seabream (*Sparus aurata* L.). The control diet (FM100) contained FM as the main protein source, while in diets FM25, FM10, FM0 and FM0+ the FM was replaced at 75%, 90% and 100%, respectively, by a protein blend consisting of Iberian pig meal (IPM), soybean, pea and sunflower meal. Diet FM0+ also contained a 5% of the microalgae *Isochrysis galbana.* Gilthead seabream juveniles (mean initial weight 64 g) were fed these diets to satiety for 114 days. Results obtained at the end of the experiment indicate that fish fed with the FM0 diet reached a final body weight and Specific Growth Rate (SGR) lower than the other treatments. Likewise, an improvement in growth was also observed in the gilthead seabream fed with the diet with total substitution due to the addition of the microalga. Regarding nutritional parameters, no differences were found in Feed Intake ratio (FI), but differences were found in the Feed Conversion Ratio (FCR), which was lower in fish with higher growth, fed the FM25 and FM100 diets. In whole-body composition, differences were found between fish fed FM100 and FM0 diets, in which observed a lower moisture contents and accordingly, a higher lipid content. No significant differences (p>0.05) were observed in the whole-body amino acid content. Retention efficiencies of protein, energy and essential amino acids were highest in fish with that were higher growth, that is, those fed the FM100 and FM25 diets. Overall, we can say that up to 75% of fishmeal can be replaced by a vegetable and animal proteins blend in diets for gilthead seabream, without compromising growth performance, feed utilization, and nutrient retention, in addition, the inclusion of microalgae *I. galbana* improves the growth and retention efficiencies in the gilthead seabream fed with a diet whitout fishmeal (FM0).

Keywords: *Sparus aurata*, *Isochrysis galbana*, Iberian pig meal, vegetal blend, amino acids.

RESUMEN

Se realizó un ensayo para evaluar el efecto de la sustitución parcial y total de harina de pescado por una mezcla de proteínas vegetales y animales, así como la inclusión de una microalga en piensos para dorada (*Sparus aurata* L.). El pienso control (FM100) contenía harina de pescado como principal fuente proteica, mientras que en los piensos FM25, FM10, FM0 y FM0+ la harina de pescado se sustituyó al 75%, 90% y 100%, respectivamente, por una mezcla constituida por harina de cerdo ibérico, harina de soja, harina de guisante y harina de girasol. El pienso FM0+ además contenía un 5% de la microalga *Isochrysis galbana*. Los juveniles de dorada (peso medio inicial 64 g) fueron alimentados con estos piensos durante 114 días. Los resultados obtenidos al final de experimento indican que los peces alimentados con el pienso FM0 alcanzaron un peso final y una Tasa de Crecimiento Instantáneo (TCI) menor que el resto de tratamientos. De igual forma también se pudo observar una mejora en el crecimiento en las doradas alimentadas con los piensos con sustitución total debida a la adición de la microalga. Respecto a los parámetros nutritivos, no se encontraron diferencias en la Tasa de Alimentación Diaria (TAD), pero sí en el Índice de Conversión Alimenticio (ICA), que fue menor en los peces con mayor crecimiento, alimentados con los piensos FM25 y FM100. En la composición corporal, se encontraron diferencias entre los peces alimentados con los piensos FM100 y FM0, en los que se observó un menor contenido en humedad y en concordancia, un mayor contenido de lípidos. No se observaron diferencias significativas (p>0,05) en el contenido de aminoácidos corporales. Las eficiencias de retención de proteína, energía y aminoácidos fueron superiores en los peces con mayor crecimiento, es decir, los alimentados con los piensos FM100 y FM25. En general, podemos decir que hasta un 75% de la harina de pescado puede ser sustituida por una mezcla de proteínas vegetales y animales en piensos para doradas, sin comprometer el crecimiento, la utilización del alimento y la retención de nutrientes, además, la inclusión de la microalga *I. galbana* mejora las eficiencias de retención y el crecimiento de la dorada alimentada con un pienso sin harina de pescado (FM0).

Palabras clave: *Sparus aurata*, *Isochrysis galbana*, harina de cerdo ibérico, mezcla vegetal, aminoácidos.

1. INTRODUCTION

Gilthead seabream (*Sparus aurata* L.) is a coastal species that inhabits in brackish and marine waters. It is distributed along the eastern coats of the Atlantic Ocean, from Great Britain to Cape Verde, and throughout the Mediterranean Sea. Total aquaculture production of seabream in Europe and the rest of the Mediterranean in 2015 was estimated in 181.442 tons (Apromar, 2016), making it great economic importance specie for the Mediterranean aquaculture industry.

With the expansion of world aquaculture production, the demand for fish feed and its main protein ingredient, fishmeal, is rapidly increasing. This has led to food constitutes the largest cost of production for commercial aquaculture. This continuous increasing demand in parallel with the decreasing supplies of fishmeal forces fish feed manufacturers to investigate alternative protein sources of good nutritional quality, which are ideally readily available and less expensive than fishmeal (Nengas et al., 1999). Consequently, the aquaculture industry is trying to become more economically sustainable focusing on improved feeding techniques (Martínez-Llorens et al., 2012).

Several works have been carried out studing the high fishmeal replacement by vegetable protein mixtures in gilthead seabream without affecting growth performance. Pereira and Oliva-Teles (2003) found that corn gluten meal can replace up to 60% fishmeal; Gómez-Requeni et al. (2004) related that up to 50–75% of fishmeal replacement seems to be feasible with EAA supplementation; De Francesco et al. (2007) indicated the possibility to use diets containing high levels (75%) of plant ingredients; Sánchez-Lozano et al. (2009) observed that fishmeal can be replaced up to 60% by a vegetable mixture supplemented with methionine and lysine; Dias et al. (2009) found that up to 60% of fishmeal can be replaced by selected plant-protein ingredients and Sánchez-Lozano et al. (2011) concluded that fishmeal can be replaced up to 32% by pea protein concentrate. Studies with high replacement of fishmeal have shown good results but this has affected other important parameters such as survival, feed efficiency and quality. In the case of survival and feed efficiency, are generally attributed to the presence of antinutritive factors present in plant sources, hence currently are studying new alternatives, which are the use of animal protein sources and feed additives.

Studies carry out with animal protein sources in diets for cultured marine fish are scarce. Animal by-products are potential alternative ingredients for fishmeal and are largely available, such as meat and bone meal, poultry by-product meal, feather meal, and blood meal. A temporary solution to decrease production costs lays on the identification of low-price food items, easily available and with no interest for human markets. Protein quality of animal by-product meals will vary depending upon the origin of raw materials; meat protein would have a better quality than other tissues such as tendon or skin; therefore, it is necessary to measure protein quality in animal by-products meals. Also, animal by-product meal contains reasonable amount of phosphorus, an important nutrient for aquatic animals (Tangendjaja, 2015).

The use of processed animal proteins (PAP) in aquafeeds is highly variable depending on the region. In the European Union (EU), its use was prohibited in 1990–2000, by the EU Commission Regulation (EC No. 999/2001) due to the arising of bovine spongiform encephalopathy in ruminants of Western Europe in the 1980–1990's. In 2013, however, this prohibition was partially lifted allowing the use of PAP derived from non-ruminant

animals (Category 3) for feeding of aquaculture animals, yet maintaining the prohibition of intra-species recycling of protein (EU Commission Regulation, EC No. 56/2013). This opened the doors to a whole new range of ingredients that can be used in aquafeeds inside the EU (Moutinho et al., 2017). The quality of these terrestrial animal protein sources depends on both raw material quality and processing. Use of more adequate processing technologies, particularly drying techniques, has helped to produce more defined and selected products for formulating fish diets (Bureau et al., 1999, 2000). For example, coextrusion and flash drying are now used to produce high quality meat and bone, and poultry by-products (Hernández et al., 2008). However, the technological process of PAP production was revised (EC No. 94/449; temperature over 133°C; pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm), which may compromise its nutritional quality. Therefore, it is necessary to thoroughly evaluate these new ingredients (Moutinho et al., 2017).

Poultry by-products meals are considered valuable sources for carnivorous species. However, compared to fishmeal, these products are reported to be deficient in one or more essential amino acids (Davies et al., 1991). Poultry by-product meals have been tested in diets for Chinook salmon (Fowler, 1982, 1991), rainbow trout (Steffens, 1994; Pfeffer et al., 1995), channel catfish (Lochmann and Phillips, 1995), Sunshine Bass (Thompson et al., 2008) and Nile tilapia (Hernández et al., 2009).

One of these animal by-products is the Iberian pig meal (IPM). The Iberian pig is the most important Mediterranean swine type, both in population size and economic importance (Juárez et al., 2009; Álvarez et al., 2014). The annual production of Iberian pigs in Spain has reached 1.800.000 animals in recent years (Daza et al., 2006). The Iberian Pig is a native breed of the Iberian Peninsula characterized for its high protein value, fat production ability, high quality products that can be obtained from them (Lopez-Bote, 1998) and its high rusticity (Martinez-Macipe et al., 2016). Although Iberian pig meal has a high protein content and a promising amino acid profile for the replacement of fishmeal, moreover has a lower methionine content. Additionally, the carcass from Iberian pigs is valued in the market according to major fatty acids proportion of its lipid depots – intramuscular and subcutaneous fat – especially subcutaneous fat. In fact, in the Iberian pig sector, a high proportion of oleic acid (C18:1 n-9) and lower proportions of palmitic (C16:0) stearic (C18:0) and linoleic (C18:2 n-6) acids in the carcass are used as quality indicators (De Pedro, 2001) (Tejerina et al., 2012).

A variety of useful feed additives, including probiotics and prebiotics having beneficial effects to the host was used in aquaculture to combat diseases such as supplements, to improve growth include increasing the size and weight gain, and in some cases, act as an alternative antimicrobial compounds (Irianto and Austin, 2002), as well as to stimulate immunity response of the host. In addition increased research for the development of new strategies of food supplementation which were assessed in various health and growth promoting compounds such as; probiotics, prebiotics, synbionts, phytobiotics and other functional food supplements were also evaluated (Denev, 2008) (Akhter et al., 2015).

The use of additives in aquaculture also benefits the palatability, improving the digestion and absorption of nutrients. One of them are the marine microalgae, which are

considered as a possible additive due to its physico-chemical properties and its facility to be cultivated.

Microalgae comprise a vast group of photosynthetic heterotrophic organisms, which are classified according to various aspects, such as cell structure, pigments and substances stored. Due to their rich nutritional properties, they are used for larval nutrition in molluscs, penaeid shrimp and fish larvae, and also for the enrichment of Rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) nauplii (Makridis et al., 2006; Spolaore et al., 2006). These properties include high protein content; capacity to synthesize all amino acids (and provide the essential ones to humans and animals); presence of carbohydrates composed of starch, glucose, sugars and non-digestible polysaccharides (agar, carrageenan and alginate); lipids in the form of glycerol and fatty acids of the ω3 and ω6 families; and a valuable content of many essentials vitamins (A, B1, B2, B6, B12, C, E, biotin, folic acid and pantothenic acid), minerals (phosphorous, zinc, iron, calcium, selenium, magnesium) and antioxidant substances (Borowitzka, 1997; Duerr et al., 1998) (Cerezuela et al., 2012b).

These characteristics have led to the increase on the research of new functional ingredients from microalgae with the aim to provide an additional health benefit besides the energetic and nutritional aspects of food (Christaki et al., 2011; Plaza et al., 2009; Spolaore et al., 2006). Employment of microalgae could generate a major immune stimulation due to its possible rich content of different immunostimulatory substances. In fish, most studies have focused on the use of seaweeds as immunostimulants and have been carried out in vitro by the incubation of immune cells with different algal extracts (Castro et al., 2004; Díaz-Rosales et al., 2007; Leiro et al., 2007), whereas information about in vivo administration of microalgal extracts or whole microalga cells is still very scarce (Duncan and Klesius, 1996; Díaz-Rosales et al., 2008; Guzmán et al., 2003) (Cerezuela et al., 2012b)

The species administrated in the present study, *Isochrysis galbana,* is known to have good nutritional qualities, particularly in its polyunsaturated fatty acid content. It is widely used in aquaculture by synthesize and accumulate large amounts of polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Yoshioka et al., 2012). In the case *I. galbana*, too many previous studies have not been carried out, to determine its efficacy as an additive in fish feed. And these studies have only focused on how it could affect larval fish (El-Sayed et al., 2014).

The aim of present work was to evaluate the effect of fishmeal substitution by a vegetable and animal proteins blend as well as the inclusión of the microalgae *Isochrysis galbana* on the growth performance, nutritive parameters and protein efficiency (protein and AA retention) of gilthead seabream (*Sparus aurata*).

2. MATERIALS AND METHODS

2.1. Growth trial and fish sampling

Gilthead seabream (*Sparus aurata*) juveniles were provided by a local fish farm (Alevines del Mediterráneo, S. L. (Blaumar), Sagunto, Spain) and transported to the

Fish Nutrition Laboratory of the Universitat Politècnica de València, Spain. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 4 weeks and were fed with a standard seabream diet (48% crude protein, CP; 23% crude lipid, CL; 11% ash; 2.2% crude fibre, CF; and 14% nitrogen free-extract, NFE). After the acclimation period, fish (with average weight 64 g/fish) were housed in 15 (three per treatment) cylindrical fibreglass tanks in groups of 24. The capacity of each tank was 1750 l.

The duration of the trial was 114 d. The trial was conducted in a recirculating marine water system (65 m^3 capacity) with a rotary mechanical filter and a gravity biofilter (approximately 6 m^3). The water temperature ranged from 21±0.82°C (mean±SD). The salinity was 33±2.15 g l^{-1}. The level of dissolved oxygen was 7.1±0.73 mg l^{-1}. The pH ranged from 8 to 8.5 during the trial. All tanks were equipped with aeration. The water temperature remained constant by a heat/cold specific pump installed in the system. The photoperiod was natural and all tanks had similar light conditions.

All fish were weighed in intervals of 30-day. Prior to weighing, the fish were anaesthetised with 30 mg l^{-1} of clove oil (Guinama®, Valencia, Spain) containing 87% of eugenol. At the end of the growth trial, all fish were individually weighed. Five fish from the initial stock and five fish from each tank at the end of the trial were randomly sacrificed by a lethal bath of clove oil (150 mg l^{-1}), and pooled for whole-body proximate composition analysis and for determination of biometric parameters. Fish length and total weight, and liver, viscera, and visceral fat weights were recorded for determination of condition factor, hepatossomatic, visceral, and visceral fat indices.

2.2. Experimental diets

Four isonitrogenous (45% crude protein) and isolipidic (20% crude lipid) experimental diets were formulated with different levels of fishmeal replacement and were named as FM25, FM10, FM0 and FM0+. In adittion, a control diet (FM100), whose ingredients were fishmeal (as the protein source), wheat, fish and soy oils and a complex of vitamins and minerals was used. In diets FM25, FM10, FM0 and FM0+ fishmeal was replaced at 75%, 90% and 100%, respectively, by an animal and vegetable proteins blend consisting in Iberian pig meal, soybean, pea, and sunflower meal. Additionally, microalgae *Isochrysis galbana* was included at 50 g kg^{-1} in FM0+. To cover the essential amino acids needs, methionine was added using the reference of AA requirements of *Sparus aurata* reported by Peres and Oliva-Teles (2009). Ingredients and chemical composition of the experimental diets are presented in Table 1.

The different feed ingredients were weighed individually and mixed to form a homogeneous mix and were prepared using a cooking-extrusion processing with a semi-industrial twin-screw extruder (CLEXTRAL BC-45, St. Etienne, France) at the UPV facilities. The processing conditions were as follows: a screw speed of 100 rpm, a temperature of 110°C and a pressure of 4–5 MPa. The experimental diets were analysed by triplicate.

Fish were fed by hand twice a day (09.00 and 16.00 hours) until apparent satiation from Monday to Saturday. Pellets were distributed slowly, allowing all fish to eat. Feed intake was recorded daily.

Table 1. Formulation and proximate composition of the experimental diets.

	Diets				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
Ingredients (g Kg^{-1})					
Fishmeal	590	150	60		
Wheat meal	259	56	14		
Soybean meal		171	206	220	206
Pea meal		101	122	129	111
Sunflower meal		101	122	129	111
Iberian pig meal[1]		237	288	328	328
Microalgae *I. galbana*[2]					50
Soybean oil	96	56	50	41	41
Fish oil	45	85	90	100	100
Mono calcium phosphate		28	33	38	38
L-Methionine[3]		5	5	5	5
Multivitamin and minerals mix[4]	10	10	10	10	10
Analyzed composition (% dry weight)					
Dry matter (%DM)	90.86	91.66	90.50	90.88	90.26
Crude Protein (% CP)	47.20	46.51	47.14	47.04	45.98
Crude Lipid (% CL)	19.89	19.06	18.56	18.67	19.53
Crude Fiber (% CF)[5]	0.80	3.30	3.81	3.99	3.49
Ash (%)	11.11	8.36	7.66	8.91	8.91
Calculated values					
Energy (kJ g^{-1})[6]	21.78	23.34	23.26	23.68	23.45
NFE (%)[7]	21.00	22.77	22.83	21.39	22.09

[1] Iberian pig meal (95.9% DM, 80.40% CP, 16.3% CL, 1.9% Ash); Slaughterhouse Guijuelo S.A. - Maguisa, Salamanca, Spain.
[2] Microalgae *I. galbana* (88.98% DM, 35% CP, 1.09% CL, 2.97% Ash); Biotechnology research group of the University of Almeria, Spain.
[3] L-Methionine: Guinama®.
[4] Multivitamin and minerals mix (values are g kg^{-1} except those in parenthesis): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1000000 IU kg^{-1}; calciferol, 500 IU kg^{-1}; DL-a-tocopherol, 10; menadione sodium bisulfite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07;ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.
[5] Crude Fiber (%CF) was calculated by FEDNA tables (2010).
[6] Energy (%) = (51.8 x (%C/100)) – (19.4 x (%N/100)). Calculated according to Brower (1965)
[7] Nitrogen-free extract, NFE (%) = 100 − %CP − %CL − %CF − %Ash.

2.3. Biometric parameters and proximate composition

At the end of the growth trial, five fish were randomly sampled from each tank to determinate the biometric parameters and to carry out the proximate composition analysis. The following indices were calculated:

$$\text{Specific Growth Rate [\% d}^{-1}\text{], SGR} = \{100 \cdot \ln[\text{Final weight/Initial weight}]\}/d$$

$$\text{Feed Intake ratio [g 100 g fish}^{-1}\text{ day}^{-1}\text{], FI} = \{100 \cdot \text{feed intake [g]}\} / \{\text{Average biomass [g]} \cdot d\}$$

Feed Conversion Ratio, FCR = Feed intake [g]/Weight gain [g]

Condition Factor [g cm^{-3}], CF = 100 x Total fish weight [g]/Total length^3 [cm^3]

Viscerosomatic Index [%], VSI = 100 x Visceral weight [g]/Total fish weight [g]

Hepatosomatic Index [%], HSI = 100 x Liver weight [g]/Total fish weight [g]

Mesenteric Fat Index [%], MFI = 100 x Mesenteric fat weight [g]/Total fish weight [g]

Chemical analyses of the dietary ingredients were performed prior to diet formulation. Fish diets, feed ingredients, and proximate composition of whole fish were analyzed according to AOAC (2002) procedures: dry matter, official method 934.01 (105°C to constant weight); ash, official method 942.05 (incinerated at 550°C for 5 h); crude protein, official method 990.03 (determinated by direct combustion method DUMAS using LECO CN628) and crude lipid, official method 920.39 (extracted with methyl-ether using ANKOM^{XT10} Extractor). All analyses were performed in triplicate.

2.4. Amino acid analysis

Following the method previously described by Bosch et al. (2006), the AA contents of the fish carcasses, ingredients and diets were determined using a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard before hydrolysation. AA were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. AA were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) and then converted to methionine and cysteine. The amino acids composition of the diets and main protein sources used can be seen in Table 2.

Table 2. Amino acids composition of the experiemtal ingredients and diets.

	Ingredients		Experimental diets				
	FM	**IPM**	**FM100**	**FM25**	**FM10**	**FM0**	**FM0+**
Essential amino acids (g 100 g^{-1} in ww)							
Arginine	5.86	4.90	2.76	2.78	2.63	2.52	2.62
Histidine	2.54	1.10	0.83	0.64	0.57	0.63	0.62
Isoleucine	3.40	2.31	1.69	1.45	1.39	1.36	1.39
Leucine	6.55	4.46	2.84	2.54	2.44	2.40	2.46
Lysine	6.01	4.12	2.55	1.89	1.92	1.89	1.73
Methyonine	2.30	0.94	1.01	1.06	0.84	0.84	0.91
Phenylalanine	3.73	2.49	1.46	1.40	1.31	1.33	1.44
Threonine	3.55	1.69	1.46	1.17	1.13	1.04	1.15
Valine	3.88	3.66	1.98	1.82	1.79	1.79	1.77
Non – essential amino acids (g 100 g^{-1} in ww)							
Alanine	4.32	6.18	2.08	2.23	2.34	2.31	2.24
Aspartate	6.97	6.28	2.97	3.38	3.53	3.52	3.24
Cystine	0.56	0.22	0.46	0.38	0.29	0.33	0.33
Glutamine	10.00	11.43	5.16	5.54	5.87	5.66	5.33
Glycine	4.26	14.30	2.25	4.02	4.16	4.23	4.35
Proline	2.87	8.40	1.62	2.66	2.83	2.84	3.00
Serine	3.41	2.45	1.46	1.40	1.33	1.37	1.39
Tyrosine	2.67	1.61	1.06	0.86	0.87	0.79	0.84
EAA	37.82	25.67	16.59	14.75	14.02	13.80	14.08
NEAA	35.06	50.87	17.08	20.47	21.23	21.04	20.73
EAA/NEAA	1.08	0.50	0.97	0.72	0.66	0.66	0.68

FM, fishmeal; IPM, Iberian pig meal; EAA, Essential amino acids; NEAA, Non-essential amino acids.

2.5. Estimation of retention efficiencies

Protein, amino acid and energy retention efficiencies were calculated as follows:

Retention efficiency of protein intake (%)

$$\text{PIR} = \frac{\text{Protein fish gain [g]}}{\text{Protein intake [g]}} \text{ x } 100$$

Retention efficiency of energy intake (%)

$$\text{EIR} = \frac{\text{Energy fish gain [kJ]}}{\text{Energy intake [kJ]}} \text{ x } 100$$

Retention efficiency of ingested Amino acid (%)

$$\text{AAIRE} = \frac{\text{AA fish gain [g]}}{\text{AA intake [g]}} \text{ x } 100$$

2.6. Ethical statement

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (BOE, 2013).

2.7. Statistical analysis

Growth data, nutrient utilization, biometric parameters, body composition and amino acid composition and retención were treated using multifactor analysis of variance (ANOVA). Values of survival were arc-sin transformed before statistical analysis. Newman–Keuls test was used to assess specific differences among diets at 0.05 significant levels (Statgraphics, Statistical Graphics System, Version Centurion XVI, Warrenton, Virginia, USA).

3. RESULTS

The results obtained on growth and biometric parameters are shown in table 3. At the end of the growth period, gilthead seabream fed the non fishmeal diet presented significantly the lowest final body weight and specific growth rate (SGR) (134.9 g and 0.65% day^{-1}, respectively), whereas fish fed the FM25 diet and fish fed the control diet (FM100) showed the highest growth (SGR, 1.03 and 1.07% day^{-1}, respectively). No differences among these variables were detected for the FM10 and FM0+ diets. The survival rate was higher than 94% in all treatments (Table 3), and no significant differences in survival rate were found among the dietary treatments (p>0.05). All diets were well accepted and no significant statistical diferences between groups for feed intake (FI) were detected. The feed conversion ratio (FCR) was significantly higher (2.86) in fish fed the FM0 diet than the obtained with fish fed the FM25, FM100 and FM0+ diets. Statistical differences were detected in Condition Factor (CF), fish fed the FM0+ diet obtain a higher value (1.91) than fish fed the FM0 diet (1.71) and similar to those fed the FM10 and FM25 diets. No differences were observed in the VSI, HSI and MFI indexes.

Table 3. Growth and biometric indexes of seabream fed with different experimental diets for 114 days (values are least-squares means ± SEM, n=3 for the growth and nutritive parameters and n=15 for the biometric parameters).

	DIETS[1]				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
Initial weight (g)	63.14 ± 1.33	64,06 ± 1.33	64.08 ± 1.33	65.40 ± 1.33	63.42 ± 1.33
Final weight (g)	217.8^{a} ± 8.6	208.2^{a} ± 11.1	163.7^{b} ± 8.3	134.9^{c} ± 8.5	177.8^{b} ± 8.5
Survival (%)	97.22 ± 2.40	94.44 ± 6.36	94.44 ± 6.36	98.61± 2.40	95.83 ± 4.16
SGR (% day^{-1}) [2]	1.07^{a} ± 0.04	1.03^{a} ± 0.05	0.81^{b} ± 0.04	0.65^{c} ± 0.04	0.89^{b} ± 0.04
FI (g 100 g $fish^{-1}$ day^{-1}) [3]	1.64 ± 0.05	1.45 ± 0.07	1.51 ± 0.05	1.44 ± 0.05	1.50 ± 0.05
FCR [4]	2.03^{c} ± 0.10	1.92^{c} ± 0.13	2.52^{ab} ± 0.10	2.86^{a} ± 0.10	2.25^{bc} ± 0.10
CF (g cm^{-3}) [5]	2.14^{a} ± 0.05	1.82^{bc} ± 0.05	1.76^{bc} ± 0.05	1.71^{c} ± 0.05	1.91^{b} ± 0.05
VSI (%) [6]	7.70 ± 0.27	7.85 ± 0.27	7.97 ± 0.27	8.34 ± 0.27	8.76 ± 0.27
HSI (%) [7]	1.26 ± 0.07	1.03 ± 0.07	1.08 ± 0.07	1.04 ± 0.07	1.02 ± 0.07
MFI (%)[8]	1.72 ± 0.20	1.37 ± 0.20	1.40 ± 0.20	2.13 ± 0.20	1.87 ± 0.20

[1]Diets explanation as in Table 1.
[2]Specific growth rate (%day^{-1}) SGR = 100 x ln (final weight/initial weight)/days.
[3] Feed Intake (g 100 g $fish^{-1}$ day^{-1}). FI = 100 x feed intake (g)/average biomass (g) x days.
[4] Feed Conversion Ratio FCR = feed intake (g)/weight gain (g).
[5] Condition factor (g cm^{-3}) CF = 100 x final weight (g)/$length^3$
[6] Viscerosomatic Index (%) VSI = 100 x visceral weight (g)/final weight (g).
[7] Hepatosomatic Index (%) HSI = 100 x liver weight (g)/final weight (g).
[8] Mesenteric Fat Index (%) MFI = 100 x mesenteric fat weight (g)/final weight (g).
Different superscripts letters indicate significant differences between treatments (p<0.05). Absence of superscript letters indicate no significant differences between treatments (p>0.05).

The proximate composition of the whole-body, expressed as percentage of the wet weight, is shown in Table 4. Fish fed the FM100 and FM0 diet exhibited the lowest moisture content (66.48 and 67.41%, respectively), and accordingly, the lipid content of those fish were the highest (12.71 and 13.02%, respectively).

Table 4. Proximate composition of gilthead seabream fed the experimental diets at the end of the trial (data are expressed as % of wet weight) (values are least-squares means ± SEM, n=3).

		DIETS[1]					
	Initial	**FM100**	**FM25**	**FM10**	**FM0**	**FM0+**	**SEM**
Analyzed composition (% ww)							
Moisture	66.50	66.48^{c}	68.77^{a}	68.05^{ab}	67.41^{bc}	68.81^{a}	0.32
Crude Protein (CP)	16.90	17.50	17.04	17.14	16.89	16,79	0.28
Crude Lipid (CL)	12.38	12.71^{a}	11.02^{b}	11.77^{b}	13.02^{a}	11.29^{b}	0.28
Ash	3.30	3.17	3.04	2.84	2.79	2.92	0.14

[1] Diets explanation as in Table 1.
Data in the same row with different superscripts (small letters) indicate significant differences between treatments (p<0.05). Absence of superscript letters indicate no significant differences between treatments (p>0.05).

No significant differences (p>0.05) were observed in the whole-body amino acid content of the fish (Table 5), except for the non-essential amino acid aspartate which had the highest value in fish fed the FM100 diet (1.41%) and the lowest value in fish fed the FM0 diet (1.22%).

Table 5. Amino acids composition of whole-body (%wet weight) of gilthead seabream after feeding experimental diets (values are least-squares means ± SEM, n=3).

		DIETS[1]				
	Initial	**FM100**	**FM25**	**FM10**	**FM0**	**FM0+**
Essential Amino acids (g 100 g^{-1}, ww)						
Arginine	1.45	1.29 ± 0.04	1.21 ± 0.15	1.17 ± 0.08	1.18 ± 0.06	1.17 ± 0.09
Histidine	0.33	0.28 ± 0.01	0.28 ± 0.07	0.33 ± 0.14	0.25 ± 0.04	0.26 ± 0.02
Isoleucine	0.49	0.68 ± 0.02	0.67 ± 0.09	0.65 ± 0.04	0.62 ± 0.04	0.62 ± 0.02
Leucine	1.29	1.17 ± 0.04	1.16 ± 0.12	1.15 ± 0.07	1.10 ± 0.04	1.11 ± 0.03
Lysine	1.27	1.08 ± 0.16	1.05 ± 0.05	1.04 ± 0.15	0.92 ± 0.02	0.99 ± 0.06
Methionine	0.43	0.39 ± 0.005	0.38 ± 0.02	0.36 ± 0.03	0.37 ± 0.03	0.37 ± 0.02
Phenylalanine	0.54	0.60 ± 0.07	0.59 ± 0.12	0.57 ± 0.04	0.58 ± 0.08	0.59 ± 0.10
Threonine	0.70	0.62 ± 0.01	0.63 ± 0.11	0.63 ± 0.03	0.59 ± 0.03	0.61 ± 0.03
Valine	0.71	0.81 ± 0.02	0.79 ± 0.09	0.77 ± 0.04	0.74 ± 0.03	0.73 ± 0.01
Non-essential amino acids (g 100 g^{-1}, ww)						
Alanine	1.31	0.87 ± 0.07	0.88 ± 0.07	0.86 ± 0.05	0.84 ± 0.05	0.85 ± 0.01
Aspartate	1.84	1.41^{a}±0.11	1.34^{ab}±0.13	1.35^{ab}±0.12	1.22^{b}±0.04	1.27^{ab}±0.09
Cystine	0.14	0.14 ± 0.01	0.13 ± 0.02	0.11 ± 0.01	0.13 ± 0.02	0.13 ± 0.01
Glutamate	2.72	2.04 ± 0.14	2.02 ± 0.17	2.04 ± 0.13	1.89 ± 0.04	1.97 ± 0.13
Glycine	1.55	1.10 ± 0.04	1.16 ± 0.09	1.07 ± 0.04	1.15 ± 0.01	1.13 ± 0.20
Proline	0.89	0.62 ± 0.04	0.62 ± 0.02	0.58 ± 0.01	0.63 ± 0.02	0.60 ± 0.05
Serine	0.81	0.61 ± 0.02	0.67 ± 0.12	0.60 ± 0.01	0.60 ± 0.04	0.59 ± 0.03
Tyrosine	0.51	0.53 ± 0.08	0.48 ± 0.09	0.49±0.005	0.47 ± 0.05	0.48 ± 0.06
EAA/NEAA	0.74	0.95 ± 0.08	0.93±0.005	0.94 ± 0.06	0.92 ± 0.05	0.92 ± 0.008

[1] Diets explanation as in Table 1.
Different superscripts letters indicate significant differences between treatments (p<0.05). Absence of superscript letters indicate no significant differences between treatments (p>0.05).

The retention efficiency of protein (PIR) and energy (EIR) intake was lowest (8.59 and 21.28%, respectively) in the fish fed the FM0 diet (Table 6); whereas fish fed the FM100 diet presented the highest values (20.7 and 54.27%, respectively). In fish fed the FM10 and FM0+ diets, the PIR values were approximately equal (12.14 and 12.76%, respectively) like the EIR values (29.04 and 29.03% respectively).

There were significants differences (p<0.05) in essential amino acids (EAA) retention efficiency of gilthead seabream fed the different experimental diets (Table 6). The retention efficiency of arginine and histidine in fish fed the FM0+ diet showed no difference with fish fed FM25 diet. Likewise, in fish fed FM0+ diet, isoleucine, leucine, methionine and threonine did not show differences with FM10 diet. Lysine showed similar values in fish fed the FM100, FM25 and FM0+ diets. Fish fed FM0 diet had the lowest values EAA retention efficiency.

Table 6. Retention efficiencies of ingested protein, energy and essential amino acids (%) of seabream fed with different experimental diets (values are least-squares means ± SEM, n=3).

	DIETS[1]				
	FM100	**FM25**	**FM10**	**FM0**	**FM0+**
PIR[2]	$20.70^{a} \pm 2.75$	$15.46^{b} \pm 4.15$	$12.14^{bc} \pm 1.96$	$8.59^{c} \pm 1.27$	$12.76^{bc} \pm 1.57$
EIR[3]	$54.27^{a} \pm 8.65$	$36.29^{b} \pm 9.81$	$29.04^{bc} \pm 3.49$	$21.28^{c} \pm 4.56$	$29.03^{bc} \pm 4.04$
			AAIRE[4]		
Arginine	$21.62^{a} \pm 3.06$	$13.33^{b} \pm 0.14$	$9.27^{bc} \pm 3.25$	$5.65^{c} \pm 3.08$	$10.40^{b} \pm 1.76$
Histidine	$15.55^{a} \pm 0.14$	$11.35^{b} \pm 0.1$	$9.17^{b} \pm 0.45$	$6.38^{c} \pm 1.81$	$9.38^{b} \pm 2.55$
Ilsoleucine	$23.50^{a} \pm 3.65$	$21.64^{ab} \pm 0.57$	$18.43^{bc} \pm 2.69$	$14.19^{c} \pm 3.07$	$17.80^{bc} \pm 1.00$
Leucine	$19.27^{a} \pm 3.43$	$15.39^{ab} \pm 0.84$	$11.98^{b} \pm 3.11$	$6.64^{c} \pm 2.52$	$11.63^{b} \pm 1.17$
Lysine	$19.23^{a} \pm 7.51$	$17.55^{a} \pm 5.6$	$11.55^{ab} \pm 5.32$	$2.73^{b} \pm 1.5$	$12.46^{a} \pm 3.9$
Methionine	$18.41^{a} \pm 2.55$	$11.99^{b} \pm 2.64$	$9.95^{bc} \pm 3.00$	$6.82^{c} \pm 3.31$	$10.67^{bc} \pm 0.94$
Phenylalanine	$21.60^{a} \pm 1.69$	$17.18^{ab} \pm 2.52$	$14.11^{b} \pm 3.34$	$11.01^{b} \pm 5.08$	$14.13^{b} \pm 3.56$
Threonine	$19.62^{a} \pm 2.26$	$18.32^{ab} \pm 2.66$	$14.37^{b} \pm 3.49$	$8.06^{c} \pm 3.5$	$14.15^{b} \pm 0.5$
Valine	$22.06^{a} \pm 3.44$	$17.95^{b} \pm 0.17$	$14.13^{c} \pm 2.27$	$10.04^{d} \pm 2.03$	$13.8^{cd} \pm 0.73$

[1] Diets explanation as in Table 1.
[2] PIR: Retention efficiency of protein intake (%) = 100 x [(final fish protein x final biomass (g)) – (initial fish protein x initial biomass (g))] / (ingested food (g) x diet crude protein)
[3] EIR: Retention efficiency of energy intake (%) = 100 x [(final fish energy x final biomass (g)) – (initial fish energy x initial biomass (g))] / (ingested food (g) x diet energy)
[4] AAIRE: Retention efficiency of ingested amino acid (%) = 100 x [(final fish amino acid x final biomass (g)) – (initial fish amino acid x initial biomass (g))] / (ingested food (g) x diet amino acid).
Different superscripts letters indicate significant differences between treatments (p<0.05).

Significant differences (p<0.05) were observed in ratio between ingested essential amino acids of the experimental diets and EAA of whole fish, except for EAA phenylalanine that no significant differences were observed (Fig. 1). Fish fed FM100 diet showed the highest values in almost all EAA. Arginine and methionine showed similar values in fish fed the FM100 and FM25 diets. Values of isoleucine, leucine, lysine, threonine and valine followed the same trend for fish fed FM25, FM10, FM0 and FM0+ diets. Except for lysine in group fed FM0+ diet and threonine in group fed FM0 diet, the ratio % EAA_{diet}/% EAA_{fish} were all higher than 0.7.

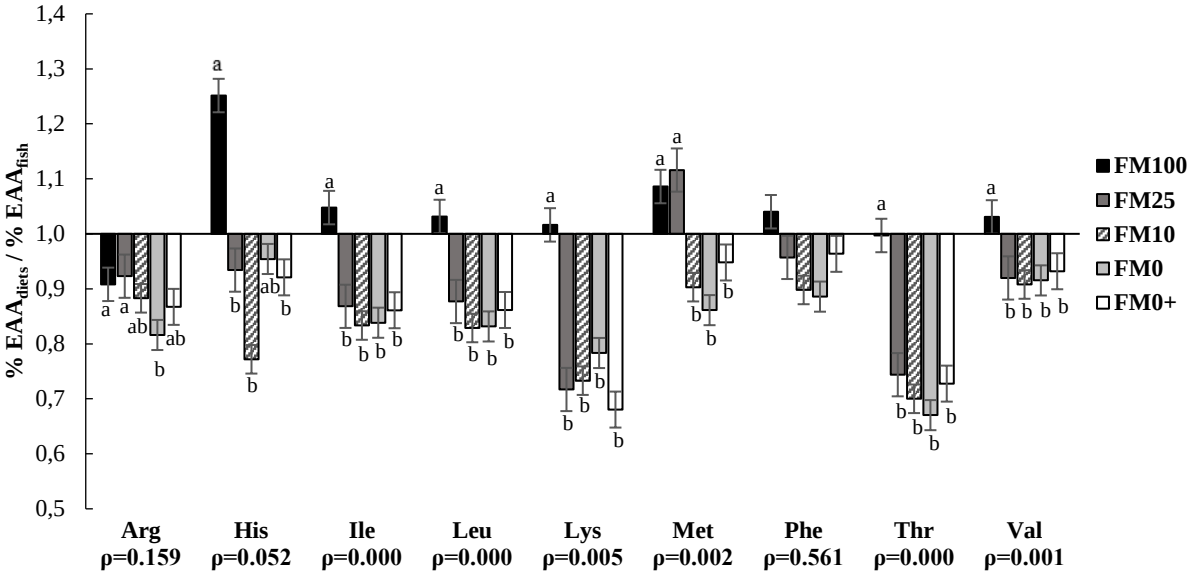

Fig. 1. Ratio between ingested essential amino acids (EAA) of the experimental diets and EAA of whole fish. Each value is the mean of triplicate groups. Significant differences are indicated by different letters (p<0.05). Absence of superscript letters indicate no significant differences between treatments (p>0.05).

4. DISCUSSION

The studies that tested the fishmeal replacement by animal-by products and vegetable protein meals for aquaculture feeds are scarce. In present study, the FM was replaced by a vegetable and animal (Iberian pig meal) proteins blend. The results indicate that up to 75% of FM can be replaced by an animal and vegetable proteins blend in diets for gilthead seabream without negative effects on growth performance and feed utilization. In addition, the Feed Conversion Factor (FCR) in fish fed the FM25 diet was the lowest (1.92). Similar to the present results, previus studies showed the feasibility to the use of animal by-products in diets for gilthead seabream: FM substitucion by blood meal (Martínez-Llorens et al., 2008), meat and bone meal (Robaina et al., 1997) and high levels of FM substitucion with meat and bone meal (Moutinho et al., 2017) and poultry meat meal (Nengas et al., 1999).

Respect to fish growth, the results obtained at the end of the experiment indicate that fish fed with the FM0 diet (total fishmeal substitution) had the lowest final body weight. Specific Growth Rate (SGR) followed the same trend observed for final body weight. Many ingredients derived from agricultural products can contain antinutritional factors that may affect animal performance. Several antinutritional factors are found in soybean (ingredient present in experimental diets) such as protease inhibitors, allergens, oligosaccharides, phytin, lipoxygenase, lectins and saponin (Tangendjaja, 2015) related with alter intestinal functions.This can be one of the reasons for the bad groth with the FM0 diet. The other reason must be relationed with the nutrient disponibility, because although the diets were formulated to cover the nutritional requeriments of the fish, as a consequence of the higher level of vegetal sources maybe has influenced negatively in the nutrient digestibility and therefore in the availability of nutrients, specifically in amino acids.

Essential amino acid (EAA) deficiency is one of the most important issues regarding FM substitution with alternative ingredients (Kaushik and Seiliez, 2010) and unbalanced EAA levels in the diets have been reported as one of the main causes for growth depression in fish fed animal by-products based diets (García-Gallego et al., 1998; Millamena, 2002; Xavier et al., 2014; Moutinho et al., 2017). The content of the EAA, especially the Lys, Met and Thr content, is generally the limiting amino acid content in economical alternative protein sources. A deficiency in one EAA will lead to poor utilization of the provided dietary protein (Wilson, 2002).

The ratio EAA_{diet}/ EAA_{fish} (of gilthead seabream fed FM0+ and FM0 diets presented the lower values for lysine and threonine, respectively. On the other hand, histidine showed the higher value in FM100 diet. In general terms, the EAA_{diet}/EAA_{fish} values are similar to those obtained by Moutinho et al. (2017) with the replacement of FM for meat and bone meal. In both studies, the values are lowest because they have been calculated for ingested EAA. In contrast, the results obtained by Sánchez-Lozano et al. (2011) and Martínez-Llorens et al. (2012), whose values are highest because the ratio has been calculated for digestible EAA.

Few studies have also looked into the potential of some of the NEAA and to the ratios between dietary essential to non-essential amino acids (EAA/NEAA ratio) (Hughes, 1985; Mambrini and Kaushik, 1994). Gómez-Requeni et al. (2003) found that the best growth performance occurs with a diet that resembles the EAA profile and EAA/NEAA muscle ratio, when fishmeal has been replaced by 35% by plant ingredients. In this study, the FM100 diet had an EAA/NEAA ratio of 0.97 and the fish a mean value of 0.93. In FM0 diet the EAA/NEAA ratio decreases to 0.66 and also fish fed with this diet showed the lowest values for retention efficiency of ingested essential amino acids (Table 6), which is related to its low final body weight gain. This is corroborated by that in gilthead seabream a dietary EAA/NEAA ratio of 1.1 to be better than a ratio of 0.8 (Gomez-Requeni et al., 2003) (Kaushik and Seiliez, 2010).

Significant differences were found in the retention efficiencies of ingested protein, energy and essential amino acids of seabream fed with different experimental diets. The diets with higher percentages of retention efficiency were FM100 and FM25, whose values are similar to those of previous studies (Moutinho et al., 2017). This shows that a fishmeal replacement up to 75% can be achieved according to the growth and retention results. In this study, values have been obtained slightly lower for the retention efficiency of methionine and arginine in control diet (18.41% and 21.62%, respectively), while in other works are obtained values close to 30% for both amino acids (Martínez-Llorens et al., 2012). FM0 diet presented the lowest retention efficiency for all amino acids, in agreement with the growth obtained with this diet. This detriment in retention efficiencies may be due to lower nutrient availability because of reduced digestibility in diets without fishmeal, which has already been proven in several species, including gilthead seabream (Lupatsch et al., 1997).

The positive effect of the microalga addition in the diets is a relevant result. Growth, the factor condition and the retention efficiencies of seabream fed with the FM0+ diet improved with respect to the FM0 diet, and some parameters equaled the results obtained with the control diet and the FM25 diet.

According to the above, it is possible to affirm that FM0+ diet showed better results. It can be due to the presence of eicosapentaenoic acid (EPA), 20: 5n-3 and docosahexaenoic acid (DHA), 22: 6n-3 in the microalgae *I. galbana*, since the quality and quantity of microalgal lipid are important to the nutrition of marine animals (Enright et al., 1986; Gallager et al., 1986; Koven et al., 1989; Sargent et al., 1989) (Fidalgo et al., 1998). *Isochrysis galbana* microalgae have 40% PUFA with a favorable ω3/ω6 ratio around 4, rich in EPA and DHA (Batista et al., 2013). These results are in accordance with findings of other authors who reported a proportion around 25% of EPA+DHA for *I. galbana* (Donato et al., 2003; Durmaz et al., 2008; Otero et al., 1997). *Isochrysis galbana* had higher values, with 4.9 g EPA and 11.6 g DHA per 100 g microalgal biomass. Recently, Nauroth et al. (2010) proved the potent anti-inflammatory activity of DPAω6 from algal source. DPAω6 can be converted into oxylipins, resolvin-like molecules, with potent anti-inflammatory activity, which could contribute to the reduction of inflammatory response in vivo. The synergistic effect of DPAω6 with DHA was also referred by the authors (Nauroth et al., 2010), suggesting that algal biomass may be a novel anti-inflammatory supplement (Batista et al., 2013). There is considerable evidence of the importance of nutritional factors such as proteins, essential fatty acids, polysaccharides, vitamins C and E and some of the trace minerals for maintaining normal immune functions in fish (Landolt, 1989). Moreover, many of the microalgae isolate components have been shown to have immunostimulating properties in fish or other animals (Amar et al., 2004; Guzmán et al., 2003; Morris et al., 2007; Ortuño et al., 2000; Puangkaew et al., 2004) (Cerezuela et al., 2012b). In mammals, different studies have shown the immunostimulating capacity of algae (both macro and microalgae) or their extracts (Guzmán et al., 2001, 2003; Leiro et al., 2007; Morris et al., 2007) and have even shown ability to reduce the damage caused by certain intestinal diseases (Bedirli et al., 2009). Macroalgae, moreover, have been studied as important sources of prebiotics for application in human and animal nutrition (O'Sullivan et al., 2010) (Cerezuela et al., 2012c). In recent years, microalgae have emerged as a very interesting natural source of new compounds with biological activity that may be used as functional ingredients (Plaza et al., 2009; Guedes et al., 2011). Most of the microalgae components are potential immunostimulants or substances with immunomodulatory capacities (Amar et al., 2004; Jha et al., 2007) (Cerezuela et al., 2012a).

No significant differences were found for feed intake, as well as the biometric indexes (VSI, HIS and MFI) of seabream fed with different experimental diets, although it is noted that the Mesenteric Fat Index is higher for fish fed FM0 diet (2.13%) which agrees with the higher lipid content of the fish. Similar results have been obtained in other studies, where even without having significant differences, there has been a slight increase in mesenteric fat of the fish on diet with greater replacement of fishmeal (Sánchez-Lozano et al., 2011). However, Kaushik et al. (2004) observed a significant increase in fat content with increasing levels of fishmeal replacement. This consequently resulted in a similar increase in whole body energy content. The high fat and energy retention values clearly suggest that there was increased lipogenesis with increasing levels of fishmeal replacement. In higher vertebrates, dietary protein level and source is known to affect lipid deposition, the fatty acid bioconversion potential and alter serum and liver lipids (Lindholm and Eklund, 1991; Terasawa et al., 1994; Potter, 1995; Aoyama et al., 2000) (Dias et al., 2005). The low protein digested might be the main reason of the high fat content of fish fed FM0 in present experiment, that produce a lower relation between protein and energy digested and therefore low growth and fish

fatness. However, despite protein digested is not shown in present manuscript, the far below values for protein efficiency registered for fish fed FM0 diet is fish (8.59%) could give a prior approximation about the low protein digestibility of this diet.

5. CONCLUSION

Major research efforts are currently underway to find new alternative protein sources for the replacement of fishmeal in aquafeeds. Likewise, a variety of useful feed additives are used in aquaculture to improve growth and to stimulate immune response of fish. It is for this reason that innovation is continuously directed towards the addition of value and the search for new additives and animal by-products that are valuable for carnivorous species.

The effect of fishmeal substitution by a vegetable and animal proteins blend as well as the inclusion of the microalgae *Isochrysis galbana* on the growth performance, nutritive parameters and protein metabolism of gilthead seabream (*Sparus aurata* L) has led to the following conclusions:

- It is possible to replace fishmeal up to 75% by a vegetable and animal proteins blend in diets for gilthead seabream without negative effects on growth performance and feed utilization.

- Fish fed FM25 diet showed optimal values (similar to control diet) for growth and retention efficiencies.

- Regarding body composition, the group fed with FM25 diet followed the same trend as the FM0+ group (high moisture values and, therefore, low crude lipid values). No significant differences were observed for protein and ash.

- The inclusion of the microalgae *I. galbana* as an additive in diets with fishmeal total substitution favors the growth and retention efficiencies of gilthead seabream.

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REFERENCES

Akhter, N., Wu, B., Mahmood Memon, A., & Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: A review. *Fish & Shellfish Immunology, 45*, 733-741.

Álvarez, R., Vicario, I.M., Meléndez-Martínez, A.J., & Alcalde M.J. (2014). Effect of different carotenoid-containing diets on the vitamin A levels and colour parameters in Iberian pigs' tissues: utility as biomarkers of traceability. *Meat Science, 98,* 187–192.

Amar, E.C., Kiron, V., Satoh, S., Watanabe, T. (2004). Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish Shellfish Immunol 16*:527–537. doi:10.1016/j.fsi.2003.09.004.

AOAC, Association of Official Analytical Chemists. (2002). Official methods of analysis of AOAC International. AOAC International, Washingyon, DC.

Aoyama, T., Fukui, K., & Takamatsu, K. (2000). Soy protein isolate and its hydrolysate reduce body fat of dietary obese rats and genetically obese mice (yellow KK*). Nutrition 16,* 349– 354.

APROMAR, Asociación Empresarial de Productores de Cultivos Marinos de España. (2016). La Acuicultura en España. Informes anuales. APROMAR, Cádiz, Spain 89 pp. URL http://apromar.es/content/la-acuicultura-en-espa%C3%B1a-2016.

Batista, A.P., Gouveia, L., Bandarra, N.M., Franco, J.M., & Raymundo, A., (2013). Comparison of microalgal biomass profiles as novel functional ingredient for food products. *Algal Research, 2,* 164–173.

Bedirli, A., Kerem, M., Ofluoglu, E., Salman, B., Katircioglu, H., Bedirli, N., Yilmazer, D., Alper, M., & Pasaoglu, H. (2009). Administration of *Chlorella* sp. microalgae reduces endotoxemia, intestinal oxidative stress and bacterial translocation in experimental biliary obstruction. *Clinical Nutrition 28,* 674–678.

BOE. (2013). Boletín Oficial del Estado Real Decreto 53/2013 sobre protección de animales utilizados en experimentación y otros fines científicos. 34 (1), 11370–11371.

Borowitzka, M.A. (1997). Microalgae for aquaculture: opportunities and constraints. *J App Phycol 9*:393–401. DOI: 10.1023/A:1007921728300.

Bosch, L., Alegria, A. & Farré, R. (2006). Application of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), reagent to the RP-HPLC determination of amino acids in infant foods. Journal of chromatography. *B, Analytical technologies in the biomedical and life sciences 831,* 176–178.

Brouwer, E. (1965). Report of sub-committee on constants and factors. In: Blaxter, K.L. (Ed.), Proceedings of the Third EAAP Symposium on Energy Metabolism. Publication No. 11. Academic Press, London, pp. 441–443.

Bureau, D.P., Harris, A.M., & Cho, C.Y., (1999). Apparent digestibility of rendered animal protein ingredients for rainbow trout. *Aquaculture 180,* 345–358.

Bureau, D.P., Harris, A.M., Beven, D.J., Simmons, L.A., Azevedo, P.A., & Cho, C.Y., (2000). Feather meals and meat and bone meals from different origins as protein sources in rainbow trout (*Oncorhynchus mykiss*) diets. *Aquaculture 181*, 281–291.

Castro, R., Zarra, I., & Lamas, J. (2004). Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. *Aquaculture 229*:67–78. DOI:10.1016/S0044-8486(03)00401-0.

Cerezuela, R., Guardiola, F.A., González, P., Meseguer, J., & Esteban, M.A. (2012a). Effects of dietary *Bacillus subtilis*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata* L.) *Fish & Shellfish Immunology 33*, 342-349.

Cerezuela, R., Guardiola, F.A., Meseguer, J., & Esteban, M.A. (2012b). Enrichment of gilthead seabream (*Sparus aurata* L.) diet with microalgae: effects on the immune system. *Fish Physiol Biochem*, DOI 10.1007/s10695-012-9670-9.

Cerezuela, R. (2012c). New probiotics and prebiotics for seabream (*Sparus aurata* L.) (Doctoral thesis). University of Murcia, Spain.

Christaki, E., Florou-Paneri, P., & Bonos, E. (2011). Microalgae: a novel ingredient in nutrition. *Int J Food Sci Nutr 62*: 794–799. doi:10.3109/09637486.2011.582460.

Davies, S.J., Nengas, I., & Alexis, M. (1991). Partial substitution of fishmeal with different meat meal products in diets for seabream (*Sparus aurata*). *Fish Nutrition in Practice*, June 24-27, 1991, INRA (Ed.), (Les Colloques, No. 6 1). Biarritz, France, pp. 907-911.

Daza, A., Mateos, A., López Carrasco, C., Rey, A., Ovejero, I., & López-Bote C.J. (2006). Effect of feeding system on the growth and carcass characteristics of Iberian pigs, and the use of ultrasound to estimate yields of joints. *Meat Science, 72*, 1–8.

De Francesco, M., Parisi, G., Pérez-Sánchez, J., Gómez-requeni, P., Médale, F., Kaushik, S.J., Mecatti, M., & Poli, B.M. (2007). Effect of high-level fishmeal replacement by plant proteins in gilthead seabream (*Sparus aurata*) on growth and body/filletquality traits. *Aquaculture Nutrition 13,* 361–372.

De Pedro, E. J. (2001). Calidad de las canales y de los productos del cerdo Ibérico: Técnicas de control y criterios de calidad. In Mundi-Prensa Madrid (Ed.), En Porcino Ibérico: Aspectos claves. C. Buxadé y A. Daza (Coordinadores) (pp. 589–622). 84-7114-876-5.

Denev, S.A. (2008). Ecological Alternatives of Antibiotic Growth Promoters in the Animal Husbandry and Aquaculture. DSc. Thesis, Department of Biochemistry Microbiology, Trakia University, Stara Zagora, Bulgaria.

Dias, J., Alvarez, M.J., Arzel, J., Corraze, G., Diez, A., Bautista, J.M., & Kaushik, S.J. (2005). Dietary protein source affects lipid metabolism in the European seabass

(*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 142,* 19-31.

Dias, J., Conceição, L.E.C., Ribeiro, A.R., Borges, P., Valente, L.M.P., & Dinis, M.T. (2009). Practical diet with low fish-derived protein is able to sustain growth performance in gilthead seabream (*Sparus aurata*) during the grow-out phase. *Aquaculture 293,* 255–262.

Díaz-Rosales, P., Felices, C., Abdala, R., Figueroa, F.L., Gómez Pinchetti, J.L., Moriñigo, M.A., & Balebona, M.C. (2007). In vitro effect of the red alga *Hydropuntia cornea* (J. Agardh) on the respiratory burst activity of sole (*Solea senegalensis*, Kaup 1858) phagocytes. *Aquac Res 38*:1411–1418. doi:10.1111/j.1365-2109.2007.01830.x.

Díaz-Rosales, P., Chabrillón, M., Abdala, R.T., Figueroa, F.L., Balebona, M.C., & Moriñigo, M.A. (2008). Effect of dietary administration of *Porphyridium cruentum* on the respiratory burst activity of sole, *Solea senegalensis* (Kaup), phagocytes. *J Fish Dis 31*:489–495. doi:10.1111/j.1365-2761.2008.00923.x.

Donato, M., Vilela, M.H., & Bandarra, N.M., (2003). Fatty acids, sterols, α-tocopherol and total carotenoids composition of *Diacronema vlkianum*. *Journal of Food Lipids, 10,* 267–276.

Duerr, E.O., Molnar, A., & Sato, V. (1998). Cultured microalgae as aquaculture feeds. *J Mar Biotechnol 6*:65–70.

Duncan, P.L., & Klesius, P.H. (1996). Effects of feeding *Spirulina* on specific and nonspecific immune responses of channel catfish. *J Aquat Anim Health 8*:308–313. DOI:10.1577/1548-8667(1996)008\0308:EOFSOS[2.3.CO;2.

Durmaz, Y., Donato, M., Monteiro, M., Gouveia, L., Nunes, M.L., Gama-Pereira, T., Gokpinar, S., & Bandarra, N.M., (2008). Effect of temperature on growth and biochemical composition (sterols, α-tocopherol, carotenoids, fatty acid profiles) of the microalga, *Isochrysis galbana*. *Israeli Journal of Aquaculture, 60,* 188–195.

El-Sayed, H.S., Ibrahim, H.A.H., Beltagy, E.A., & Khairy, H.M. (2014). Effects of short term feeding of some marine microalgae on the microbial profile associated with *Dicentrarchus labrax* post larvae. *Egyptian Journal of Aquatic Research 40*, 251–260.

Enright, C.T., Newkirk, G.F., Craigie, J.S., & Castell, J.D., (1986). Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. *J. Exp. Mar. Biol. Ecol. 96,* 1–13.

FEDNA, Fundación Española para el Desarrollo de la Nutrición Animal. (2010). Tablas FEDNA de composición y valor nutritivo de alimentos para la fabricación de piensos compuestos (3ª edición). C. de Blas, G.G. Mateos y P. García-Rebollar. Fundación Española para el Desarrollo de la Nutrición Animal. Madrid. 502 pp. URL http://fundacionfedna.org/tablas-fedna-composicion-alimentos-valor-nutritivo

Fidalgo, J.P., Cid, A., Torres, E., Sukenik, A., & Herrero, C. (1998). Effects of nitrogen source and growth phase on proximate biochemical composition, lipid clases and fatty acid profile of the marine microalga *Isochrysis galbana. Aquaculture, 166*, 105–116.

Fowler, L.G. (1982). Tests with poultry by-product meal in fall Chinook salmon diets. *Abernathy salmon cultural development centre.* Tech. Transfer Series No. 82-1, 13 pp.

Fowler, L.G. (1991). Poultry by-product meal as a dietary protein source in fall Chinook salmon diets. *Aquaculture 99,* 309–321.

Gallager, S.M., Mann, R., & Sasaki, G.C., (1986). Lipid as an index of growth and viability in three species of bivalve larvae. *Aquaculture 56,* 81–103.

García-Gallego, M., Akharbach, H., & de la Higuera, M. (1998). Use of protein sources alternative to fishmeal in diets with amino acids supplementation for the European eel (*Anguilla anguilla*). *Anim. Sci. 66,* 285–292.

Gómez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., Medale, F., Corraze, G., Panserat, S., Martín, S.A.M., Houlihan, D.F., Kaushik, S.J., & Pérez-Sánchez, J. (2003). Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotropic axis responsiveness of gilthead seabream (*Sparus aurata*). *Aquaculture, 220*, 749-767.

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., & Pérez-Sánchez, J. (2004). Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fishmeal replacement by plant protein sources in gilthead seabream (*Sparus aurata*). *Aquaculture 232,* 493 – 510.

Guedes, A.C., Amaro, H.M., & Malcata, F.X. (2011). Microalgae as sources of high added-value compoundsea brief review of recent work. *Biotechnol Progr 27*, 597-613.

Guzmán, S., Gato, A., & Calleja, J.M. (2001). Antiinflammatory, analgesic and free radical scavenging activities of the marine microalgae *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother Res 15*:224–230. DOI: 10.1002/ptr.715.

Guzmán, S., Gato, A., Lamela, M., Freire-Garabal, M., & Calleja, J.M. (2003). Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother Res 17*:665–670.DOI:10.1002/ptr.1227.

Hernández, C., Olvera-Novoa, M.A., Aguilar-Vejar, K., González-Rodríguez, B., & Abdo de la Parra, I. (2008). Partial replacement of fishmeal by porcine meat meal in practical diets for Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture, 277*, 244–250.

Hernández, C., Olvera-Novoa, M.A., Hardy, R.W., Hermosillo, A., Reyes, C., & González, B. (2009). Complete replacement of fishmeal by porcine and poultry by-

product meals in practical diets for fingerling Nile tilapia *Oreochromis niloticus*: digestibility and growth performance. Aquaculture Nutrition 10. DOI: 10.1111/j.1365-2095.2008.00639.x

Hughes, S.G. (1985). Evaluation of glutamic acid and glycine as sources of non-essential amino acids for lake trout (*Salvelinus namaycush*) and rainbow trout (*Salmo gairdneri*). 81A, 669-671.

Irianto, A., & Austin, B. (2002). Probiotics in aquaculture. *J. Fish Dis., 25*, 633-642.

Jha, A.K., Pal, A.K., Sahu, N.P., Kumar, S., & Mukherjee, S.C. (2007). Haemato-immunological responses to dietary yeast RNA, omega-3 fatty acid and beta-carotene in *Catla catla* juveniles. *Fish Shellfish Immunol 23*, 917-27.

Juárez, M., Clemente, I., Polvillo, O., & Molina, A. (2009). Meat quality of tenderloin from Iberian pigs as affected by breed strain and crossbreeding. *Meat Science, 81*, 573–579.

Kaushik, S.J., Covès, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax. Aquaculture 230*, 391 – 404.

Kaushik, S.J., & Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research 41*, 322–332.

Koven, W.M., Kissil, G.Wm., & Tandler, A., (1989). Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. *Aquaculture 79*, 185–191.

Landolt, M. (1989). The relationship between diet and the immune response of fish. *Aquaculture 79:*193–206. DOI:10.1016/0044-8486(89)90461-4.

Leiro, J.M., Castro, R., Arranz, J.A., & Lamas, J. (2007). Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. *Agardh. Int Immunopharmacol 7*:879–888. doi:10.1016/j.intimp.2007.02.007.

Lindholm, M., & Eklund, A. (1991). The effects of dietary protein on the fatty acid composition and D6-desaturase activity on rat hepatic microsomes. *Lipids 26*, 107–110.

Lochmann, R.T., & Phillips, H. (1995). Comparison of rice bran oil, poultry fat and cod liver oil as supplemental lipids in feeds for channel catfish and golden shiner. *J. Appl. Aquacult. 5*, 47–56.

Lopez-Bote, C.J. (1998). Sustained utilization of the Iberian pig breed. *Meat Science, 49*(1), S17–S27.

Lupatsch, I., Kissil, G.W., Sklan, D., & Pfeffer, E., (1997). Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, (*Sparus aurata* L.). *Aquaculture Nutrition, 4*, 165-174.

Makridis, P. Costa, R.A., & Dinis, M.T. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched Artemia metanauplii. *Aquaculture 255*:76–81. doi:10.1016/j.aquaculture.2005. 12.010.

Mambrini, M., & Kaushik, S.J. (1994), Partial replacement of dietary protein nitrogen with dispensable amino acids in diets of Nile Tilapia, *Oreochromis Niloticus*. *Comparative Biochemistry and Physiology A - Comparative Physiology 109*, 469-477.

Martínez-Llorens, S., Tomás, A., Moñino, A.V., Gómez J.A., Pla, M., & Jover, M. (2008). Blood and haemoglobin meal as protein sources in diets for gilthead seabream (*Sparus aurata*): effects on growth, nutritive efficiency and fillet sensory differences. *Aquaculture Research, 39,* 1028–1037.

Martínez-Llorens, S., Baeza-Ariño, R., Nogales-Mérida, S., Jover-Cerdá, M., & Tomás-Vidal, A. (2012). Carob seed germ meal as a partial substitute in gilthead seabream (*Sparus aurata*) diets: Amino acid retention, digestibility, gut and liver histology. *Aquaculture, 338-341,* 124–133

Martinez-Macipe, M., Rodríguez, P., Izquierdo, M., Gispert, M., Manteca, X., Mainau, E., Hernández, F.I., Claret, A., Guerrero, L., & Dalmau, A. (2016). Comparison of meat quality parameters in surgical castrated versus vaccinated against gonadotrophin-releasing factor male and female Iberian pigs reared in free-ranging conditions. *Meat Science, 111,* 116–121.

Millamena, O.M. (2002). Replacement of fishmeal by animal by-product meals in a practical diet for grow-out culture of grouper *Epinephelus coioides*. *Aquaculture 204*, 75–84.

Morris, H.J., Carrillo, O., Almarales, A., Bermúdez, R.C., Lebeque, Y., Fontaine, R., Llauradó, G., & Beltrán, Y. (2007). Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* on undernourished mice. *Enzyme Microb Technol 40:*456–460. doi:10.1016/j.enzmictec.2006.07.021

Moutinho, S., Martínez-Llorens, S., Tomás-Vidal, A., Jover-Cerdá, M., Oliva-Teles, A., & Peres, H. (2017). Meat and bone meal as partial replacement for fishmeal in diets for gilthead seabream (*Sparus aurata*) juveniles: Growth, feed efficiency, amino acid utilization, and economic efficiency. *Aquaculture, 468,* 271–277.

Nauroth, J.M., Liu, Y.C., van Elswyk, M., Bell, R., Hall, E.B., Chung, G., & Arterburn, L.M., (2010). Docosahexaenoic acid (DHA) and docosapentaenoic acid (DPAn−6) algal oils reduce inflammatory mediators in human peripheral mononuclear cells in vitro and paw edema in vivo. *Lipids, 45,* 375–384.

Nengas, I., Alexis, M.N., & Davies S.J. (1999). High inclusion levels of poultry meals and related byproducts in diets for gilthead seabream *Sparus aurata* L. *Aquaculture 179*, 13–23.

Ortuño, J., Esteban, M.A., & Meseguer, J. (2000). High dietary intake of alpha-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol 10:*293–307. DOI:10.1006/fsim. 1999.0238.

Otero, A., García, D., & Fábregas, J., (1997). Factors controlling eicosapentaenoic acid production in semicontinuous cultures of marine microalgae. *Journal of Applied Phycology, 9,* 465–469.

O'Sullivan, L., Murphy, B., McLoughlin, P., Duggan, P., Lawlor, P.G., Hughes, H., & Gardiner, G.E. (2010). Prebiotics from marine macroalgae for human and animal health applications. *Marine Drugs 8,* 2038–2064.

Pereira, T.G., & Oliva-Teles, A. (2003). Evaluation of corn gluten meal as a protein sourcein diets for gilthead seabream (*Sparus aurata* L.) juveniles. *Aquaculture Research, 34,* 1111-1117.

Peres, H., & Oliva-Teles, A. (2009). The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture, 296*:81–86.

Pfeffer, E., Kinzinger, S., & Rodehutscord, M. (1995). Influence of the proportion of poultry slaughter by-products and of untreated or hydrothermically treated legume seeds in diets for rainbow trout, *Oncorhynchus mykiss* on apparent digestibilities of their energy and organic compaunds. *Aquacult. Nutr. 1,* 111–117.

Plaza, M., Herrero, M., Cifuentes, A., & Ibáñez, E. (2009). Innovative natural functional ingredients from microalgae. *J Agr Food Chem 57,* 7159-70.

Potter, S. (1995). Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *J. Nutr. 125,* 606S– 611S.

Puangkaew, J., Kiron, V., Somamoto, T., Okamoto, N., Satoh, S., Takeuchi, T., & Watanabe, T. (2004). Nonspecific immune response of rainbow trout (*Oncorhynchus mykiss* Walbaum) in relation to different status of vitamin E and highly unsaturated fatty acids. *Fish Shellfish Immunol 16*:25–39. DOI:10.1016/S1050-4648(03)00028-7.

Robaina, L., Moyano, F.J., Izquierdo, M.S., Socorro, J., Vergara, J.M., & Montero, D. (1997). Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (*Sparus aurata*): Nutritional and histological implications. *Aquaculture 157,* 347-359.

Sánchez-Lozano, N.B., Martínez-Llorens, S., Tomás-Vidal, A., & Jover-Cerdá, M. (2009). Effect of high-level fishmeal replacement by pea and rice concentrate protein on growth, nutrient utilization and fillet quality in gilthead seabream (*Sparus aurata,* L.). *Aquaculture 298,* 83–89.

Sánchez-Lozano, N., Martínez-Llorens, S., Tomás-Vidal, A., & Jover-Cerdá, M., (2011). Amino acid retention of gilthead seabream (*Sparus aurata,* L.) fed with pea protein concentrate. *Aquaculture nutrition, 17 (2),* e604-e614.

Sargent, J.R., Henderson, R.J., & Tocher, D.R., (1989). The lipids. *In: Halver, J.E. Ed., Fish Nutrition. Academic Press*, pp. 153–218.

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *J Biosci Bioeng 101*:87–96. doi:10.1263/jbb.101.87.

Steffens, W. (1994). Replacing fishmeal with poultry by-product meal in diets for rainbow trout *Oncorhynchus mykiss*. *Aquaculture 124*, 27–34.

Tangendjaja, B. (2015). Quality control of feed ingredients for aquaculture. *Feed and Feeding Practices in Aquaculture, Elsevier, 141–169.*

Tejerina, D., García-Torres, S., Cabeza de Vaca, M., Vázquez, F.M., & Cava, R. (2012). Study of variability in antioxidant composition and fatty acids profile of *Longissimus dorsi* and *Serratus ventralis* muscles from Iberian pigs reared in two different Montanera seasons. *Meat Science, 90,* 414–419.

Terasawa, F., Hirano, Y., Wada, M., Takita, T., Nakamura, K., & Innami, S. (1994). Effects of dietary casein and soy-protein on metabolic conversion of eicosapentaenoic acid to docosahexaenoic acid in the liver of rat. *J. Nutr. Sci. Vitaminol. 40,* 353–362.

Thompson, K.R., Rawles, S.D., Metts, L.S., Smith, R., Wimsatt, A., Gannam, A.L., Twibell, R.G., Johnson, R.B., Brady, Y.J., & Webster, C.D. (2008). Digestibility of Dry Matter, Protein, Lipid, and Organic Matter of Two Fish Meals, Two Poultry By-product Meals, Soybean Meal, and Distiller's Dried Grains with Solubles in Practical Diets for Sunshine Bass, *Morone chrysops* × *M. saxatilis*. World Aquaculture Society. DOI: 10.1111/j.1749-7345.2008.00174.x

Wilson, R.P. (2002). Amino acids and protein, In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd edn. Elsevier, Amsterdam, the Netherlands, pp. 309–365.

Xavier, T.O., Michelato, M., Vidal, L.V.O., Furuya, V.R.B., & Furuya, W.M. (2014). Apparent protein and energy digestibility and amino acid availability of commercial meat and bone meal for Nile tilapia, *Oreochromis niloticus*. *J.World Aquacult. Soc. 45*, 439–446.

Yoshioka, M., Yago, T., Yoshie-Stark, Y., Arakawa, H., & Morinaga, T. (2012). Effect of high frequency of intermittent light on the growth and fatty acid profile of *Isochrysis galbana*. *Aquaculture, 338-341*, 111–117.