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Development of innovative feed products and feed concepts for marine species in aquaculture

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PhD Thesis

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Summary

The sustainability challenges of the aquaculture sector will probably be closely linked with continuing interest in ingredients as alternatives to fishmeal for use within aquafeeds. Among the many protein sources available, plant proteins appear to be the most appropriate alternatives. Different aspects on the health implications of using vegetable feed ingredients on the digestive tract, plus possible effects on quality of the fish, are some of the most relevant problems currently confronting the aqua industry.

The need to understand this phenomenon leads to the present thesis, in which the investigation on the feasibility of the inclusion of plant protein in aquafeed was undertaken through a multidisciplinary approach.

The first study highlighted that inclusion of soybean meal in combination with different blends of plant protein maintains optimal growth in 20% fish meal dietary level for the on-growing of European sea bass. Despite fiber is considered as a component that cannot be utilized by most fish, inclusion up to 9.3% has no effects on growth, feed efficiency and digesta transit time in European sea bass. It was encouraging to note that inclusion of high levels of plant protein sources and fiber did not affect health and nutritional status of the animals. When replacing fish meal in aquafeeds, besides the research on alternative ingredients, also the nutritional profile should be considered. In the third study, it was demonstrated that taurine is a required nutrient for juvenile Southern flounder. The fourth study, highlighted that increasing the inclusion of plant ingredients, in substitution of fish meal, had no effects on quality traits of the commercial product.

However, it is not yet an easy or economically feasible task to reduce the aquaculture dependence from marine feedstuff, the present thesis has contributed to provide some insights on the feasibility of the replacement of fish meal in aquafeed.

Table of contents

Introduction

In developing the vision of aquaculture, a focus on the global challenge of doubling food production is fundamental, to feed in a sustainable manner the nine billion people predicted to be on the planet in 2050. Global aquaculture production has more than doubled since 2000, increasing from 41.7 million tonnes to a new high of over 90.4 million tonnes in 2012, with production growing at an annual average rate of 6.7% since then (FAO, 2014). For the finfish and crustacean aquaculture sector to maintain its current growth rate, the supply of nutrient and feed inputs will have to grow at a similar rate (Tacon and Metian, 2015). In fact, aquaculture, as like other intensive terrestrial animal production activities, is totally dependent upon the external provision of feed or nutrient inputs to the culture system (Tacon and Metian, 2008).

In particular, continued growth and intensification of the world finfish production depends upon the development of sustainable protein sources to replace fish meal in aquafeeds (Gatlin et al., 2007). For several years, there has been continuing interest in identifying and developing ingredients as alternatives to fish meal for use within aquafeeds in particular due to the concern raised about the negative impact of fish meal production on global fish stocks has heightened this interest (Tacon et al., 1998; Naylor et al., 2000). Notwithstanding the above, fish meal and fish oil are not essential feed ingredients *per se*, but rather represent ideal feed ingredients for the aquaculture sector by possessing a nutritional profile approximating to the nutritional requirements of most farmed aquatic species (Tacon and Metian, 2015).

Fish diets of the future will include a wider range of alterative ingredients to fishmeal, therefore is currently the case to identify new ingredients as a potential alternative to fishmeal for use in aquafeed. Among the many protein sources available for animal feeds, plant proteins appear to be the most appropriate alternatives for fish meal in fish diets (Fournier et al., 2004).

There are several key components that should be considered in ingredient assessment including ingredient characterization, ingredient digestibility, palatability, nutrient utilization, functionality and trade commodities/market availability. Ingredient nutritional characterization is the first part of any evaluation process. As reported by Glencross (2007), chemical composition, variability in composition, source and species of origin are all important factors that need to be documented so as to allow any meaningful assessment and reporting of that assessment. Determination of ingredient palatability is the second key component of knowledge required about an ingredient before it can be successfully used. Palatability is defined as the combination of both attractiveness and ingestion of a diet and therefore of most relevance to feed development. This is important because, irrespective of how digestible and available the nutrients and energy from an ingredient might be, if the ingredient reduces feed intake then it will have limited value (Glencross 2007). Commonly, a decrease of feed intake is observed in fish fed diets containing plant protein, due to a reduced feed palatability. Ingredient functionality is another crucial aspect of ingredient evaluation. If an ingredient cannot be functionally introduced into a feed in a manner that allows its processing in a suitable manner, then it is of diminished value as a feed ingredient. Alternatively, some ingredients may add additional value to a diet, based on some functionality features that they contribute to a formulation (Glencross, 2007). Trade commodities are another aspect to be considered in aquafeed formulation. Feed production relies on a basket of common input plant protein ingredients, and other sectors compete for same feed resources (such as animal husbandry or human consumption) (Rana et al., 2009). Since many key ingredients traditionally used in recipes for commercial feeds are internationally traded commodities, aquafeed production is also subjected to any common global market shocks and volatility, thus the increase in the cost of raw ingredients for commercially manufactured or on-farm

aquafeeds resulted in an increase in aquafeed prices (Rana et al., 2009; Lall and Dumas, 2015). Thus, feed ingredient selection by aquaculture feed compounders is usually based upon series of different considerations, these ranging from market availability and cost, nutritional composition and quality, processing/handling requirements and limitations, target species acceptability, to market acceptability for use (Tacon and Metian, 2015). Once identified some products as alternative ingredients, a key area of investigation includes the evaluation of varying proportions and combinations of these ingredients to identify mixtures and formulation that can be modeled based on sustainability, availability and price volatility of ingredients (Tacon and Metian, 2015; De Santis et al., 2016).

Besides the cost of feed, there is substantial pressure to develop formulations that maintain efficient growth at lower cost per unit gain (Hardy, 2010). Thus, with a growing interest in non-fishmeal protein sources for aquafeeds there are several challenges that must be overcome to maintain and enhance growth rates and feed efficiency values at higher levels of substitution of fishmeal (Li et al., 2009).

Moreover, proper nutrition is critical not only to achieve optimal growth rates but also to maintain the health of cultured fish (Sealey and Gatlin, 2001). In the past, in the aquaculture industry relatively little attention has been given so far to the optimal functioning of the digestive system of fish and shrimp and the fish nutrition field focused mainly on establishing the minimum nutrient requirements for normal growth of different fish species (NRC, 2011). Many aquafeed formulations were mainly focused on nutritional specifications and ingredient choices, whereas the optimal utilization of the nutrients by the fish and the health status of the digestive tract are two areas that are not often taken into account. Indeed, nowadays some of the most relevant problems currently confronting the aqua industry are related to the fish health status. The gastro intestinal tract adjusts to changing diet composition, and the mucosal defence system provided by the gastro

intestinal tract must protect the body from injurious agents and at the same time develop oral tolerance to antigens from the diet and commensal microbiota (Chehade and Mayer, 2005). Furthermore, in farmed species, dry diets impose an extra osmotic stress on the intestine, triggering post-prandial drinking and water influx in the chyme from the extracellular fluid in order to moisturize feed to an adequate level for its digestion (Ruohonen et al., 1997; Kristiansen and Rankin, 2001). Histology of the digestive system is one of the tool used to evaluate the health status of fish because it can assess pathological changes that can be induced by nutritional factors. Histopathology can provide additional information on the zootechnical performances to better explore mechanisms involved in digestion, feed utilization, metabolism, and monitoring the overall health of reared fish.

Besides the study of blood metabolites, histological analysis of the digestive system is considered a valid tool to evaluate the health status of fish and the effects of diet formulation on haematological parameters recently have received particular attention. For terrestrial animals, with the development of routine blood tests, evaluation of nutritional status became more practical, sophisticated and precise, allowing an earlier detection of impaired malnutrition and health. Indeed, for terrestrial animals, blood analysis is usually one of the routine evaluation tools, providing important diagnostic and prognostic information of pathological and metabolic disorders and of therapeutic approaches (Kerr, 2008). In fish, impair feeding, specific nutritional deficiencies, stress and disease induce changes in the blood constituents. Thus, the use of blood biochemistry as diagnostic is becoming a useful tool to assess the nutritional condition of animal under intensive aquaculture conditions, also for its characteristics of not being lethal and non-invasive.

With the growth of global aquaculture production, another aspect to be considered is the effect of commercial feeds on fish quality, since consumers are raising their

expectations on quality, nutritional value, healthiness, taste and freshness. It is generally accepted that farmed fish quality can be influenced by the formulation of composition of their feed. As reported by Hardy and Lee (2010), this is a potential advantage for farmed fish over wild fish, but one that the aquaculture industry has been slow to exploit. By using science-based nutritional approaches, farmed fish can be produced to meet target quality characteristics on a consistent basis. Altering nutrient content of feed has an important impact on several parameters, directly influencing the quality of the fish, such as colour and appearance, smell and taste, texture, nutritional quality, shelf life, and level of contaminants (Lie, 2001). The quality of farmed fish can be controlled to a large extent by feeding level, nutrient level in feed, ingredient selection, and protein/lipid ratio of the feed during the latter stages of production.

Given these considerations, behind the importance to find sustainable protein sources to replace fish meal in aquafeed, is necessary to consider all the aspects of these ingredients, with a particular attention to health status and the quality of the animals.

The overall objective of this thesis was to gain further knowledge on the development of innovative feed products and feed concepts for marine species in aquaculture with estimation of the nutritional value of different plant protein ingredients.

The diet manipulation can actively play a role in reducing the fish meal content and the products derived from soybean are some of the most studied plant feedstuffs. On the other hand, soybean meal has been found to induce a variety of histological and functional changes in the gastrointestinal tracts of some finfish species. Thus, the first study in this thesis was the evaluation of varying proportions and combinations of soy ingredients to identify mixtures that are more efficiently utilized by the fish. In Chapter 1, the effects of increasing levels of soybean meal by replacing a mix of plant ingredients (wheat meal, wheat gluten, corn gluten and sunflower meal) in low fishmeal diets, on growth, blood

biochemistry profile and gut histology in European sea bass (*Dicentrarchus labrax*) were investigated.

Changing trends in fish feed formulation, with progressively higher inclusion levels of plant ingredients, will invariably introduce more fiber despite this part of the carbohydrate component of plant ingredients cannot be utilized by most fish. Thus, the effects of increasing the dietary fiber level (2.8-9.3%) on growth, nutrient utilization, blood parameters and gut health in European sea bass (*Dicentrarchus labrax* L.) were studied over 117 days (Chapter 2). Moreover, investigation on digesta transit time through gastrointestinal evacuation pattern and on digesta characteristics was conducted.

Commercial food producers have been trying to substitute fishmeal using alternative plant protein sources, but these products are often devoid or contain very low concentrations of Taurine compared to fishmeal. This amino acid is considered an essential nutrient in some teleost species and comparison among finfish species leads to the conclusion that the response to dietary taurine seems to be species specific. Hence, the following chapter (Chapter 3) was focusing on the understanding of the role of taurine in Southern flounder (*Paralichthys lethostigma*), an important cultured fish species both for stock enhancement and food fish production, in order to evaluate if this amino acid is an essential nutrient for this species.

It is generally accepted that farmed fish quality can be influenced by the formulation of composition of their feed. For this reason, besides the nutritional properties, another aspect to be considered is if a novel dietary formulation could affect certain quality attributes of the whole fish or flesh, since consumers are raising their expectations on nutritional value, healthiness, taste and freshness. Therefore, in Chapter 4 were studied the effects of increasing levels of a blend of plant proteins, in substitution of fish meal, on growth and quality indexes.

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Chapter 1

Effects of feeding low fishmeal diets with increasing soybean meal levels on growth, gut histology and plasma biochemistry of European sea bass (*Dicentrarchus labrax*)

Under review in "Animal"

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Abstract

The aquaculture industry depends upon the development of sustainable protein sources to replace fishmeal (FM) in aquafeeds and the products derived from soybeans are some of the most studied plant feedstuffs. A key area of investigation for continuing to improve modern aquafeeds includes the evaluation of varying proportions and combinations of plant ingredients to identify mixtures that are more efficiently utilized by the fish. This study investigated the effects of increasing soybean meal (SBM) by replacing a mix of plant ingredients in low FM (20%) diets on growth, blood biochemistry profile and gut histology on European sea bass. Five isonitrogenous and isolipidic experimental diets were formulated: four diets containing increasing SBM levels (0, 10, 20 and 30%; 0SBM, 10SBM, 20SBM and 30SBM, respectively) with a low content of FM (20%) and one control diet (CD) (0% SBM; 35% FM). Diets containing SBM brought to comparable performance and protein utilization to CD, while 0SBM had negative impact on feed conversion rate and protein utilization. Blood parameters suggested an optimal nutritional status under all feeding treatments, even though slightly decreased values were reported at increasing dietary SBM. Histology examination did not show any changes indicative of soy-induced enteritis. We can conclude that for European sea bass: i) different blends of plant protein did not affect feed intake despite the 20% FM dietary level; ii) the inclusion of SBM maintains optimal growth and feed utilization in low FM diets; iii) blood biochemistry profile showed a good nutritional status under all feeding regimes; iv) no evidence of soy-induced enteritis was reported in any group fed low FM diets. For formulation of practical diets in on-growing of European sea bass, SBM up to 30% can be successfully incorporated into feeds containing low FM inclusion.

1.1 Introduction

For several years, there has been continuing interest in identifying and developing ingredients as alternatives to fish meal (FM) for use within aquafeeds, resulting in a substantial decrease of this ingredient in the feed formulation of many species (Tacon et al., 1998). Nowadays aquaculture relies on a basket of common input plant protein ingredients such as soybean meal (SBM), gluten, sunflower meal, for which it competes in the marketplace with the animal husbandry sector as well as with use for direct human consumption (Rana et al., 2009). Based on evidence indicating that mixed dietary plant proteins outperform single sources, a key area of investigation for continuing to improve modern aquafeeds includes the evaluation of varying proportions and combinations of plant ingredients to identify mixtures that are more efficiently utilized by the fish (De Santis et al., 2016). Among the different plant ingredients, SBM is one of the most interesting alternatives to FM because of the advantages of supply, price, protein and amino acid composition (Rossi et al., 2013; Parma et al., 2016). However, SBM has been found to induce a variety of histological and functional changes in the gastrointestinal tracts of several species, especially in salmonids, such as subacute enteritis of the distal epithelial mucosa including morphological alteration and inflammation (Krogdahl et al., 2010).

To our knowledge, only a few studies have explored in European sea bass (*Dicentrarchus labrax*) the effects of increasing levels of soybean meal on performance, and most of the data in the literature were restricted to replacing FM with SBM. Hence, this study investigated the effects of increasing SBM by replacing a mix of plant ingredients (wheat meal, wheat gluten, corn gluten and sunflower meal) in low fishmeal FM diets, on growth, blood biochemistry profile and gut histology in European sea bass.

1.2 Materials and methods

1.2.1 Experimental diets

Five isonitrogenous and isolipidic experimental diets were formulated: four diets containing increasing SBM levels (0, 10, 20 and 30%; 0SBM, 10SBM, 20SBM and 30SBM, respectively) with a low content of FM (20%) and one control diet (CD) (0% SBM; 35% FM). Ingredients and proximate composition of the experimental diets are presented in Table 1.1.

Table 1.1. Ingredients and proximate composition of the experimental diets.

	Experimental diets				
	CD	0SBM	10SBM	20SBM	30SBM
Ingredient, % of the diet					
FM North-Atlantic	35.00	20.00	20.00	20.00	20.00
Hi-pro SMB	0.00	0.00	10.00	20.00	30.00
Wheat	21.43	19.31	15.13	10.94	6.75
Corn gluten	12.00	18.00	16.00	14.00	12.00
Wheat gluten	12.05	18.07	15.98	13.89	11.80
Sunflower meal	4.00	8.00	6.00	4.00	2.00
Fish oil North-Atlantic	15.02	16.12	16.40	16.67	16.95
Vit/Min premix[1]	0.50	0.50	0.50	0.50	0.50
Proximate composition[2]					
Protein	45.41	46.10	47.00	46.63	46.57
Lipid	19.56	19.13	19.19	19.83	20.17
Ash	5.64	4.42	4.72	5.00	5.31
Moisture	6.26	6.61	6.66	6.30	7.22
Energy (MJ)	22.0	22.4	21.9	22.1	22.0

[1]Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011).
[2]Values are reported as mean of duplicate analyses.

SBM was replaced by adding a combination of wheat meal, wheat gluten, corn gluten and sunflower meal, as recently described by Parma et al. (2016) on gilthead seabream (*Sparus aurata* L.). The diets were produced by Skretting Aquaculture Research Centre, Stavanger, Norway. According to the feed manufacturer, protein and lipid levels were within the range of commercial diets for sea bass. The high FM level (35%) in the CD group, was

designed as a low plant protein control diet compared to the rest of experimental diets. The diameter of the pellet was 4 mm.

1.2.2 Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea bass juveniles were obtained from Ecloserie Marine de Gravelines (Gravelines, France). One week before the beginning of the trial, fish were fed with a mixture of the five experimental diets. At the beginning of the trial, 60 fish (initial average weight 68.9 ± 1.7 g) per tank were randomly distributed into fifteen 1000 L square tanks with a conical bottom. Each diet was administered to triplicate groups, assigned in a completely random manner, over 91 days. Tanks were provided with natural seawater and connected to a closed recirculation system (overall water volume: 18 m^3). The rearing system consisted of a mechanical sand filter (Astralpool, Spain), ultraviolet lights (Philips, the Netherlands) and a biofilter (Astralpool, Spain). The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept constant at 22 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant at 100% saturation by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 ppm), nitrite (≤ 0.2 ppm) and salinity (25 g L^{-1}) were spectrophotometrically monitored daily (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8–8.0. Feed was provided to 10% overfeeding by automatic feeders, twice a day for six days a week, while one meal was supplied on Sundays, as reported by Mongile et al. (2014). Each meal lasted 1 hour, after

that the uneaten pellets in each tank were gathered, dried overnight at 105°C, and their weight was deducted for overall calculation.

1.2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were anesthetized and individually weighed. In case of any mortality, fish were immediately removed and the weight was recorded for overall calculation. Specific growth rate, voluntary feed intake and feed conversion rate were calculated. Survival rate was calculated as a percentage of the initial number of fish. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and on pooled samples of 5 fish per tank at the end of the trial. Protein efficiency rate, gross protein efficiency and gross lipid efficiency were calculated. Furthermore, at the end of the trial, wet weight, viscera, liver and visceral fat weight were individually recorded for 5 fish per tank to determine viscerosomatic index, hepatosomatic index, fat index and fillet yield. At the end of the trial five fish per tank (15 fish per dietary treatment) were sampled for intestine histology examination. After the end of the trial, the fish left were kept in the same rearing and feeding conditions for three more days and then were sampled to perform blood analyses of hematocrit, serum total protein, triglycerides and cholesterol. Blood from 5 fish per tank was collected 5 h postprandial from the caudal vein. Samples were then centrifuged (3000g for 10 min at 4°C) and serum aliquots were stored at 4°C and analyzed during the same day.

All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

1.2.4 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Voluntary Feed Intake (VFI) (g feed $fish^{-1}$) = g feed ingested / fish number. Feed conversion ratio (FCR) = feed intake / weight gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight). Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight / body weight). Fillet yield (FY) (%) = 100 * (fillet weight / body weight). Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.

1.2.5 Histology

After euthanasia, the gut was removed and the intestine was divided into three segments (proximal, intermediate and distal). From each segment, a 5 mm-long piece was sectioned and fixed in 10% buffered formalin. Samples were then processed for routine histology to obtain a transversal section, which was stained with hematoxylin and eosin (H&E). Sections were evaluated blind under a light microscope (Nikon Eclipse 80i, Nikon Corporation, Japan) for degenerative and inflammatory changes. Regarding diet CD and 30SBM, also morphometry and scoring were conducted for proximal and distal tracts. Morphometry consisted in measurements at 100x magnification of five randomly selected fields per intestinal tract. Width of *lamina propria*, width of mucosal layer in the middle of the folds, height of mucosal layer at the base of the folds and height of *submucosa* were measured. Goblet cells were counted in three fields per intestinal tract at 40x magnification. A continuous scale scoring system, with the range of scores set at 0–3, was used for evaluation of inflammatory cells infiltration, number of mast cells and number of intraepithelial lymphocytes. This scoring system was adapted from that used for Atlantic

salmon by Penn et al. (2011). Values of each separate parameter of scoring and morphometry were calculated by averaging.

1.2.6 Analytical methods

Diets and whole body were analyzed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450 °C. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, USA).

The hematocrit value was obtained as packed cell volume % using microhematocrit tubes on a dedicated centrifuge (Hettich Haematokrit 210, Tuttingen, Germany). Serum total protein, cholesterol and triglycerides were measured using colorimetric methods (Total protein OSR6232, Cholesterol OSR6216, Triglyceride OSR61118; Beckman Coulter, Brea, CA, USA) on an automated analyzer (AU 400, Beckman Coulter, Brea CA, USA).

1.2.7 Statistical analysis

All data are presented as mean ± standard deviation (SD). Tank was used as the experimental unit for analyzing growth and performance, a pool of five sampled fish was considered the experimental unit for analyzing carcass composition, whereas individual fish was used for analyzing VSI, HSI, FaI, FY and all blood parameters. All data, except for histological, were analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA). The differences among treatments were considered significant at $P \leq 0.05$. Histological data were analyzed using software Statistica 8 (StatSoft Inc., Tulsa, Oklahoma). Normal distribution was tested by Shapiro-

Wilk. If normality criteria were not met, Mann-Whitney U-testing was run. Significance was set at $P \leq 0.05$.

1.3 Results

1.3.1 Growth

Growth performances are summarized in Table 1.2.

Table 1.2. Growth performance, intake and survival of European sea bass fed experimental diets over 91 days.

	Experimental diets					
	CD	0SBM	10SBM	20SBM	30SBM	*P* value
IBW (g)	68.2 ± 1.3	68.7 ± 0.4	70.0 ± 0.9	69.4 ± 3.2	68.3 ± 2.1	0.7138
FBW (g)	231.6 ± 8.0	219.3 ± 2.5	230.7 ± 3.4	228.3 ± 4.4	228.4 ± 10.4	0.2239
SGR (day^{-1})	1.34 ± 0.06	1.28 ± 0.01	1.31 ± 0.03	1.31 ± 0.03	1.33 ± 0.04	0.3275
VFI	210 ± 2.9	211 ± 4.5	219 ± 4.8	210 ± 3.7	215 ± 7.7	0.1586
FCR	1.28^{a} ± 0.06	1.38^{b} ± 0.03	1.35^{ab} ± 0.02	1.31^{ab} ± 0.02	1.35^{ab} ± 0.01	0.0234
Survival (%)	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	99 ± 1.0	0.4516

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights).
VFI = Voluntary Feed Intake (g feed/fish) = g feed ingested/fish
FCR = Feed conversion rate = feed intake / weight gain.

No significant differences between treatments were found in final body weight, specific growth rate and voluntary feed intake. Fish fed diet CD showed lower feed conversion rate in comparison to those fed diet 0SBM. No significant differences were recorded in survival rate among groups.

Data on biometric indices, body composition and nutritional indices are shown in Table 1.3. No significant differences were found in viscerosomatic indices, fat index and fillet yield. Within the groups fed low FM, hepatosomatic index significantly decreased from 0SBM to 20SBM, while fish fed diet CD showed higher value in comparison to that fed 10SBM, 20SBM and 30SBM. No significant differences were found in the whole body

proximate composition. Fish fed diet CD showed higher protein efficiency rate in comparison to those fed diet 0SBM and 10SBM and higher gross protein efficiency in comparison to 0SBM. No significant differences among treatments were found in gross lipid efficiency.

Table 1.3. Biometric indices, body composition and nutritional indices of European sea bass fed the experimental diets.

	Experimental diets					
	CD	0SBM	10SBM	20SBM	30SBM	*P* value
Biometric indices						
VSI	12.7 ± 1.9	12.4 ± 2.2	11.8 ± 1.7	11.0 ± 2.3	12.1 ± 2.0	0.2000
HIS	2.2^{d} ± 0.3	2.1^{cd} ± 0.4	1.9^{bc} ± 0.3	1.6^{a} ± 0.3	1.7^{ab} ± 0.4	<0.0001
MFI	9.2 ± 1.6	9.2 ± 2.3	8.4 ± 2.0	7.8 ± 1.9	8.4 ± 1.8	0.2413
FY	48.5 ± 2.0	47.5 ± 1.4	49.0 ± 1.7	47.7 ± 4.7	49.0 ± 3.5	0.2625
Whole body composition, as-is basis						
Protein	16.1 ± 0.3	16.0 ± 0.4	16.2 ± 0.4	16.3 ± 0.2	16.0 ± 0.6	0.8391
Lipid	18.5 ± 1.1	19.0 ± 1.2	18.6 ± 1.0	18.8 ± 1.2	17.8 ± 0.3	0.6606
Ash	3.4 ± 0.3	3.0 ± 0.2	3.1 ± 0.2	3.3 ± 0.2	3.2 ± 0.1	0.7511
Moisture	59.2 ± 0.9	59.2 ± 0.9	58.7 ± 1.3	59.9 ± 1.3	57.9 ± 3.6	0.3324
Nutritional indices						
PER	1.72^{b} ± 0.08	1.55^{a} ± 0.04	1.56^{a} ± 0.02	1.63^{ab} ± 0.02	1.60^{ab} ± 0.04	0.0105
GPE	26.6^{b} ± 0.96	23.6^{a} ± 0.35	24.3^{ab} ± 1.08	25.7^{ab} ± 0.26	24.6^{ab} ± 1.46	0.0210
GLE	79.1 ± 6.69	77.0 ± 5.21	76.3 ± 5.65	77.9 ± 6.43	69.5 ± 2.83	0.3023

Data are given as the mean (n=3; n=15 for VSI, HSI, FaI, FY) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
VSI = Viscerosomatic index (%) = 100 * (viscera weight/body weight).
HSI = Hepatosomatic index (%) = 100 * (liver weight/body weight).
MFI = Mesenteric fat index (%) = 100 * (mesenteric fat weight / FBW).
FY = Fillet yield (%) = 100 * (fillet weight / FBW).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = (100*[(%final body protein * FBW)-(%initial body protein * IBW)]/total protein intake fish $^{-1}$).
GLE = Gross lipid efficiency = (100*[(%final body lipid * FBW)-(%initial body lipid * IBW)]/total lipid intake fish^{-1}).

1.3.2 Blood biochemistry

Hematocrit, serum total protein, triglycerides and cholesterol levels are shown in Table 1.4. No significant differences among treatments were found in hematocrit (*P*=0.0503) and cholesterol (*P*=0.0666) levels, even though the values decreased with the increasing of SBM inclusion within low FM diets (0SBM, 10SBM, 20SBM, 30SBM). Fish fed diet CD showed higher serum total protein in comparison to 30SBM and lower serum triglycerides in comparison to 0SBM and 20SBM.

Table 1.4. Hematocrit, serum total protein, triglycerides and cholesterol concentrations of European sea bass fed the experimental diets.

	Experimental diets					
	CD	0SBM	10SBM	20SBM	30SBM	*P* value
Hematocrit (%)	44.3 ± 4.5	46.2 ± 5.5	45 ± 4.5	41.7 ± 7.0	41.4 ± 3.6	0.0503
Total Protein (g/dl)	5.7^{b} ± 0.8	5.6^{ab} ± 0.6	5.3^{ab} ± 0.4	5.5^{ab} ± 0.5	5.1^{a} ± 0.4	0.0293
Triglycerides (mg/dl)	923^{a} ± 370	1885^{b} ± 809	1630^{ab} ± 746	1649^{b} ± 826	1339^{ab} ± 638	0.0042
Cholesterol (mg/dl)	335 ± 65	369 ± 108	354 ± 81	350 ± 78	288 ± 56	0.0666

Each value is mean from 15 samples ± SD. In each line, different superscript letters indicate significant differences between treatments ($P \leq 0.05$).

1.3.3 Histology

Inflammatory and/or degenerative changes indicative of soy-induced enteritis were not present in any histological section from all subjects examined (Fig. 1.1).

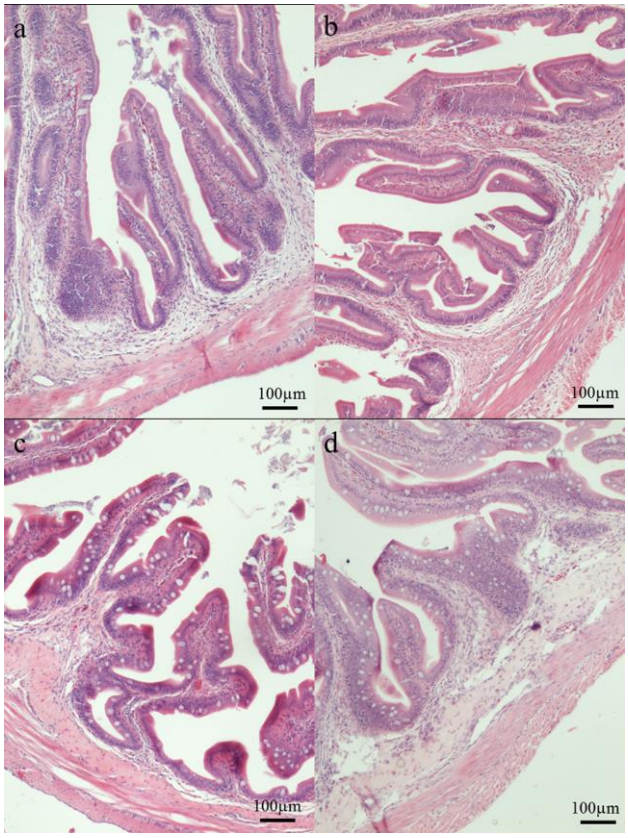

Fig 1.1. *Histomorphology of the proximal and distal intestine of sea bass fed the control diet CD (a,c) and 30SBM diet (b,d). Sections shows a normal architecture of the mucosal (regular columnar epithelium with polarized and basally located nuclei), submucosal (loose connective tissue rich in capillary network) and muscular layer for both treatments (H&E, bar=100 µm).*

Regarding proximal tract, no differences were recorded for morphometry and scoring while a depletion of goblet cells was found in animals fed 30SBM in comparison to CD. Considering distal tract, no differences were recorded for all the parameters evaluated. All data are shown in Table 1.5.

Table 1.5. Morphometry and histopathological scoring of European sea bass fed diets CD and 30SBM.

	Proximal				
	CD		30SBM		
	Median	Range	Median	Range	*P* value
Morphometry (µ)					
Lamina propria	56.2	26.1-71.1	50.5	35.2-88.1	0.4428
Width mucosa	39.0	27.2-53.4	33.2	25.0-46.9	0.1408
Height mucosa	37.3	30.5-47.3	35.4	28.5-51.2	0.8519
Submucosa	145.1	37.9-266.6	152.2	75.3-238.2	0.9174
Goblet cells (number)	56.3[b]	24.0-116.3	24[a]	6-96.7	0.0016
Scoring (0-3)					
Inflammatory cells	1	0-2	1	0-2	0.4553
Mast cells	0	0-2	1	0-2	0.4553
Intraepithelial lymphocytes	1	0-2	1	0-3	0.5069
	Distal				
	CD		30SBM		
	Median	Range	Median	Range	*P* value
Morphometry (µ)					
Lamina propria	46.2	22.8-62.2	45.9	38.4-72.1	0.5755
Width mucosa	30.5	25.6-45.5	31.5	24.9-51.9	0.5755
Height mucosa	37.1	28.1-49.8	36.8	28.4-56.3	0.7874
Submucosa	124.7	83.5-404.8	162.6	82.0-220.1	0.3296
Goblet cells (number)	147.3	43.3-265.7	103.0	6.3-298.0	0.2058
Scoring (0-3)					
Inflammatory cells	1	1-3	1	0-3	0.4306
Mast cells	1	0-3	1	0-3	0.8034
Intraepithelial lymphocytes	1	0-3	1	0-2	0.4428

Each value is median from 15 samples. In each line, different superscript letters indicate significant differences between treatments ($P < 0.05$).

1.4 Discussion

Few studies have investigated the utilization of plant dietary inclusion in European sea bass, but mainly as a FM replacement. To our knowledge, this is the first study to compare the effects of diets containing the same amount of FM (20%) and different plant protein blends, on the performance of European sea bass.

As reported by Médale and Kaushik (2009), a blend of plant protein sources can replace 75 to 95% of FM in almost all species, thus reducing the pressure of aquaculture on marine resources. The authors also reported that to limit the negative effects of each raw material, the strategy adopted is to replace FM with a mixture of vegetable protein sources. Kaushik et al. (2004) observed that an almost total replacement of FM by a mixture of several plant protein sources had no influence on final weight at commercial size in European sea bass. In the same direction, Geay et al. (2011) found that European sea bass fed an exclusively vegetable-based diet exhibited significantly lower final weight and daily growth index than those fed a fish-based diet. Overall, one of the reasons of reduced growth in fish fed diets containing plant protein is a decrease of feed intake, due to a reduced feed palatability. Interestingly, in the present study no differences were recorded in voluntary feed intake among all feeding regimes. As reported by Fournier et al. (2004) The use of several mixtures of plant protein could reduce the potential inhibition of feed consumption due to the specific effect of a single ingredient (Fournier et al., 2004; Bonaldo et al., 2011; Bonaldo et al, 2015). Hence, in our study, despite the low FM level, the inclusion of different blends of different plant feedstuff did not cause palatability problems in European sea bass.

Considering the low FM treatments, all the diets containing SBM brought to comparable performance and protein utilization to CD. On the other hand, the diet without SBM (0SBM) had negative impact on feed conversion rate and protein utilization. Previous studies reported that SBM can substitute up to 25% of the total protein of the diet without any negative effect on European sea bass performance (Lanari and D'Agaro, 2005). As reported by Bonaldo et al. (2008), SBM was successfully incorporated up to a level of 30% without any deleterious effects on feed intake, growth and protein utilization in commercial diets for European sea bass juveniles with 35% of FM. According to our

results, even in diets containing 20% FM the inclusion of SBM up to 30% maintains optimal growth and feed utilization.

No significant differences were found in VSI and this result is in agreement with those previously reported for this species (Kaushik et al., 2004). The authors reported no differences in VSI in European sea bass fed diets containing different levels of FM incorporation. Geay et al. (2011) suggested no influence on VSI in European sea bass fed a plant-based diet, but it seems that this index was regulated only by genetic factors. Instead, HSI values decreased on increasing the inclusion of SBM. Geay et al. (2011) found lower HSI values in European sea bass fed vegetable-based diets in comparison to fish-based diets; on the contrary, Kaushik et al. (2004) reported no differences in European sea bass fed diets with graded levels of FM replacement. Another possible explanation of the trend in HSI, found in our study, can be related to the decrease in level of wheat in SBM diets. As reported by Bonaldo et al. (2008), increasing starch level can increase HSI values, related to an inverse proportion between wheat and SBM in the diets. Carnivorous fish may seem, in general, to have limited capability to digest and utilize dietary starch. Diets high in digestible carbohydrates have been found to increase liver glycogen in salmon and rainbow trout (Krogdahl et al., 2004). In European sea bass, an increase in liver glycogen content with the increase of dietary starch level is usually observed (Enes et al., 2011).

Information on the nutritional status and health in fish species can be achieved through the study of blood metabolites (Peres et al., 2014; Bonvini et al., 2015). To our knowledge, few studies have assessed blood parameters in response to SBM inclusion in low FM diets in marine species. In aquaculture, this practice is not widely spread and it is to be expected that, as occurs in relation to land animals, blood analysis will become a useful tool (Peres et al., 2014). Data obtained in our study are within the ranges reported for European sea bass under good nutritional status (Coeurdacier et al. 2011; Peres et al.

2014; Faggio et al., 2014). However, we found some differences between feeding treatments. Hematocrit averaged between 41.4 and 46.2. Even though no statistical differences were reported (P=0.0503), lower values were found in fish fed diet 20SBM and 30SBM. As reported for Persian sturgeon (*Acipenser persicus*) (Imanpoor et al., 2010) and juvenile beluga (*Huso huso*) (Hosseini and Khajepour, 2013), hematocrit was significantly lowered by increasing SBM of diets. On the contrary, Moradi et al. (2013) reported that hematocrit concentration decreased parallel to plant protein inclusion in diet in *Cyprinus carpio*. In rats, raw soy bean reduced red cell osmotic fragility and could also reduce hematocrit depending on the processing methods applied (Olaleye et al. 1999; Hosseini and Khajepour, 2013). These effects were ascribed to the presence of trypsin inhibitor in soy protein. On the other hand, Alada et al. (2004), reported that consumption of soya bean diet preparations by rats caused gradual but significant increases in hematocrit as the concentration of soya bean in the diet increased. Plasma proteins averaged between 5.7 and 5.1 g dl-1, with the lowest value recorded in diet 30SBM (5.1±0.4 g/dl). Plasma protein level is usually very stable in well-nourished animals but decreases under fasting conditions (Coeurdacier et al. 2011; Peres et al., 2014). Indeed, under malnutrition or stress conditions, altered plasma total protein levels often occur as a consequence of amino acid oxidation or peripheral proteolysis (Peres et al., 2014). There are no clear relationships between the level of SBM in the diet and change in blood protein level of the cultured fish; further investigations will be necessary to determine and understand the effects of the diets on variations of this parameter. Triglycerides and cholesterol levels are in agreement with previously reported values for this species (Couto et al., 2015; Guerreiro et al., 2015), but higher than those reported by Peres et al. (2014). In low FM diets, triglycerides concentration slightly decreased with the increasing of SBM. It has been reported that dietary soy product suppresses lipogenic enzyme activities and may consequently lower

liver and plasma triglyceride levels in different fish species (Dias et al., 2005; Lim and Lee, 2009; Lim et al., 2011). Regarding cholesterol, no statistical differences were detected (P=0.0666), even though the values decreased with the increasing of SBM inclusion within low FM diets. Indeed, several researches reported that soybean components have a hypocholesterolemic effect in fish (Kaushik et al., 1995; Gómez-Requeni et al., 2004), including European sea bass (Couto et al., 2015; Guerreiro et al., 2015).

Regarding histology, no inflammatory and/or degenerative changes were recorded in any of the histological sections. Soybean meal has been found to induce a variety of histological/functional changes in the gastrointestinal tracts of salmonids (Krogdahl et al., 2010). European sea bass seems to be less sensitive to certain soy-anti-nutritional factors, which induce intestinal disturbances in salmonids (Tibaldi et al., 2006). Bonaldo et al. (2008) too reported no evidence of soy-induced enteritis in European sea bass juveniles fed diets with 35% of FM and 30% of SBM. Our results support the thesis that SBM, even at the high inclusion rate of 30% in a diet with only 20% of FM, did not lead to soy-induced enteritis in European sea bass. Further, no differences were recorded in measurement and scoring in proximal and distal tracts in CD and 30SBM diets, and a slight depletion of goblet cells in proximal tracts in 30SBM diet was the only difference reported. Few references are available on this subject. Olsen et al. (2007) observed in cod (*Gadus morhua*) goblet cell hypertrophy and hyperplasia in the distal parts of the gastrointestinal tract in fish fed high plant protein levels. Notwithstanding the above, few studies reported a depletion in number of goblet cells in fish fed a diet with other vegetable raw materials like Jojoba meal (Saleh and Toutou, 2015) and vegetable oil (Perez-Sanchez et al., 2013), but to our knowledge the reasons for this decrement are still unknown. Further investigations will be necessary to determine and understand the effects of the diets on the depletion of these cells.

In conclusion, from our findings we can assess that for European sea bass: i) different blends of plant protein did not affect feed intake despite the 20% FM dietary level; ii) the inclusion of SBM maintains optimal growth and feed utilization in low FM diets; iii) blood biochemistry profile showed a good nutritional status under all feeding regimes; iv) no evidence of soy-induced enteritis was reported in any group fed low FM diets. For formulation of practical diets in on-growing of European sea bass, SBM up to 30% can be successfully incorporated in low FM.

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Chapter 2

Feeding European see bass (*Dicentrarchus labrax*) with increasing dietary fiber levels: impact on growth, blood biochemistry, gut histology, gut evacuation

Under review in "Animal feed science and technology"

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Abstract

Changing trends in fish feed formulation, with progressively higher inclusion levels of plant ingredients, are invariably introducing more fiber despite this component cannot be utilized by most fish. Thus, the effects of increasing the dietary fiber level (2.8-9.3%) on growth, nutrient utilization, blood parameters and gut health in European sea bass (*Dicentrarchus labrax* L.) were studied over 117 days. Moreover, investigation on digesta transit time through gastrointestinal evacuation pattern and digesta characteristics (gastrointestinal water content and moisture of digesta) were studied. No significant differences due to fiber inclusion levels were observed in final body weight, specific growth rate, feed intake, feed conversion rate, protein and lipid efficiency. No significant differences in serum total protein, glucose, triglycerides, alkaline phosphatase and inorganic phosphorous were found. All the histological section showed a normal intestinal architecture, inflammatory and/or degenerative changes were not present in any histological section from all subjects examined. The investigation on gastrointestinal evacuation pattern revealed no significant differences between treatments, despite increasing fiber levels in the diets tended to an increasing in time required to empty the stomach, (22 h *vs* 35 h to empty the 90% of the stomach content in diet F2.8 and F9.3, respectively). On the other hand, the time required to empty the 90% of the hindgut content was similar in all the treatments, ranging around 46-47 h. No differences were found between diets in digesta characteristics (water content and moisture of digesta). We can conclude that, the different fiber levels tested in this trial have no effects on overall performances and feed efficiency in European sea bass. Results from blood biochemistry profile and histology confirm a good nutritional and health status of fish under all feeding treatments. Fiber had no influence also on digesta transit time and digesta characteristics. However, increasing fiber levels in the diets tended to an increasing in time required to

empty the stomach. In formulation of feed for the on-growing of European sea bass fiber can be included up to 9.3%.

2.1 Introduction

Dietary fiber is the edible part of plants, or similar carbohydrates, that are resistant to digestion and absorption in the small intestine (Lattimer and Haub, 2010). Cellulose and other fibrous carbohydrates are found in the structural components of plants and are indigestible to monogastric (simple-stomach) animals, as fish. Changing trends in fish feed formulation, with progressively higher inclusion levels of plant ingredients, are invariably introducing more fiber.

The fiber content in some plant proteins might alter intestinal function and can interfere with nutrient digestibility in fish (Francis et al., 2001; Krogdahl et al., 2005). Moreover, the presence of fiber can affect gastrointestinal transit time of feed (Storebakken et al., 1999; Zhou et al., 2004). There is little published information regarding the effects of dietary fiber inclusion in fish. Moreover, as reported by Altan and Korkut (2011), low dietary concentrations of dietary fibre (3–5%) may have a beneficial effect on fish growth, but on the contrary, high dietary fibre (>8%) may decrease dry matter digestibility of the diet and reduce the availability of other nutrients. Some discrepancies between values of maximum dietary fiber level have been reported for fish: less than 7% (Altan and Korkut, 2011); less than 8% (Eusebio et al., 2004); as low as possible and not exceed 10% (NRC, 2011). For the on-growing of the European sea bass, as reported by Kousoulaki et al. (2015), the fiber contents in commercial feeds ranged between 1.5-3.2%.

Thus, the effects of increasing the dietary fiber level (2.8-9.3%) on growth, nutrient utilization, blood parameters, gut health, gastrointestinal evacuation pattern and digesta characteristics in European sea bass (*Dicentrarchus labrax* L.) were studied over 117 days.

2.2 Materials and methods

2.2.1 Experimental diets

Five iso-proteic and iso-energetic diets were formulated to contain increasing fiber levels (2.8, 4.5, 6, 7.1 and 9.3%; F2.8, F4.5, F6, F7.1 and F9.3 respectively). The fiber content was increased by increasing levels of a combination of sunflower hulls and soya bean hulls to provide same proportion of fiber from each ingredient. The diets were produced by extrusion process by Skretting Aquaculture Research Centre, Stavanger, Norway. The diameter of the pellet was 4 mm. Ingredients and proximate composition of the experimental diets are presented in Table 2.1.

Table 2.1. Ingredients and proximate composition of the experimental diets.

	Experimental diets				
	F2.8	F4.5	F6	F7.1	F9.3
Ingredient, % of the diet					
Sunflower Hulls	0.0	1.5	3.2	4.8	6.4
Soybean Hulls	0.0	2	4.2	6.4	8.6
Fish meal	20	20	20	20	20
SBM concentrate	13	13	13	13	13
Wheat	28.4	23.3	17.9	12.4	6.9
Corn gluten	8	8	8	8	8
Wheat gluten	15.2	15.6	16.1	16.6	17.0
Fish oil	7.5	8	8.6	9.2	9.8
Rapeseed oil	7.5	8	8.6	9.2	9.8
Vit/Min premix[1]	5	5	5	5	5
Proximate composition[2]					
Protein	43.6	43.2	43.5	43.8	43.7
Lipid	22.5	24.3	24.8	25.4	27.1
Ash	4.42	4.47	4.53	4.63	4.73
Moisture	7.28	7.77	7.71	7.49	7.74
NFE[3]	23.1	20.5	17.4	14.5	11.6
ADF	2.8	4.5	6.0	7.1	9.3
Gross Energy[4] (MJ)	22.0	21.9	21.9	22.0	22.0

[1] Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011).
[2]Values are reported as mean of duplicate analyses.
[3]Nitrogen-free extract
[4] Calculated using following caloric values: protein 23.6 kg/J; lipid 38.9 kg/J; NFE 16.7 kg/J (Miglavs and Jobling, 1988)

2.2.2 Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea

bass juveniles were obtained from Maricoltura Mattinatese (Mattinata, Foggia, Italy). At the beginning of the trial, 60 fish (initial average weight: 69.4 ± 2.3 g) per tank were randomly distributed into fifteen 900 L square tanks with a conical bottom. Each diet was administered to triplicate groups, assigned in a completely random manner, over 117 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume: 18 m^3). The rearing system consisted of a mechanical sand filter (Astralpool, Spain), ultraviolet lights (Philips, the Netherlands) and a biofilter (Astralpool, Spain). The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept constant at 22 ± 1.0°C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant at 100% saturation by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 ppm), nitrite (≤ 0.2 ppm) and salinity (25 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on daily basis to keep pH constant at 7.8–8.0. Feed was provided to 10% overfeeding by automatic feeders, twice a day for six days a week, while one meal was supplied on Sundays. Each meal lasted 1 hour, after that the uneaten pellets of each tank were gathered, dried overnight at 105°C and their weight was deducted for overall calculation.

2.2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were anesthetized and individually weighed. Specific growth rate (SGR), voluntary feed intake (VFI) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER),

gross protein efficiency (GPE) and gross lipid efficiency (GLE) were calculated. Furthermore, at the end of the trial, wet weight, viscera, liver and mesenteric fat weight were individually recorded for 10 fish per tank to determine viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat index (MFI). At the end of the trial, five fish per tank (15 fish per dietary treatment) were sampled for intestine histology examination. At the end of the trial, the fish left were kept in the same rearing and feeding conditions for three more days and then were sampled to perform blood analyses of serum total protein (TP), triglycerides (TRIG), glucose (GLU), alkaline phosphatase (ALP) and inorganic phosphorus (P). Blood from 4 fish per tank was collected 5 h postprandial from the caudal vein. Samples were then centrifuged (3000 g for 10 min at 4°C), serum aliquots were stored at 4°C and analysed during the same day. All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.2.4 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Voluntary Feed Intake (VFI) (g feed $fish^{-1}$) = g feed ingested / fish number. Feed conversion ratio (FCR) = feed intake / weight gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight). Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight / body weight). Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross lipid

efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.

2.2.5 Histology

After euthanasia, the gut was removed and the intestine was divided into two segments (proximal and distal). From each segment, a 5 mm-long piece was sectioned and fixed in 10% buffered formalin. Samples were processed for routine histology to obtain a transversal section, which was stained with haematoxylin and eosin (H&E). Sections were evaluated blind under a light microscope (Nikon Eclipse 80i, Nikon Corporation, Japan) to verify the preservation of the normal intestinal architecture. In particular, the histological investigation was focused on the main cells constituents of the mucosal layer (goblet cells, supranuclear absorption vacuoles in the enterocytes), capillary within the intestinal folds, lymphoplasmacytic cells within lamina propria (GALT-like tissue). Moreover, any possible degenerative and diets adaptive induced changes were taken in consideration.

2.2.6 Gastric evacuation, time and digesta characteristics

Following the feeding trial, sampling for gastrointestinal evacuation pattern and digesta characteristics was conducted. We adopted the approach used in the studies by Adamidou et al. (2009a) and Nikolopoulou et al. (2011). Fish were fasted for 72 h before being fed to make sure that the gastrointestinal tract was empty. Four fish per treatment were sacrificed at 1, 4, 10, 16, 28 and 48 h after feeding a single meal to satiation. Each sampled fish was weighted, then the abdominal cavity was opened and the digestive tract was carefully removed and separated into three parts: stomach, foregut and hindgut. Foregut was defined as the section from the pyloric sphincter to the ileocecal valve and hindgut from the ileocecal valve to the anus. Stomach and intestinal contents from the above sections were collected in pre-weighed dishes, weighed for each fish separately, dried and reweighed. The measured weights were used for the calculations described below. The geometric

means of stomach and intestinal dry digesta content divided by the fish weight were regressed against time separately for each diet, in order to fit to a model for calculating gastric evacuation rate (GER) for the stomach, gastric filling time (GFT) for foregut and hindgut and gastric evacuation time (GET).

2.2.6.1 Stomach gastric evacuation pattern calculation

In the case of stomach, GER was calculated according to the formula described by Elliot (1972). The geometric mean of the stomach dry digesta content divided by the fish weight was regressed against time separately for each diet. GER is estimated as the value of r of the regression model:

$W_t = A\, e^{-rt}$

that is equivalent to the semi-logarithmic model:

$lnW_t = lnA - rt$

where Wt is the geometric mean weight of stomach dry matter digesta at time t, A is an intercept estimated from the model regression and r is the rate of gastric evacuation.

Gastric evacuation time (GET 50%, GET 75% and GET 90%) is the evacuation time (expressed in hours) required to empty 50%, 75% and 90% of each gastrointestinal tract. It was computed through:

$$\text{GET } p\% = \frac{\ln 100 - \ln (100 - p)}{r}$$

where *p* is the digestible organic matter to be evacuated from the stomach.

2.2.6.2 Foregut and Hindgut gastric evacuation pattern calculation

In the case of foregut and hindgut the best model is the quadratic regression c, represented as a parabola. It was not possible to calculate GER, because the trend of digesta in foregut/hindgut was not linear. Points from each curve were estimated to determine the

time of maximum foregut/hindgut content and evacuation time. The GFT (maximum filling time) was calculate as the vertex of the parabola.

In these cases, the equation used were:

$W_t = A + r_1 t + r_2 t^2$

The vertex of the parabola:

$$\left(-\frac{r_1}{2r_2}, \frac{4Ar_2 - r_1^2}{4r_2} \right)$$

and the gastric filling time (GFT):

$$-\frac{r_1}{2r_2}$$

The gastric evacuation time (GET 50%, GET 75% and GET 90%) was computed through:

$$(1-p)\frac{4Ar_2 - r_1^2}{4r_2} = A + r_1 t + r_2 t^2$$

$$\left(A - (1-p)\frac{4Ar_2 - r_1^2}{4r_2} \right) + r_1 t + r_2 t^2 = 0$$

2.2.6.3 Digesta characteristics calculation

The weights of stomach and intestine contents, at each sampling time, were used to calculate the following digesta characteristics, according to Nikolopoulou et al. (2011):

- Water content in each gastrointestinal segment as percentage of the total water present in the whole gastrointestinal tract;
- Moisture of digesta as percentage of digesta weight in each gastrointestinal segment.

2.2.7 Analytical methods

Diets and whole body were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105°C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl

method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450°C. Acid detergent fiber (ADF) was analysed according to AOAC (2004; procedure number 973.18). Serum glucose, alkaline phosphatase, inorganic phosphorus, triglycerides and total protein were measured using colorimetric methods (Total protein OSR6232, Triglyceride OSR61118, Glucose OSR6121, Alkaline phosphatase OSR6004, Inorganic phosphorus OSR6122; Beckman Coulter, Brea, CA, USA) on an automated analyzer (AU 400, Beckman Coulter, Brea CA, USA).

2.2.8 Statistical analysis

Data of growth performance, VSI, HSI, MFI, nutritional indices and blood parameters are presented as mean ± standard deviation (SD) and were analysed by a one-way ANOVA followed by a Tukey's multiple comparison test. Digesta characteristics were analyzed by two-way ANOVA using diet and time as independent factors. Statistical analysis was performed using the software R version 3.1.0 (Revolution analytics, Palo Alto, CA, USA). The differences among treatments were considered significant at $P \leq 0.05$.

2.3 Results

2.3.1 Growth and blood biochemistry

Growth performances are summarized in Table 2.2.

Table.2.2. Growth performance and feed intake of European sea bass fed experimental diets over 117 days.

	Experimental diets					
	F2.8	F4.5	F6	F7.1	F9.3	*P* value
IBW (g)	69.5 ± 2.1	68.1 ± 4.0	68.7 ± 1.1	69.7 ± 1.3	71.2 ± 2.5	0.5944
FBW (g)	215 ± 11.7	204 ± 14.9	215 ± 15.9	223 ± 6.3	219 ± 15.8	0.5369
SGR (% day^{-1})	0.97 ± 0.03	0.94 ± 0.02	0.97 ± 0.07	0.99 ± 0.01	0.96 ± 0.04	0.5136
VFI (gfeed $fish^{-1}$)	237 ± 7.7	219 ± 7.7	230 ± 11.6	232 ± 8.1	237 ± 13.4	0.2759
FCR	1.63 ± 0.06	1.43 ± 0.24	1.58 ± 0.11	1.51 ± 0.03	1.60 ± 0.06	0.3773

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days.
VFI = Voluntary Feed Intake (g feed/fish) = g feed ingested / number of fish
FCR = Feed conversion rate = feed intake / weight gain.

No significant differences due to fiber inclusion levels were observed after the 117 days in terms of growth performance (final body weight and SGR), feed intake (VFI) or feed utilization (FCR). Data on biometric indices, body composition and nutritional indices are shown in Table 2.3.

Table 2.3. Biometric indices, body composition and nutritional indices of European sea bass fed the experimental diets.

	Experimental diets					
	F2.8	F4.5	F6	F7.1	F9.3	*P* value
Biometric indices						
VSI	$12.1^{b} \pm 1.5$	$11.4^{ab} \pm 1.8$	$11.3^{ab} \pm 2.5$	$11.1^{ab} \pm 1.4$	$10.5^{a} \pm 1.9$	0.0303
HSI	$3.2^{c} \pm 0.5$	$3.1^{c} \pm 0.5$	$2.7^{b} \pm 0.6$	$2.6^{ab} \pm 0.5$	$2.3^{a} \pm 0.5$	<0.0001
MFI	6.7 ± 1.5	6.1 ± 1.8	6.4 ± 2.5	6.3 ± 1.5	5.8 ± 1.6	0.4604
Whole body composition						
Protein	$17.1^{b} \pm 0.1$	$16.4^{ab} \pm 0.3$	$16.6^{ab} \pm 0.3$	$16.2^{a} \pm 0.5$	$16.3^{ab} \pm 0.3$	0.0465
Lipid	17.6 ± 1.3	18.6 ± 1.4	17.6 ± 2.3	17.8 ± 1.9	19.0 ± 1.1	0.7498
Ash	2.8 ± 0.21	2.7 ± 0.03	2.7 ± 0.07	2.7 ± 0.04	2.8 ± 0.15	0.7554
Moisture	61.6 ± 0.8	61.2 ± 0.4	60.9 ± 0.5	60.3 ± 0.5	60.0 ± 1.9	0.3485
Nutritional indices						
PER	1.41 ± 0.05	1.43 ± 0.08	1.46 ± 0.11	1.51 ± 0.03	1.43 ± 0.05	0.4484
GPE	24.4 ± 0.97	23.5 ± 1.78	24.2 ± 1.24	24.2 ± 0.62	23.2 ± 0.65	0.6601
GLE	56.4 ± 7.1	57.2 ± 8.2	52.9 ± 11.7	54.2 ± 6.3	52.7 ± 4.1	0.9343

Data are given as the mean (n=3; n=30 for VSI, HSI, MFI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
MFI = Mesenteric fat index (%) = 100*(mesenteric fat weight/FBW).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
GLE = Gross lipid efficiency = 100*[(%final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish.

VSI and HSI values significantly decreased with increasing fiber dietary levels, while no significant differences were found in MFI. Regarding whole body protein content, fish fed diet F2.8 showed higher value in comparison to that fed F7.1, while no significant differences were found for the other parameters.

No significant differences among treatments were found in PER, GPE and GLE. Serum TP, GLU, TRIG, ALP and P levels are shown in Table 2.4. No significant differences among treatments were found, except for P.

Table 2.4. Blood biochemistry of European sea bass fed the experimental diets.

	Experimental diets					
	F2.8	F4.5	F6	F7.1	F9.3	*P* value
TP (g dL^{-1})	5.5 ± 0.40	5.6 ± 0.98	5.3 ± 0.70	5.5 ± 0.63	5.1 ± 1.16	0.5586
TRIG (mg dL^{-1})	1884 ± 818	2023 ± 600	1769 ± 788	1790 ± 715	1797 ± 670	0.9064
GLU (mg dL^{-1})	98 ± 55.1	90 ± 30.1	77 ± 17.3	89 ± 26.0	111 ± 33.8	0.2544
ALP (U L^{-1})	235 ± 58.9	252 ± 59.1	227 ± 56.7	207 ± 49.7	210 ± 51.9	0.2300
P (mg dL^{-1})	8.4 ± 0.85	9.4 ± 1.21	8.5 ± 0.72	8.4 ± 1.01	8.9 ± 1.00	0.0469

Each value is mean from 12 samples ± SD. In each line, different superscript letters indicate significant differences between treatments ($P \leq 0.05$).
TP = total protein
TRIG = triglycerides
GLU = glucose
ALP = alkaline phosphatase
P = inorganic phosphorus

2.3.2 Histology

All the histological section showed a normal intestinal architecture (Fig. 2.1).

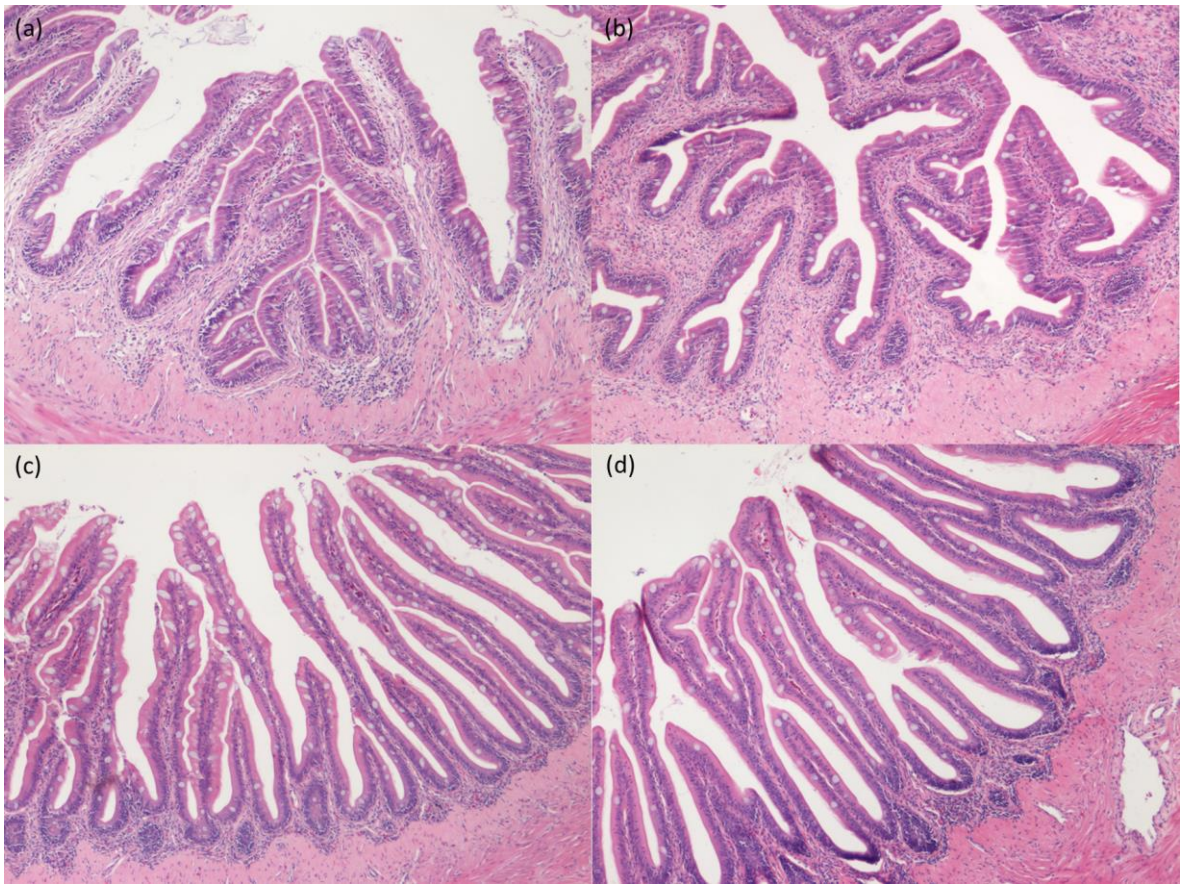


Figure 2.1. *Histology of the proximal (a,b) and distal (c,d) intestine of European sea bass fed diets F2.8 (a,c) and F9.3 (b,d). All the histological sections showed a normal architecture of the mucosal (regular columnar epithelium with polarized and basally located nuclei), submucosal (loose connective tissue rich in capillary network) and muscular layer (H&E, 10x objective).*

No differences were found in the cells constituent of the mucosal layer (goblet cells, supranuclear absorption vacuoles in the enterocytes), capillarity within the intestinal folds, lymphoplasmacytic cells within lamina propria. Moreover, inflammatory and/or degenerative changes were not present in any histological section from all subjects examined.

2.3.3 Gut evacuation rate and time

Data on gastric evacuation rate (GER), gastric filling time (GFT) for foregut and hindgut and gastric evacuation time (GET) are presented in Table 2.5.

Table 2.5. Get evacuation rate (GER), gut filling time (GFT) and evacuation time (GET, expressed in hours) of European sea bass fed the experimental diets.

	Experimental diets				
	F2.8	F4.5	F6	F7.1	F9.3
Stomach					
GER	0.1031	0.0945	0.0899	0.0607	0.0657
GET 50% (h)	6.72	7.33	7.71	11.43	10.55
GET 75% (h)	13.44	14.66	15.41	22.86	21.09
GET 90% (h)	22.32	24.36	25.60	37.96	35.04
R^2	0.82	0.92	0.92	0.98	0.77
Foregut					
GFT (h)	12.99	21.01	12.52	19.45	19.99
GET 50% (h)	35.63	39.50	35.39	38.65	38.88
GET 75% (h)	40.72	43.65	35.39	42.97	43.12
GET 90% (h)	43.36	45.81	43.20	45.21	45.33
R^2	0.33	0.55	0.33	0.43	0.53
Hindgut					
GFT (h)	25.71	23.28	23.71	25.66	25.34
GET 50% (h)	41.60	41.00	40.07	41.54	41.20
GET 75% (h)	45.71	44.99	43.74	45.11	44.76
GET 90% (h)	47.03	47.06	45.66	46.97	46.62
R^2	0.71	0.42	0.65	0.70	0.60

Dry weight of the stomach content decreased with time as shown in Fig. 2.2. Increasing fiber levels in the diets tended to an increasing in time required to empty the stomach, but differences were not statistically significant. Diet F2.8 required 22 h to evacuate the 90% of the initial digesta content, while diet F7.1 and F9.3 37 h and 35 h respectively.

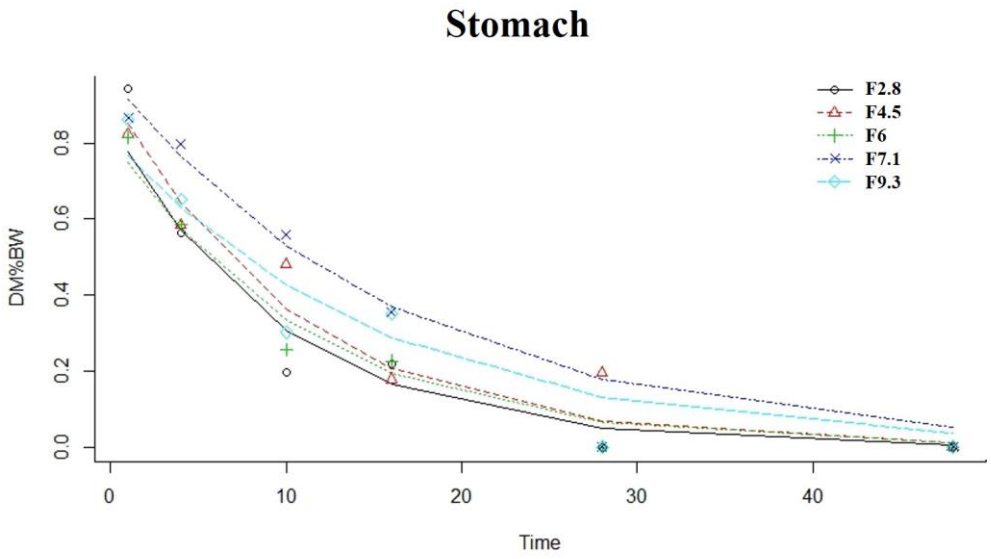
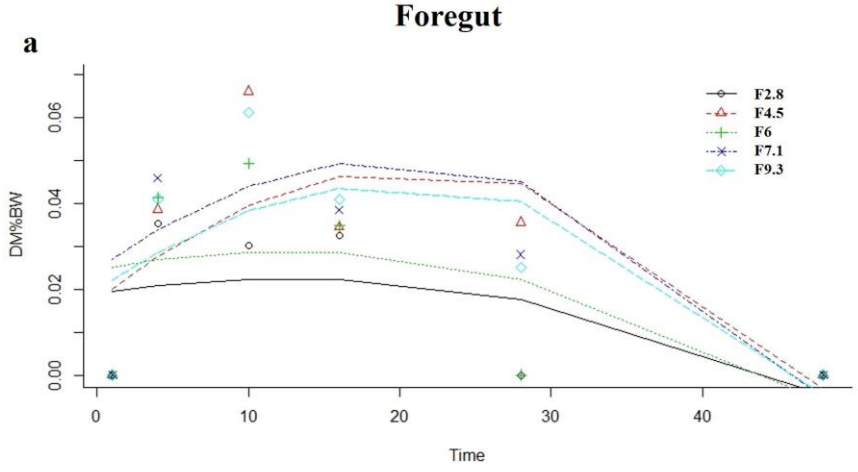


Figure 2.2. *Exponential curves showing stomach evacuation of g digesta dry matter % body weight (DM %BW) over the 48 h sampling period of European sea bass fed the experimental diets.*

The data extracted for the foregut and for the hindgut evacuation rate fitted a quadratic model (Fig. 2.3 a, b). Despite fish fed high fiber levels required more time to empty the stomach content, the time required to empty the 90% of the hindgut was similar in all the treatments. According to the equations, time required to empty the whole gastrointestinal tracts was estimated to be around 46-47 h for fish fed under all treatments.



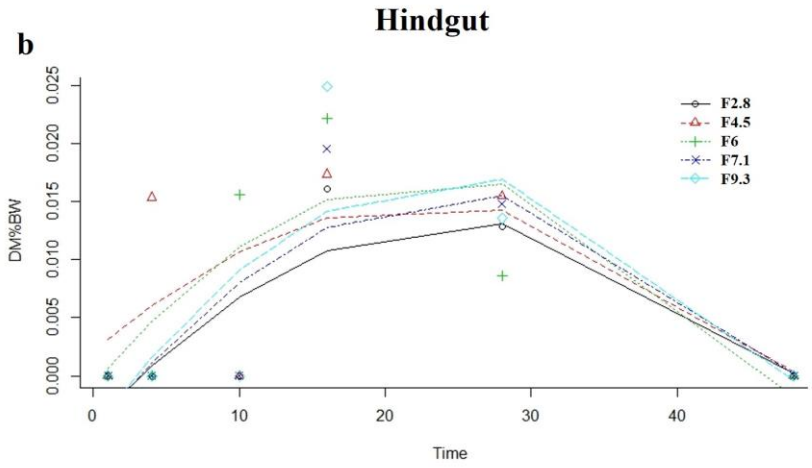


Figure 2.3. *Foregut (**a**) and hindgut (**b**) quadratic curves describing the evacuation of g digesta dry matter % body weight (DM %BW) over the 48 h sampling period of European sea bass fed the experimental diets.*

Data on digesta characteristics (water content and moisture of digesta) are shown in Table 2.6. No differences were found between diets, but digesta characteristics changed with time. Moisture of stomach digesta increased with time, from the first hours after feeding and remained constant until 20 h postprandial. for the first 20h. Regarding water content of the stomach showed higher values were reported during the first 4 h post-prandial. The amount remained constant until 15-16 h postprandial and then rapidly decreased.

Table 2.6. Statistical significance of results for gastrointestinal water content and moisture of digesta obtained for each gastrointestinal tract of European sea bass by two-way ANOVA using time and diet as independent factors. The numbers indicate the proportion of total variation explained by the main effects and their interactions.

	Time	Diet	Time*Diet
Stomach			
Water content	74.1***	0.2	0.1
Moisture	53.1***	0.1	0.2
Foregut			
Water content	17.2***	1.0	4.3**
Moisture	7.2***	0.1	0.6
Hindgut			
Water content	64.2***	0.1	0.8
Moisture	2.5**	0.0	3.0

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$

2.4 Discussion

To our knowledge, this is the first study to compare the effects of dietary fiber on performance, health and digesta transit time of European sea bass. Fiber is basically cellulose, which does not have any value in the nutrition of carnivorous fish and according to Altan and Korkut (2011) should be restricted to less than 7% in fish diet. The results of our study demonstrated that dietary fiber up to level of 9.3 % had no effects on growth performance, feed intake and protein/lipid utilization. The performance registered in this trial, are similar than those found in other studies on European sea bass (Tulli et al., 2010; Guerreiro et al., 2015).

Few studies have investigated the effects of fiber in marine fish species. Hansen and Hemre (2013) reported that plant protein can contain soluble fibres and antinutrients that can interfere with nutrient digestibility. Soluble fibres increase viscosity of gut content, which potentially can reduce digestible enzyme activities and negatively affect nutrient digestion and absorption (Leenhouwers et al., 2006). Recently, Adamidou et al. (2011) reported that an inclusion of fiber up to a level of 5 g $100g^{-1}$ did not affect the growth performance or nutrient digestibility by sharpsnout seabream (*Diplodus puntazzo*). Even Bou et al. (2014) have demonstrated that using diets with the inclusion of fiber up to 18% at expenses of carbohydrate did not affect growth performance in gilthead sea bream (*Sparus aurata*). Both sharpsnout and gilthead sea bream belong to the family of Sparidae, that are considered as omnivorous fish and more predisposed to digest plant-based ingredients then carnivorous species, as European sea bass (Stone, 2003; Tacon and Metian, 2015).

VSI and HSI values increased in the diets with the lowest fiber inclusion. In literature, no evidence is reported about any influence of fiber levels on somatic indices. One possible explanation of the trend in HSI, found in our study, can be related to the decrease in level

of wheat in diets at the increasing of fiber. Previous studies on European sea bass reported that increasing starch and wheat level in the diets can increase HSI values (Bonaldo et al., 2008; Bonvini et al., 2017- Chapter1).

Information on the nutritional status and health in fish species can be achieved through the study of blood metabolites (Peres et al., 2014; Bonvini et al., 2015). To our knowledge, no studies have assessed blood parameters in response to fiber inclusion in diets in marine species. Among the blood parameters, TP, TRIG, GLU, ALP and P seem to have potential as predicative diagnostic tools for evaluation of European sea bass nutritional status (Peres et al., 2014). In this trial, no differences were found among treatments in all parameters analyzed. TP level is usually very stable in well-nourished animals but decreases under fasting conditions (Coeurdacier et al., 2011; Peres et al., 2014). Structural liver alterations and impaired control of fluid balance may increase concentrations of total protein in fish, while failure of protein synthesis due to malnutrition (i.e. starvation) decreases total protein concentrations (Bernet et al., 2001). In this study, plasma TP averaged between 5.6 and 5.1 g dl^{-1} and these values are within the ranges reported for European sea bass under good nutritional status (Peres et al., 2014; Bonvini et al., 2017 – Chapter 1). TRIG levels are in agreement with previously reported values for this species (Bonvini et al., 2017 - Chapter 1), but higher than those reported by Peres et al. (2014). Difference with the latter study can be related to the different lipid level and formulation of the diet used, in comparison to our study. GLU levels are in agreement with previously reported values for this species (Adamidou et al., 2009b; Peres et al., 2014). Adamidou et al. (2009a), reported that glucose levels in European sea bass serum was also affected by the type of carbohydrate ingested with wheat starch showing the most rapid increase and decrease in serum glucose compared to fish fed pea and chickpea diets, while faba bean starch had a delay in the serum glucose peak and a lower range of glucose

values. ALP levels are higher in comparison to Peres et al. (2014). Alkaline phosphatase is involved in the absorption and transport of lipid and carbohydrates from the intestine, and intestinal activities are positively correlated with food ingestion and growth rate (Lemieux et al., 1999). Finally, inorganic phosphorus levels are in agreement with previously reported values for this species (Peres et al., 2014). Plasma electrolytes (univalent and bivalent) are considered to be valuable indicators of the secondary stress and osmoregulation ability in fish (Roque et al., 2010). Plasma phosphorus has been identified as good indicator of stress (i.e. starvation and stocking density) and pathological situations (Roque et al., 2010; Peres et al., 2014).

Besides the study of blood metabolites, histological analysis of the digestive system is considered a valid tool to evaluate the health status. No inflammatory and/or degenerative changes were recorded in any of the histological sections of gut. The examination revealed no differences in the cells constituent of the mucosal layer (goblet cells and supranuclear absorption vacuoles in the enterocytes) capillary within the intestinal folds and lymphoplasmacytic cells within lamina propria. Few references are available on the effects of fiber on the intestine. Olsen et al. (2007) reported that is possible that goblet cell changes can be related to a significant amount of fibres in the diets. In mammals, these compounds tend to increase intestinal size in general, and goblet cell volume and numbers in particular (Lundin et al., 1993).

Dietary fiber can affect gastrointestinal transit time of feed (Zhou et al., 2004). The investigation on gastrointestinal evacuation rate/time in this study reported no significant differences between treatments, but a trend was evident. In fact, increasing fiber levels in the diets tended to an increasing in time required to empty the stomach, (22 h *vs* 35 h to empty the 90% of the stomach content in diet F2.8 and F9.3, respectively). On the other hand, the evacuation time of hindgut was similar in all the treatments, ranging around 46-

47 h to empty the 90% of the hindgut content. There is little published information regarding the effects of fiber on gastric evacuation. When plant-based ingredients are included in the diet, gastric evacuation time is higher, increasing more with legumes than with cereals (Adamidou et al., 2009a). Moreover, Venou et al. (2003) reported that differences in ingredient processing can also modify gastric evacuation time, which is higher for extruded cereals than for raw cereals in gilthead sea bream. In the same species, García-Meilán et al. (2014) found that the differences in diet composition, such as high lipid levels or high starch content, may be involved in the differential transit rate and Fountoulaki et al. (2005) found that a low transit rate was related to a high lipid content.

Moisture of digesta and water content in the gastrointestinal tract showed no differences between treatments. Moisture of stomach digesta increased with time, from the first hours after feeding and remained constant for the first 20h. While water content of the stomach, higher values were reported during the first 4 h post-prandial. The amount remained constant until 15-16 h postprandial and then rapidly decreased. The water required for feed moisturization originates from feed water, initial water absorption of pellets, drinking and stomach secretions (Kristiansen and Rankin, 2001). In this contest, dietary fiber behaves within the gastrointestinal tract as a polymer matrix with variable physicochemical properties including water-holding capacity (Kay, 1982). Dietary fiber inclusion up to 9.3%, did not determine an extra water intake is required.

In conclusion, the different fiber levels tested in this trial have no effects on overall performances and feed efficiency in European sea bass. Results from blood biochemistry profile and histology confirm a good nutritional and health status of fish under all feeding treatments. Fiber had no influence also on digesta transit time and digesta characteristics. However, increasing fiber levels in the diets tended to an increasing in time required to

empty the stomach. We can conclude that, in formulation of feed for the on-growing of European sea bass fiber can be included up to 9.3%.

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Chapter 3

Effect of different dietary taurine levels in plant protein based diets on growth performance in juvenile Southern flounder (*Paralichthys lethostigma*)

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Abstract

Taurine (Tau) is an amino acid abundant in fish meal and animal products, but absent in plant products, and considered an essential nutrient in some teleost species. The present study is focusing on the understanding of the effects of Tau in juvenile Southern flounder (*Paralichthys lethostigma*), an important cultured fish species both for stock enhancement and food fish production, in order to evaluate if this amino acid is an essential nutrient for this species. The experimental diets consisted of a plant-based formula, were devoid of protein ingredients of fish origin and composed only by plant protein sources, with two levels of Tau, 0 and 2%. Fish fed the diet with 2% of Tau supplementation showed highest final body weight in comparison to those fed diet without Tau (P = 0.0198). During the first 11 weeks, no differences were reported between the treatments; whereas, after week 12 body weight of fish fed the diet without Tau did not apparently increase. No significant differences were recorded in terms of feed intake (VFI) and feed efficiency (FE). According to our findings, taurine is an essential nutrient for juvenile Southern flounder.

3.1 Introduction

Taurine (Tau) is an amino acid abundant in fish meal and animal products, but absent in plant products (Li et al., 2009). Research related to Tau has gained much attention over the past years, because several studies have demonstrated that in some teleost species it is an essential nutrient (Salze and Davis, 2015). This amino acid plays an important role in promoting growth, enhancing feed utilization, stimulating immune status and intestinal development/structure of fish larvae (Kim et al., 2008; Zhu et al., 2011; Velasquez et al, 2015). Taurine biosynthesis ability varies greatly among species (Hayes and Sturman, 1981; Yokoyama et al., 2001; Wang et al., 2016). Livers from dog and rat have a high concentration of all enzymes required for taurine biosynthesis while those from man, monkey and cat exhibit extremely low activity of the key enzymes involved in Tau biosynthesis (Hayes et al., 1980; Sturman and Hayes, 1980). In fish, a wide range of the enzymes involved in Tau biosynthesis is described and comparison among finfish species leads to the conclusion that the response to dietary Tau seems to be species specific (Salze and Davis, 2015; Wang et al., 2016).

Continued growth and intensification of the world finfish production depends upon the development of sustainable protein sources to replace fish meal in aquafeeds, which resulted in a substantial decrease of this ingredient in the feed formulation of many species (Tacon and Metian, 2008; Gatlin et al., 2007). Among the many protein sources available for animal feeds, plant proteins appear to be the most appropriate alternatives to fish meal in fish diets (Fournier et al., 2004). However, such alternative ingredients are often devoid or contain very low concentrations of Tau compared to fish meal (Spitze et al., 2003). Thus, as taurine-rich ingredients are removed from practical diet formulations several studies are focused on supplementation of this amino acid in aquafeeds.

The present study is focusing on the understanding of the role of Tau in Southern flounder (*Paralichthys lethostigma*), an important cultured fish species both for stock enhancement and food fish production, in order to evaluate if this amino acid is an essential nutrient for this species. Therefore, we evaluated the effect of two diets with different levels of dietary Tau concentration (0 and 2%) on growth of juvenile Southern flounder.

3.2 Materials and methods

3.2.1 Experimental diets

Two practical iso-energetic and iso-nitrogenous diets were formulated. The experimental diets consisted of a plant-based formula, were devoid of protein ingredients of fish origin and composed only by plant protein sources, with two levels of Tau, 0 and 2%. All diets were processed with a twin-screw extruder (DNDL-44, Buhler Inc., Plymouth, MN) at the U.S. Fish and Wildlife Service, Fish Technology Center, Bozeman, Montana. Ingredients and proximate composition of the experimental diets are presented in Table 3.1.

3.2.2 Fish and feeding trial

The experiment was carried out at the Texas A&M Aquacultural Research and Teaching Facility, Department of Wildlife and Fisheries Sciences, Texas A&M University. One week before the beginning of the trial, fish were subjected to a conditioning period and fed with the diet without Tau inclusion. At the beginning of the trial, 8 fish (initial average weight 3.4 ± 0.1 g) per tank were randomly distributed into 110-L square flat bottom tanks. Each diet was administered to triplicate groups, assigned in a completely random manner, over 16 weeks. Tanks were connected to a closed recirculating system, whereby waste water gravity flowed to a settling chamber, then to a biological filter and was pumped through an ultraviolet light chamber and sand filter before being returned to the tank.

Table 3.1. Ingredients and proximate composition of the experimental diets.

	Experimental diets	
	0 Tau	2% Tau
Ingredient, % of the diets		
Corn protein concentrate	17.00	17.00
Soybean meal	15.00	15.00
Soy protein concentrate	17.00	17.00
Wheat meal	19.96	17.96
Stay-C	0.15	0.15
Vitamin premix	1.00	1.00
TM ARS 640	0.10	0.10
NaCl	0.28	0.28
Magnesium oxide	0.06	0.06
Potassium Chloride	0.56	0.56
Monocalcium phosphate	3.80	3.80
Choline chloride	1.00	1.00
DL-Methionine	0.70	0.70
L-Lysine	3.16	3.16
L-Thereonine	0.82	0.82
Lecithin	1.00	1.00
Menhaden fish oil internal	5.00	5.00
Menhaden fish oil coat	13.41	13.41
Taurine	0	2
Proximate composition[1]		
Protein	40.5	41.8
Lipid	18.9	19.2
Moisture	4.1	3.8
Ash	6.1	6.2
Gross Energy (MJ)	23	23
Taurine	0.00	0.24

[1]Values are reported as mean of duplicate analyses.

The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. Salinity was maintained at 6-8 g/L by combining a stock salt (Stock Salt, United Salt Corp., Houston, TX, USA) and commercial concentrated synthetic salt mix (Fritz Supersalt Concentrate, Fritz Industries Inc., Dallas, TX, USA). Low-pressure electrical blowers provided aeration via air stones to maintain dissolved oxygen levels near saturation. During the trial, water temperature was maintained at 24 ± 1 °C by controlling ambient temperature with dual air-conditioning units. Photoperiod was maintained at 12 h light and 12 h dark through fluorescent lights controlled by an automatic timer. Ammonia (total ammonia nitrogen ≤ 0.1 ppm) and nitrite

(≤ 0.2 ppm) were daily monitored. Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8-8.0. The fish were hand-fed twice a day at a rate approaching apparent satiation with pre-weighed rations based on a percentage of total fish weight per tank (4-5% of total body weight) and visual feeding cues. Fish in each tank were individually weighed every week and feed rations adjusted accordingly.

3.2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were individually weighed. In case of any mortality, fish were immediately removed and the weight was recorded for overall calculation. Specific growth rate (SGR), voluntary feed intake (VFI) and feed efficiency (FE) were calculated. Furthermore, at the end of the trial, wet weight, viscera, liver and fillet weight were individually recorded for all the fish to determine viscerosomatic index (VSI), hepatosomatic index (HSI) and fillet yield (FY).

3.2.4 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Voluntary Feed Intake (VFI) (g feed/fish) = g feed ingested/fish number. Feed efficiency (FE) = biomass gain / feed ingested. Viscerosomatic index (VSI) (%) = 100 * (viscera weight/body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight/body weight). Fillet yield (FY) (%) = 100 * (fillet weight / FBW).

3.2.5 Analytical methods

Crude protein (N× 6.25) was determined in diets by the Dumas method (AOAC, 2006) on a Leco TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, Michigan, USA). Lipid was determined by petroleum ether extraction using an AnkomXT10 (AnkomTechnologies, Macedon, New York, USA). Gross energy was determined by

isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, Illinois, USA). Moisture content was obtained by weight loss after drying samples in an oven at 105°C until a constant weight was achieved. Ash content was estimated by incineration to a constant weight in a muffle oven at 450°C. Tau concentrations were analysed by Eurofins Scientific Inc. (Nutrition Analysis Center, Des Moines, IA) using standard amino acid analysis procedure (AOAC 982.30 mod., 2006).

3.2.6 Statistical analysis

All data are presented as mean ± standard deviation (SD) and were analyzed by t-test. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA). The differences among treatments were considered significant at $P \leq 0.05$.

3.3 Results

Growth performances are summarized in Table 3.2.

Table 3.2. Growth performance and feed intake of Southern flounder fed experimental diets over 16 weeks.

	Experimental diets		
	0 Tau	2% Tau	*P* value
IBW (g)	3.4 ± 0.1	3.5 ± 0.2	0.4676
FBW (g)	9.6 ± 0.37[a]	16.8 ± 8.32[b]	0.0198
SGR (%day^{-1})	0.94 ± 0.04	1.34 ± 0.42	0.1730
VFI (g feed/fish)	45.9 ± 4.2	49.2 ± 10.5	0.6451
FE	0.17 ± 0.01	0.32 ± 0.13	0.1197

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days.
VFI = Voluntary Feed Intake (g feed/fish) = g feed ingested / number of fish
FE = Feed efficiency = biomass gain / feed ingested.

Fish fed diet with the inclusion of 2% Tau showed highest final body weight in comparison to those fed the diet without Tau (P = 0.0198). During the first 11 weeks, no differences

were reported between the treatments; whereas, after week 12 body weight of fish fed diet without Tau did not apparently increase (Fig. 3.1).

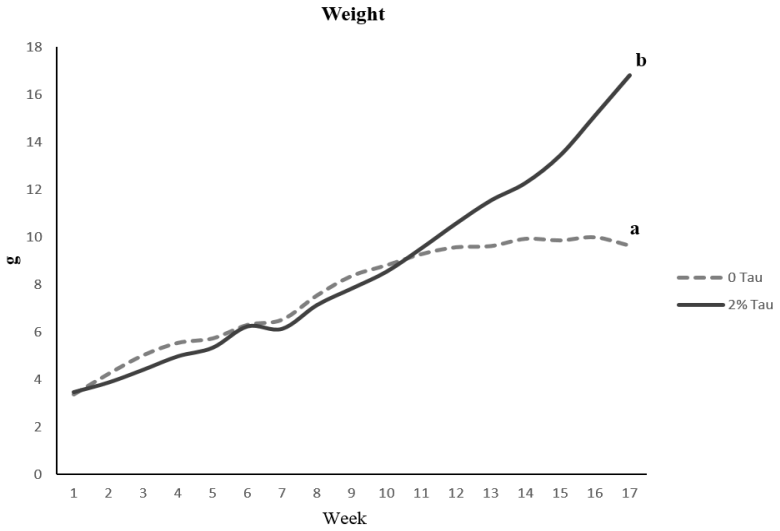

Figure 3.1 *Juvenile Southern flounder weight (g) recorded during the trial. Different letters denote significant differences among the treatments ($P \leq 0.05$).*

No significant differences were recorded between treatments in terms of feed intake (VFI) and feed efficiency (FE).

Data on biometric indices are shown in Table 3.3. Fish fed diet with 2% of Tau supplementation showed highest VSI in comparison to those fed the diet without Tau. No significant differences in HSI and FY were observed.

Table 3.3. Biometric indices of Southern flounder fed the experimental diets.

	Experimental diets		
	0 Tau	2% Tau	*P* value
Biometric indices			
VSI	5.0 ± 0.76^{a}	6.0 ± 0.52^{b}	0.0293
HSI	2.4 ± 0.45	2.6 ± 0.3	0.4023
FY	20 ± 3	22 ± 6	0.4857

Data are given as the mean ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
VSI = Viscerosomatic index (%) = 100*(viscera weight/body weight).
HSI = Hepatosomatic index (%) = 100*(liver weight/body weight).
FY = Fillet yield (%) = 100 * (fillet weight / FBW).

3.4 Discussion

Southern flounder (*Paralichthys lethostigma*), is a left-eyed benthic flatfish of the family Bothidae (Drake et al., 2006). It can be found in coastal waters from Albemarle Sound, North Carolina, through South Atlantic states to Corpus Christi Pass, Texas, with the exception of South Florida (Alam et al., 2009). The development of intensive culture methods for Southern flounder is of great interest because of its status as a highly desirable food and recreational species and its potential for commercial culture (Alam et al., 2009). To our knowledge, this is the first study to investigate whether Tau can affect growth performance of juvenile Southern flounder.

Taurine was reported to affect growth performance and feed utilization of juvenile Japanese flounder (Park et al., 2002). Further, Tau supplementation in high plant protein diets fed to sea bream (*Sparus aurata*), red sea bream (*Pagrus major*) and red drum (*Sciaenops ocellatus*) improved feed performance, increased the mean voluntary feed intake and thus growth (Matsunari et al., 2008; Chatzifotis et al., 2009; Velasquez et al., 2015). On the other hand, Kim et al. (2008) reported that exogenous taurine in the common carp (*Cyprinus carpio*) had no growth-promoting effects and Espe et al. (2012) reported that the addition of Tau to high plant protein diets had a negative effect on growth performance in juvenile Atlantic salmon (*Salmo salar*). The results of our study demonstrated that inclusion of Tau in diets for juvenile Southern flounder enhanced growth performance.

Moreover, in our trial until week 11, fish fed diet without Tau showed the same growth in comparison to those fed the diet with 2% of Tau, revealing a deficiency of this amino acid after almost 3 months. It is possible that Tau might have been accumulated from the commercial diet the fish were fed before the experimental feeding trial. Thus, the deficiency of this nutrient in the basal diet might have depleted their taurine reserves.

These results also can be related to a low taurine biosynthesis ability of Southern flounder in the juvenile stage. Indeed, Li et al. (2009) suggested a suboptimal de novo synthesis of taurine by certain species. Pathways of methionine transmethylation, remethylation, transsulfuration for the synthesis of taurine are likely present in fish, despite possible quantitative differences among species (Li et al., 2009). As reported by Wang et al. (2016), Tau biosynthesis is high in rainbow trout (*Oncorhynchus mykiss*) but low in Japanese flounder (*Paralichthys olivaceus*) and turbot (*Psetta maxima*). Espe et al. (2012) reported that juvenile Atlantic salmon fed a low fish meal diet without taurine supplementation maintained liver taurine concentration at similar levels as did the fish meal fed control fish. Atlantic salmon is able to produce taurine through transsulphuration and to increase transsulphuration when taurine intake is low (Espe et al., 2008; Espe et al., 2012). Because a nutrient is considered a required nutrient in the diet if endogenous production from precursors is absent or insufficient to meet physiological needs (Salze and Davis, 2015), according to our findings, we can suppose that taurine is a required nutrient for Southern flounder.

The overall feed intake and efficiency registered in this trial are lower than those found in other studies on Southern flounder (Gao et al., 2005; González et al., 2005; Alam et al., 2009). In our studies the diet were plant-based, without the inclusion of fish meal or other animal products, while in those other studies the fish meal content ranged between 40 and 70%. In the same direction, Geay et al. (2011) found that European sea bass (*Dicentrarchus labrax*) fed an exclusively vegetable-based diet exhibited lower final weight and daily growth index than those fed a fish-based diet. Even Espe et al. (2012) found reduced performance in juvenile Atlantic salmon fed plant-based diets, most likely due to a reduced feed intake due to the different physical properties of the feeds (Espe et al., 2012). As reported by Glencross (2007), one of the reasons of reduced growth in fish

fed diets containing plant protein is a decrease in feed intake, due to a reduced feed palatability. Southern flounder are carnivorous fishes that are generally considered to be top or near-top predators. Hence, the plant-based formula without protein ingredients of animal origin utilized in this study, might have reduced the feed palatability, explaining the low feed intake registered in comparison to other studies.

In conclusion, the results of our study recognized taurine as an essential nutrient for Southern flounder and quantitative requirement levels of this amino acid should be determined. Moreover, further studies are necessary to investigate the feasibility of plant-based diet for this species.

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Chapter 4

Feeding European sea bass (*Dicentrarchus labrax*) with different dietary plant protein levels: impact on growth and quality

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Abstract

The global consumption of fish and derived fish products has greatly increased during recent decades. Along with the issue of fish meal replacement, another aspect to be considered is the quality of the fish, since consumers are raising their expectations on nutritional value, healthiness, taste and freshness. It is generally accepted that farmed fish quality can be influenced by the formulation of composition of their feed. Altering nutrient content of feed can directly improve product quality in terms of colour and appearance, smell, taste, texture, nutritional quality and shelf life. Therefore, the aim of this study was to evaluate the effects of increasing levels of a blend of plant proteins, in substitution of fish meal, on growth and quality. Three diets were formulated to contain increasing plant protein levels (50, 67 and 84%; 50PP, 67PP and 84PP, respectively), with fish meal dietary levels at 30, 20 and 10%, respectively. No significant differences due to reducing fish meal content were observed after 118 days in terms of growth performance (final body weight and specific growth rate) and feed intake. Fish fed diet 50PP showed lower feed conversion rate in comparison to those fed diet 84PP, while no differences were recorded between diet 50PP and 67PP. No significant differences in biometric indices and fillet composition were observed. No significant differences were found in pH, liquid holding capacity and skin colour measurements between treatments. While, regarding fillet color, significant differences were found only for $H°_{ab}$. In conclusion, our results confirm the possibility to use diets containing high percentage (67%) of a blend of plant ingredients in European sea bass. Our findings also demonstrate that high dietary plan content (up 50% to 84%) had no effects on quality traits of the commercial product.

4.1 Introduction

Aquaculture, the farming of aquatic plants and animals, is no different from any other terrestrial farming activity in that production is totally dependent upon the provision and supply of nutrient inputs (Tacon and Metian, 2008). Clearly, for the finfish and crustacean aquaculture sector to maintain its current growth rate, the supply of nutrient and feed inputs will have to grow at a similar rate (Tacon and Metian, 2015) Thus, continued growth and intensification of aquaculture production depends upon the identification and development of sustainable protein sources to replace fish meal in aquafeeds, which resulted in a substantial decrease of this ingredient in the feed formulation of many species (Naylor et al., 2000; Gatlin et al., 2007).

Besides the global consumption of fish and derived, fish products have greatly increased during recent decades. Along with the issue of fish meal replacement, another aspect to be considered is the quality of the fish, since consumers are raising their expectations on quality, nutritional value, healthiness, taste and freshness. It is generally accepted that farmed fish quality can be influenced by the formulation of composition of their feed. This is a potential advantage for farmed fish over wild fish, but one that the aquaculture industry has been slow to exploit, by using science-based nutritional approaches farmed fish can be produced to meet target quality characteristics on a consistent basis (Hardy and Lee, 2010). Altering composition of feed has an important impact on several parameters, directly influencing the quality of the fish, such as colour and appearance, smell and taste, texture, nutritional quality, shelf life, and level of contaminants (Lie, 2001). European sea bass (*Dicentrarchus labrax*), is an important aquaculture marine finfish species in Europe, particularly in the Mediterranean, cultured for food production. Several studies have compared wild *vs* cultured (Alasalvar et al.,

2002; Fasolato et al., 2010; Fuentes et al., 2010) and organic *vs* conventionally-farmed (Trocino et al., 2012; Di Marco et al., 2017) European sea bass in terms of quality traits.

Given the lack of information on the effects of dietary plant protein sources on fillet quality of European sea bass, the aim of the present study was to study if fish meal replacement by a mixture of plant protein can affect growth and quality.

4.2 Materials and methods

4.2.1 Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated to contain increasing plant protein levels (50, 67 and 84%; 50PP, 67PP and 84PP, respectively), with fish meal dietary levels at 30, 20 and 10%, respectively. Ingredients and proximate composition of the experimental diets are presented in Table 4.1

Table 4.1. Ingredients and proximate composition of the experimental diets.

	Experimental diets		
	50PP	67PP	84PP
Ingredient, % of the diet			
Wheat gluten	8.00	10.00	16.19
SPC 60%	8.00	13.00	17.00
Fish meal LT	30.00	20.00	10.0
Corn gluten	9.62	8.91	9.00
Soya extr.	18.18	17.00	15.94
Wheat	11.62	8.60	7.00
Fish oil	9.62	10.11	10.59
Rapeseed oil	9.62	10.11	10.59
DL-Methionine	0.06	0.12	0.20
Phosphate	0.23	1.11	2.02
L-Lysine	0.20	0.59	1.00
Vit/Min premix[1]	0.46	0.46	0.46
Proximate composition[2]			
Protein	44.4	43.0	43.5
Lipid	30.2	31.5	30.4
Ash	6.2	5.5	4.7
Moisture	5.7	6.1	6.5
Gross Energy (MJ)	23.3	23.5	23.2

[1]Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011).
[2]Values are reported as mean of duplicate analyses.

The diets were produced by extruded process by Skretting Aquaculture Research Centre, Stavanger, Norway. The diameter of the pellet was 4 mm.

4.2.2 Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea bass juveniles were obtained from Panittica Pugliese (Torre Canne di Fasano, Brindisi, Italy). At the beginning of the trial 60 fish (initial average weight: 66.2 ± 1.7 g) per tank were randomly distributed into fifteen 900 L square tanks with a conical bottom. Each diet was administered to triplicate groups, assigned in a completely random manner, over 118 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume: 18 m^3). The rearing system consisted of a mechanical sand filter (Astralpool, Spain), ultraviolet lights (Philips, the Netherlands) and a biofilter (Astralpool, Spain). The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept constant at 22 ± 1.0°C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant at 100% saturation by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 ppm), nitrite (≤ 0.2 ppm) and salinity (25 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on daily basis to keep pH constant at 7.8–8.0. Feed was provided to 10% overfeeding by automatic feeders, twice a day for six days a week, while one meal was supplied on Sundays. Each meal lasted 1 hour, after that the uneaten pellets of each tank were gathered, dried overnight at 105°C and their weight was deducted for overall calculation.

4.2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were anesthetized and individually weighed. In case of any mortality, fish were immediately removed and the weight was recorded for overall calculation. Specific growth rate (SGR), voluntary feed intake (VFI) and feed conversion rate (FCR) were calculated. Survival rate was calculated as a percentage of the initial number of fish. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER) and gross protein efficiency (GPE) were calculated. Furthermore, at the end of the trial, wet weight, viscera, liver, mesenteric fat and fillet weight were individually recorded for 10 fish per tank to determine viscerosomatic index (VSI), hepatosomatic index (HSI), mesenteric fat index (MFI) and fillet yield (FY). The proximate composition of the fillet was determined at the end of the trial on a pooled sample of 5 fish per tank. Moreover, at the end of the trial, quality assessment was performed. All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

4.2.4 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Voluntary Feed Intake (VFI) (g feed/fish) = g feed ingested / fish number. Feed conversion ratio (FCR) = feed intake / weight gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight). Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight / body weight). Fillet yield (FY) (%) = 100 *

(skinned fillet weight / body weight). Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish.

4.2.5 Quality assessment

4.2.5.1 Determination of fillet pH, Liquid holding capacity (LHC) and Allo-Kramer shear

We adopted the approach used in the studies by Álvarez et al. (2008) for pH and Veiseth-Kent et al. (2010) for LHC. For pH, sample from 3 fish per tank was collected from the dorsal-left skinned fillet, homogenized and then pooled into one sample (one pool per tank). Ten grams of each sample were blended with 100 ml distilled water and the pH value of the fish homogenate was measured using a pH-meter (Crison pH Meter Basic 20, Crison Instruments S.A., Barcelona, Spain), standardized at pH 4.01 and 7.00.

For Liquid Holding Capacity, sample from 5 fish per tank was collected from the dorsal-left skinned fillet and individually analysed. Muscles samples (15 g) were weighed and placed in a tube with a weighted absorbing paper (Schleicher & Schuell GmbH, Germany) (V1). The tubes were centrifuged at 500 rpm for 10 min at 10°C (TJ-25 Centrifuge, Beckman Coulter) and the wet paper was weighed (V2) before drying at 50 °C until constant weight (V3). The formulae employed were as follows:

Liquid loss (LL) = 100* (V2 - V1) * S^{-1} (where S = weight of muscle sample). Water loss (WL) = 100 * (V2 - V3) * S^{-1}. Fat loss = (FL) as 100 * (V3 - V1) * S^{-1}. All losses were expressed as percentage of muscle wet weight.

For Allo-Kramer share test, sample from 3 fish per tank was collected from the dorsal skinned fillet near the central backbone and then pooled into one sample (one pool per tank). Fillets were diced (5 × 5 × 5 mm) and formed into bricks (33 × 24 × 15 mm), which were tested either raw or after moist-heating (core temperature 80°C). Two bricks were

prepared for each tank. Texture was analysed using an Instron Model 3365 software Bluehill 2, version 2.19 (Instron Engineering Corp., Canton, Mass., U.S.A.). The five-blade Allo-Kramer shear compression cell (adapted to a 2 kN load cell) was operated at 100 mm/min over a fixed distance to determine peak shear force. Allo-Kramer shear values were reported as kilograms shear per gram of sample.

4.2.5.2 Skin and fillet color

Colour measurements were evaluated throughout a Minolta Chroma Meter CR400 (Minolta, Osaka, Japan), according to Álvarez et al. (2008). Colours were expressed as CIELab coordinates. In this system, L^* denotes lightness on a 0–100 scale of black to white; a^*, (+) red or (−) green; b^*, (+) yellow or (−) blue. Colour intensity is expressed by chroma (C^*_{ab}) value and hue ($H°_{ab}$) is the name of a colour as it is found in its pure state on the spectrum. Values were calculated using the formulae: $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$ and $H°_{ab} = \arctan (b^*/ a^*)$.

Skin colour measurements were estimated on 5 fish per tank. Four colour measurements were performed on each individual on the left side, as described by Pavlidis et al. (2006): two on the dorsal skin area, (i) at the positions where the vertical line to the longitudinal body axis passes through the anterior margin of the dorsal fin (D1) and (ii) through the anus (D2); two at the ventral skin area, (i) below the pelvic fin (V1) and (ii) at the position where the vertical line to the longitudinal body axis passes through the anus, crossing the parallel line to the longitudinal body axis as it passes through the ventral margin of the caudal peduncle (V2) (Fig. 4.1).

Fillet colour measurements were estimated on 5 fish per tank. Two colour measurements were performed on each individual on the left side, both on the dorsal side: (i) at the positions where the vertical line to the longitudinal body axis passes through the anterior margin of the dorsal fin (D1); (ii) through the anus (D2). European sea bass is

characterized by a white light colour of his fillet, thus two Whiteness index (Wtn1 and Wtn2) were performed on the fillet of each fish, according to Schubring (2010). Values were calculated using the formulae: $Wtn1 = L^* - 3b^*$ and $Wtn2 = 100 – ((100 - L^*)^2 + a^{*2} + b^{*2})^{1/2}$.

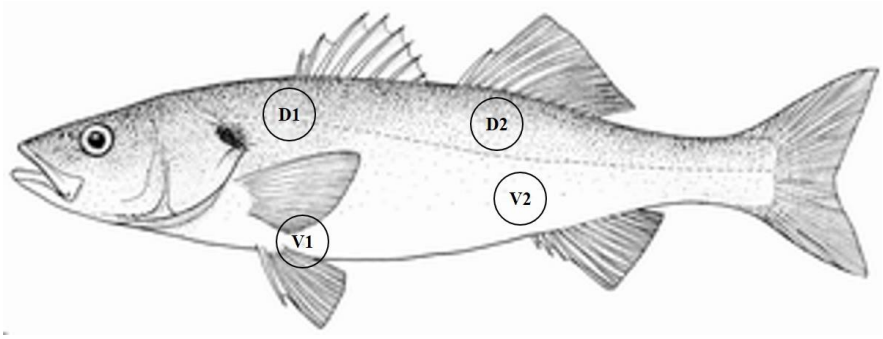

Figure 4.1. *Representation of the points on the sea bass where color was measured.*

4.2.6 Analytical methods

Diets, whole body and fillets were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450 °C. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, USA).

4.2.7 Statistical analysis

All data are presented as mean ± standard deviation (SD). Data were analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. Allo-Kramer shear values were analyzed by two-way ANOVA using diet and state (raw and cooked) as independent factors. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA). The differences among treatments were considered significant at $P \leq 0.05$.

4.3 Results

4.3.1 Growth

Growth performances and nutritional indices are summarized in Table 4.2.

Table 4.2. Growth performance, feed intake and nutritional indices of European sea bass fed experimental diets over 118 days.

	Experimental diets			
	50PP	67PP	84PP	*P* value
IBW (g)	65.6±1.0	66.2±1.1	66.8±2.7	0.7153
FBW (g)	214.2±15.1	213.3±8.8	199.7±6.2	0.2597
SGR (%day^{-1})	1.00±0.05	0.99±0.04	0.93±0.03	0.1511
VFI (g feed/fish)	190±7.3	201±5.9	193±5.3	0.1721
FCR	1.28±0.08^{a}	1.37±0.04^{ab}	1.45±0.05^{b}	0.0372
Survival (%)	99.4±1.0	96.7±4.4	99.4±1.0	0.3955
Nutritional indices				
PER	1.76±0.11	1.70±0.05	1.58±0.06	0.0722
GPE	29.6±1.10^{b}	28.8±0.79^{b}	25.2±1.17^{a}	0.0045

Data are given as the mean (n=3; n=60 for IBW, FBW, FBL and CF) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights).
VFI = Voluntary Feed Intake (g feed/fish) = g feed ingested / number of fish
FCR = Feed conversion rate = feed intake / weight gain.
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

No significant differences due to reducing fish meal content were observed after the 118 days in terms of growth performance (final body weight and SGR) and feed intake (VFI), even though values decreased with the increasing of plant protein. Fish fed diet 50PP showed lower feed conversion rate (FCR) in comparison to those fed diet 84PP, while no differences were recorded between diet 50PP and 67PP. No significant differences among treatments were found in PER. On the contrary, fish fed diet 84PP showed lower gross protein efficiency (GPE) in comparison to those fed diet 50PP and 67PP.

4.3.2 Quality assessment

Data on biometric indices and fillet composition are shown in Table 4.3.

Table 4.3. Biometric indices and fillet composition of European sea bass fed the experimental diets.

	Experimental diets			
	50PP	67PP	84PP	*P* value
Biometric indices				
VSI	11.1. ±1.7	11.5±1.4	11.3±1.5	0.5691
HSI	2.9±1.5	2.7±0.5	2.3±0.6	0.0677
MFI	5.7±1.7	5.8±1.6	5.8±1.5	0.9292
FY	48±4.3	49±3.5	47±3.9	0.2214
Fillet proximate composition				
Protein	19.2±0.4	19.0±0.4	18.6±0.4	0.3158
Lipid	11.7±0.7	12.3±1.1	12.6±1.8	0.7057
Ash	1.3±0.0	1.2±0.0	1.2±0.1	0.1250
Moisture	68.8±0.4	68.7±0.9	67.7±1.6	0.4456

Data are given as the mean (n=3; n=30 for VSI, HSI, MFI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
MFI = Mesenteric fat index (%) = 100*(mesenteric fat weight/FBW).
FY = Fillet yield (%) = 100 * (skinned fillet weight/FBW).

No significant differences in VSI, HIS, MFI and FY were observed. Regarding fillet proximate composition, no significant differences were found between fish fed all the diets.

pH and LHC of the fillets are shown in Table 4.4.

Table 4.4. pH and LHC (liquid holding capacity) of fillet of European sea bass fed experimental diets.

	Experimental diets			
	50PP	67PP	84PP	*P* value
pH	6.54±0.06	6.48±0.02	6.54±0.04	0.2255
Liquid holding capacity				
LL (%)	10.33±1.82	10.37±0.87	8.68±0.43	0.2203
WL (%)	0.92±0.56	0.91±0.07	0.60±0.24	0.4951
FL (%)	9.41±1.27	9.46±0.84	8.08±0.38	0.1878

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
LL = Liquid loss.
WL = Water loss.
FL = Fat loss.

No significant differences were found in pH and in the three parameters analyzed for the Liquid holding capacity (Liquid loss; Water loss; Fat loss).

Allo-Kramer shear values are shown in Table 4.5.

Table 4.5. Allo-Kramer shear values of the fillets at the raw and cooked state, expressed in kg/g.

	Experimental diets				
	50PP	67PP	84PP	*P* value	Interaction
Raw	0.54±0.06	0.52±0.07	0.52±0.07	0.9155	0.4434
Cooked	1.00±0.12	1.13±0.07	1.10±0.18	0.4895	

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

No differences were found between diets, but shear values changed with the state, cooked fillet shown highest values in comparison to the row fillet. Skin and fillet colour measurements are shown in Table 4.6 and 4.7 respectively.

Table 4.6. Skin colour measurements of European sea bass fed experimental diets.

	Experimental diets			
	50PP	67PP	84PP	*P* value
Dorsal				
L*	49.0 ± 9.1	50.4 ± 9.6	47.8 ± 8.1	0.6198
a*	-0.77 ± 0.60	-0.70 ± 0.59	-0.91 ± 0.56	0.4957
b*	6.09 ± 1.76	6.48 ± 1.10	6.57 ± 1.03	0.4509
C^*_{ab}	6.19 ± 1.64	6.56 ± 1.03	6.68 ± 0.94	0.4077
$H°_{ab}$	-1.03 ± 0.39	-1.02 ± 0.49	-1.09 ± 0.36	0.8490
Ventral				
L*	84.6 ± 2.3	84.6 ± 2.9	84.6 ± 1.7	0.6146
a*	0.04 ± 0.97	0.05 ± 0.88	-0.16 ± 1.03	0.8057
b*	7.05 ± 2.65	7.12 ± 2.71	7.24 ± 2.38	0.9683
C^*_{ab0}	7.14 ± 2.60	7.20 ± 2.66	7.36 ± 2.33	0.9571
$H°_{ab}$	-0.21 ± 1.01	-0.29 ± 1.03	-0.38 ± 1.00	0.8497

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
C^*_{ab} = chroma = $(a^{*2}+b^{*2})^{1/2}$.
$H°_{ab}$ = hue = arctan (b^*/a^*).

No significant differences among treatments were found in skin colour measurements. While, regarding fillet color, significant differences were found only for $H°_{ab}$, fish fed diet 84PP showed lower values in comparison to those fed diet 67PP and 50PP.

Table 4.7. Fillet colour measurements of European sea bass fed experimental diets.

	Experimental diets			
	50PP	67PP	84PP	*P* value
L*	42.9 ± 1.1	43.1 ± 2.2	43.8 ± 1.6	0.6471
a*	0.87 ± 0.93	0.88 ± 0.94	0.68 ± 0.66	0.9009
b*	-1.36 ± 0.80	-1.56 ± 1.09	-1.07 ± 1.02	0.6896
C^*_{ab}	1.95 ± 0.11	2.14 ± 0.37	1.87 ± 0.30	0.2824
$H°_{ab}$	-0.41 ± 0.15^b	-0.44 ± 0.08^b	-0.10 ± 0.28^a	0.0139
Wtn1	47.2 ± 0.9	47.8 ± 1.1	47.0 ± 1.4	0.5107
Wtn2	42.8 ± 1.2	43.0 ± 2.2	43.7 ± 1.6	0.6471

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
C^*_{ab} = chroma = $(a^{*2} + b^{*2})^{1/2}$.
$H°_{ab}$ = hue = arctan (b^*/ a^*).
Wtn1 = whiteness1 = $L^* - 3b^*$.
Wtn2 = whiteness2 = $100 - ((100 - L^*)^2 + a^{*2} + b^{*2})^{1/2}$.

4.4. Discussion

Several studies have investigated the utilization of plant dietary inclusion in European sea bass, as a fish meal replacement, but to our knowledge, few studies investigated the effects on both growth and quality traits. Hence, in this study the effects of different dietary plant protein as fish meal replacement were studied to understand if fish quality can be influenced by the composition of feed.

In the present study, increasing levels of plant protein up to 84% did not lead to a depletion in terms of body weight and specific growth rate. The growth performance registered in this trial are similar than those found in other studies on European sea bass (Guerreiro et al., 2015; Bonvini et al., 2017 – Chapter 2). As reported by Médale and Kaushik (2009), a blend of plant protein sources can replace 75 to 95% of fish meal in almost all species, thus reducing the pressure of aquaculture on marine resources. Kaushik et al. (2004) observed that an almost total replacement of fish meal by a mixture of several plant protein sources had no influence on final weight at commercial size in European sea bass.

Concerning feed conversion rate, in fish fed diet 67PP and 50PP values registered are similar than those found in other studies on European sea bass. As previously reported, European sea bass fed blends of plant protein at 20% fish meal inclusion had no negative effects on growth, feed intake and feed efficiency (Bonvini et al., 2017 – Chapter 1). On the other hand, despite the same growth and feed intake, the 84% plant protein dietary inclusion had a negative impact on feed conversion rate and protein utilization. Generally, one of the reasons of reduced performances in fish fed diets containing plant ingredients is a decrease of feed intake, due to a reduced feed palatability. Interestingly, according to our findings, highest inclusion of plant protein had an effect only on feed utilization and no problem of palatability was recorded. Robaina et al. (1999) reported high digestibility coefficient of protein from wheat gluten in European sea bass. Moreover, Messina et al. (2013), showed that replacing up to 70% fish meal protein by wheat gluten in diets supplemented with the most limiting amino acids did not adversely affect feed intake, growth, feed and nutrient conversion efficiency in European sea bass. In these studies, the fish meal level ranged between 19 and 45 %, a higher inclusion in comparison to diet 84PP tested in our trial, where fish meal inclusion was 10%. Despite plant protein utilized in our trial are good ingredients for aquatic feeds, we hypothesize that if incorporated in diet with a low fish meal level (10%) they can compromise the feed efficiency and protein utilization. Moreover, the biology of the species should be considered. European sea bass is a strictly carnivorous marine fish, mainly a predatory, and in nature feed on small fish, prawns, crabs, and cuttlefish (Stickney, 2000). Hence this specie utilizes dietary plant protein poorly or not at all and is less flexible in terms of vegetable ingredients use (Stone, 2003; Tacon and Metian, 2015). Furthermore, when plant protein ingredients are included at high concentrations, seems that feeding frequency must also be considered, with a preferable 12 h interval between each meal, assuming all fish are fed to satiation within a

given meal (Peres et al., 1999; Kousoulaki et al., 2015). In our trial, maybe also the interval of 7 h between each meal may have compromised the efficiency in the diet with the highest plant inclusion.

It is also encouraging to note that inclusion of high levels of plant protein sources did not affect morphological traits and fillet proximate composition. In particular fillet yield ranged between 47% and 49%. Those values are comparable to those reported by Poli et al. (2001) and Tibaldi et al. (2015) (calculated on fillet with skin) and by Bonvini et al. (2017 – Chapter 2) (calculated on skinned fillet). Moreover, fillet protein composition values found in this trial are comparable to those reported for organic, conventionally-farmed and fed experimental diets, ranging between 18.6% and 19.2% (Trocino et al., 2012; Tibaldi et al., 2015). The quality of the edible portion, destined to the consumers, seems not to be affected by high plant protein inclusion. Varying the protein sources did not affect also Allo-Kramer shear values of the fillet. Differences were only recorded between the raw and cooked fillet, with increasing values for the cooked state in all the treatments. Comparison in the texture of flesh is not easy due to the different analysis methods utilized in the researches. Moreover, low references are available on the effects of diet composition on this characteristic and generally are correlated with the dietary lipid level. In fact, high fat content and lipid sources could have an effect and modify the texture. Different authors found that an increase in fat content led to a decrease in firmness (Fuentes et al., 2010).

The colour deserves particular attention from the point of view of consumer acceptance, because with price and quality, influences purchasing decision criteria. The skin and fillet colour of fish fed the different diets were similar and were not substantially affected by the difference in amount of dietary plant protein. To our knowledge, few references are available on the effects of a blend of plant protein on skin and fillet colour

and generally are focused on the effects on a specific ingredient. In salmonids and gilthead sea bream, dietary corn gluten meal has a notable effect on fillet colour (Robaina et al., 1999; Hardy 1996; de Francesco et al., 2004). In European sea bass, increased greenish skin pigmentation was associated to a slightly enhanced yellowish flesh in fish fed the diets containing dried microalgae (Tibaldi et al., 2015). On the other hand, fish colour could also be influenced by other factors such as rearing environment and temperature, swimming possibilities, feeding regime, storage period (Fuentes et al., 2010). In European sea bass, differences in moisture content may explain the changes in colour between fish, higher moisture content contributes to the creation of refractive indices within the food matrix leading to a lighter colour (Fuentes et al., 2010: Trocino et al., 2012). In this trial, only a slightly decrease in $H°_{ab}$ in fish fed diet 84PP were found. Despite the significant difference, such as slight change in colour can not be detected by human eye.

In conclusion, our results confirm the possibility to use diets containing high percentage (67%) of plant protein sources for the on-growing of European sea bass. Our findings also demonstrate that increasing the inclusion of vegetable ingredients in substitution of fish meal had no effects on quality traits of the commercial product.

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Overall considerations

The overall objective of this thesis was to gain further knowledge on the development of innovative feed products and feed concepts for marine species in aquaculture.

The sustainability challenges of the aquaculture sector will probably be closely linked with continuing interest in identifying and developing ingredients as alternatives to fish meal for use within aquafeeds. The attention is focused on the nutritional specifications and ingredient choices to find the optimal combination for an optimal utilization of the nutrients by the fish. More attention has been given to the use of plant protein sources, which are available in large amounts on the market. The main challenges in using plant protein sources in diets for carnivorous fish lie in their often lower levels of protein and high levels of unfavourable amino acid, high levels of fiber and starch, mineral profiles, and, perhaps most consequential, the presence of antinutritional factors and/or antigens. The investigation in this sector on the effects that various alternative protein-rich ingredients have on fish has largely been restricted to fish growth, feed conversion efficiency and digestibility of the feed nutrients to understand if different formulation and some component can compromise normal growth of different fish species. Different aspects on the health implications of using vegetable feed ingredients on the digestive tract, plus possible effects on quality of the fish, nowadays are some of the most relevant problems currently confronting the aqua industry.

The need to understand this phenomenon leads to the present thesis, in which the investigation on the feasibility of the inclusion of plant protein in aquafeed was undertaken through a multidisciplinary approach.

In the cases studied, it was highlighted that the inclusion of soybean meal in combination with different blends of plant protein maintains optimal growth and feed utilization, in 20% fish meal dietary level, for the on-growing of European sea bass (Chapter 1). Whilst, when fish meal level is reduced up to 10%, the feed efficiency is compromised (Chapter 4).

Despite fiber is considered as a component that cannot be utilized by most fish and that can compromise gut health and digestive process, inclusion up to 9.3% has no effects on overall performances and feed efficiency in European sea bass (Chapter 2). Moreover, the different fiber levels tested had no influence also on digesta transit time and digesta characteristics despite, increasing fiber levels in the diets tended to an increasing in time required to empty the stomach.

It was encouraging to note that inclusion of high levels of plant protein sources and fiber up to 9.3% did not affect health status of the animals (Chapter 1 and 2). Blood biochemistry profile showed a good nutritional status under all feeding regimes and histological examinations showed a normal intestinal architecture, inflammatory and/or degenerative changes were not present in any histological section from all subjects examined.

When replacing fish meal in aquafeeds, besides the research on alternative ingredients, also the nutritional profile should be considered. In the case studied, it was demonstrated that taurine is a required nutrient for juvenile Southern flounder (Chapter 3). Quantitative requirement levels of this amino acid should be determined for the farmed carnivorous finfish thus, taurine biosynthesis ability varies greatly among species.

Moreover, the case studied shown that increasing the inclusion of plant ingredients in substitution of fish meal had no effects on quality traits of the commercial product (Chapter 4). The inclusion of alternative ingredients will did not compromise some of the main marketable indexes of the European sea bass.

However, it is not yet an easy or economically feasible task to remove fish meal in aquaculture feeds without affecting the growth and health of the fish. Further efforts are needed to reduce the aquaculture dependence from marine feedstuff and thereby enhance the sustainability of the sector. Moreover, the biology of the species should be considered,

since different species with the same carnivorous trophic profile, perform differently when fed plant-based aquafeeds, given physiological and metabolic differences. The present thesis has contributed to provide some insights on the feasibility of the replacement of fish meal in aquafeed, without compromising growth and chiefly, fish health and quality. The progressive reduction of fish meal in aquafeed will improve the sustainability and allow the growth of the finfish production of the aquaculture industry.