Potential use of high levels of vegetal proteins in diets for Gilthead Sea Bream (*Sparus aurata*)

Raquel Monge[1], Silvia Martínez-Llorens[1], Lorenzo Márquez[3], Francisco Javier Moyano[2], Miguel Jover-Cerdá [1], Ana Tomás-Vidal[1]

[1]Research Group of Aquaculture and Biodiversity. Institute of Animal Science and Technology. Universitat Politècnica València. Camino de Vera, 14. 46071-Valencia, Spain

[2]Dpto. de Biología Aplicada. Universidad de Almería, España.

[3]Núcleo de Investigación en Producción Alimentaria / Escuela de Acuicultura. Facultad de Recursos Naturales. Universidad Católica de Temuco. Avda. Rudecindo Ortega 02950. PO Box 15-D. Temuco. Chile.

***Corresponding author:** Raquel Monge Ortiz, Universitat Politècnica València. Camino de Vera, 14. 46071-Valencia (Spain). Tel.: 34-96-3879752; Fax: 34-96-3877439. E-mail: ramonor@upv.es.

Abstract

The effect of a diet partially or totally substituting fishmeal (FM) by vegetal protein sources (soybean meal, rapeseed meal and wheat gluten) supplemented with crystalline amino acids on growth and feed efficiency was carried out in on-growing gilthead sea bream (initial average weight 131 g). The vegetal protein diets also contained 47g/kg krill meal (KM) in order to improve palatability and therefore feed acceptance. At the end of the trial (158 days), fish survival was above 90%. Final weight and the specific growth rate were statistically different in fish fed a control diet, (361g and 0.64%/d), compared with 395-390 g and 0.70-0.69 %/d in the FM-free diet. No significant differences were found in the feed intake ratio (FI), which was 0.91 to 1.04 kg/ 100 kg fish•d and the feed conversion ratio ranged from 1.6 to 1.8 kg/kg. Simultaneously, a digestibility analysis with chromium oxide was carried out to determine the digestibility coefficient, which was similar in all diets. The blood parameters did not showed significant differences between treatments groups and were not correlated with FM substitution. The enzymatic analysis concerning in trypsin and pepsin showed significant differences between the control diet and the experimental diets. In the distal intestine the villi length in fish fed FM25 was significantly longer, and the intestine of the fish fed the FM100 diet exhibited a smaller number of goblet cells. Total FM substitution by a vegetal mix supplemented with synthetic amino acids in on-growing sea bream is feasible.

Key words: *Sparus aurata;* plant protein; fishmeal; feed utilisation; amino acids.

1. Introduction

At present, the fishmeal (FM) contents in commercial feeds for different fish species varies greatly; 250–450 g/kg in diets for *Salmo salar*, 150-500 g/kg in trout, 250-500 g/kg in *Sparus aurata* and *Dicentrarchus labrax*, and 50-200 g/kg in the common carp (Tacon and Metian 2009).

Results of most studies indicate that inclusion of up to 30-40% of such vegetal protein sources as a single ingredient may result in good growth. However, most of these studies were performed using small fish and for short experimental periods, without fish reaching their commercial weight.

When using mixtures of different vegetal ingredients, FM substitutions of 50-60% in seabream (De Francesco et al. 2007; Dias et al. 2009; Sánchez-Lozano et al. 2009) and even of 95-100% have been reported (Kissil & Lupatch 2004) in fish with an initial weigh of 40 g.

Furthermore, the cost of feed is above 40% of the total production spendings of an aquaculture farm, and mostly spent during the fattening stage, when there is a greater biomass and it is necessary to economize. Therefore, studying the effects of substitution in the fattening stage is imperative to reduce feed spending.

It seems reasonable to think that the synergy obtained with the mixture of different protein sources is due to the fact that the amino acids present in several ingredients complement each other. However, total FM replacement can only be achieved when diets are supplemented with an adequate balance of crystalline amino acids (CAA) simulating the FM amino acid profile (Kissil and Lupatsch 2004) in on-growing seabream. It has been

demonstrated that good results in the simulation of the FM amino acid profile may be reached using mixtures of several plant-derived meals or commercial amino acid preparations. Also, total FM substitution by plant-derived meal, according to Espe et al. (2007) and Burr et al. (2012), does not have a negative effect on growth in Atlantic salmon if the amino acid profile is well balanced.

It is well-known that the substitution of fishmeal by a mixture of protein sources decreases digestive activity in trout and sea bream (Santigosa et al. 2008), and especially trout trypsin is highly sensitive to plant protease inhibitors (Krogdahl et al. 1994) affecting protein and energy digestibility, reducing growth and feed utilisation. Moreover, to cover the nutritional requirements with non-fishmeal diets and to not interfere with enzymatic processes, it is essential to check if any damage to the digestive system has occurred, mostly histological alterations in the gut layers when feeding sea bream a vegetable protein substitution diet (Sitjà-Bobadilla et al. 2005; Santigosa et al. 2008, Baeza-Ariño et al. 2014).

The absence of taurine in plant ingredients must be considered as it is abundant in FM and animal marine products. Taurine is not incorporated into proteins, but plays an important role in fat digestion, antioxidative defence and cellular osmoregulation, as well as in the development of visual, neural and muscular systems (Saltze 2015). The objective of this study was to evaluate the effect of high and total FM substitution by vegetable proteins supplemented with amino acids on the growth performance, feed parameters, protein metabolism (enzymatic activity and amino acid retention) and fish health (intestinal damage and blood parameters) of sea bream (*Sparus aurata*).

2. Materials and methods

2.1. Experimental diets

Three iso-energetic and iso-nitrogenous experimental diets were formulated based on proximate analysis of five protein feed ingredients: fishmeal (FM), wheat gluten (WG), soybean meal (SBM), rapeseed meal (RM) and krill meal (KM). The FM100 diet served as a control diet containing FM as the primary protein source, while 75 and 100% of the FM protein in the FM25 and FM0 diets, respectively, were substituted by a vegetable protein mixture consisting of WG, SBM and RM, and 47 g/kg KM (Table 1).

Deficiencies in some essential amino acids (methionine, lysine, arginine, histidine and threonine) were corrected with crystalline amino acids, taking the amino acid requirements of sea bream as a reference (Peres and Oliva-Teles 2009), being below 10% in the present study (Table 2).

The different ingredients of the diets were weighed individually and mixed to form a homogeneous dough, and were prepared using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, St. Etienne, France) at the university facilities. The processing conditions were as follows: a screw speed of 100 rpm, a temperature of 110 ºC, and a pressure of 40-50 atm. The experimental diets were assayed in triplicate.

In addition, to study the proteolytic ability when the gastric digestion of vegetal-based diets takes place, the buffer capacity of all diets was determined. Samples of 500 mg of each diet were suspended in 5 ml of distilled water and stirred magnetically. Small amounts of HCl 50 mM were added progressively and the pH was recorded 30 s after each

addition, according to Márquez et al. (2013). All the measurements were made using a Crison pH25 pH-meter, equipped with a Crison 5208 microelectrode. Table 1 shows the mEq/kg required to reach pH 3.5.

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

[Table 2. Amino acid composition of experimental diets in dry matter.]

2.2 Growth trial and fish sampling

On-growing gilthead sea bream (*S. aurata*) were obtained from a local fish farm (Piscimar, S.L., Castellón, Spain) and transported live to the Fish Nutrition Laboratory of Polytechnic University of Valencia, in Gandia, Spain. Prior to the feeding trial, all fish were acclimatised to the indoor rearing conditions for two weeks and fed a standard sea bream diet (480 g/kg crude protein, CP; 230 g/kg ether extract, EE; 110 g/kg ash; 22 g/kg crude fibre, CF; and 140 g/kg nitrogen free-extract, NFE).

The trial was conducted in a recirculating marine water system (20 m^3 capacity) with a pressure filter and a gravity biofilter (approximately 2 m^3), water temperature was 20.5 ± 2.1ºC (mean ± SD), salinity was round 38 ± 1 g/l. All tanks were equipped with aeration and the level of dissolved oxygen was 6.9 ± 0.5 mg/l. pH ranged from 7.5 to 8 during the experimental period. The photoperiod was natural and all tanks had similar light conditions.

After the acclimation period, groups of 25 fish (average weight 131 g/fish) were housed in nine cylindrical fibreglass tanks (750 l), three tanks per treatment. The fish were

fed during 158 days by hand, twice a day (9.00h and 16.00h) until apparent satiation. The pellets were distributed slowly to allow all fish to eat. The uneaten diet was collected and dried to determine feed intake (FI). All fish were individually weighed at intervals of 30 days. Prior to weighing, the fish were anaesthetised with 30 mg/l clove oil (Guinama®, Valencia, Spain) containing 87% eugenol. At the end of the growth trial, all fish were individually weighed.

2.3. Biometric parameters and proximate composition.

Five fish were randomly sampled from each tank to determinate their biometric parameters and to carry out the proximate composition analysis.

Main performances and biometric parameters were analyzed following these formulas:

SGR (Specific Growth Rate) [%/d] = {100 • ln [final weight/initial weight]}/d.

FI (Feed Intake Ratio) [g 100 g/fish•d]= {100 • feed consumption [g]} / {average biomass [g] • d}.

FCR (Feed Conversion Ratio) = feed intake [g] / weight gain [g].

CF (Condition Factor) = 100 • total weight [g]/ total length [cm3].

VSI (Viscerosomatic Index) [%] = 100 • visceral weight [g]/ empty fish weight [g].

HIS (Hepatosomatic Index) [%] =100 • liver weight [g]/ fish weight [g].

MFI (Mesenteric Fat Index) [%] = 100 • visceral fat [g]/ empty fish weight [g].

DP (Dressout Percentage) [%] = 100 • [total fish weight- visceral weight- head weight (g)]/ fish weight (g).

PIR (Retention efficiency of protein intake) = fish protein gain [g]/ protein intake [g] • 100.

PDR (Retention efficiency of digested protein) = fish protein gain [g]/ protein digested [g] • 100.

Chemical analysis of the dietary ingredients was performed prior to diet formulation. Diets and their ingredients, as well as the whole fish, were analysed according to AOAC (1990) procedures: dry matter (105 ºC to constant weight), ash (incinerated at 550 ºC to constant weight), and crude protein (N x 6.25) by the Kjeldahl method after acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden), ether extract extracted with methyl-ether (Soxtec 1043 extraction unit, Tecator) and crude fibre by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator). The buffering capacity and the pH of the diets were measured according to Márquez et al. (2013). All analyses were performed in triplicate.

2.4 Digestibility and retention estimations.

The digestibility of the diets was determined in a concurrent study, before extrusion; diets were supplemented with 5g/ kg chromium oxide as an inert indicator.

12 fish were used in a trial designed to determine the apparent digestibility of the experimental diets. The digestibility system was constructed according to the Guelph System protocol (Cho et al. 1982), using three digestibility tanks (four fish/ tank). The water temperature averaged 20.5 ± 2.1ºC (mean ± SD). The fish groups were fed the experimental diets,during a 30-35 day period, and faeces were collected from each tank daily. In samples of digestibility, no replicas were made; faeces of the different tanks of the same diet were pooled.

177 Chromium oxide was determined in the diets and in faeces using an atomic
178 absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA) after acid
179 digestion.

180 The apparent digestibility coefficients (ADCs) for protein and amino acids for the
181 diets tested were calculated with the following formula:

182 $$ADC\ [\%] = 100 \bullet [1-(F/D \bullet DCr/ FCr)]$$

183 Where F is the percentage of nutrient or energy in faeces, D is the percentage of
184 nutrient or energy in the diet, DCr is the percentage of chromic oxide in the diet and FCr is
185 the percentage of chromic oxide in faeces (Cho and Kaushik 1990).

186 Protein and amino acid retention efficiencies were calculated as follows:

187 Protein retention efficiency (PRE) or digestible protein retention efficiency (DPRE) [%]:

188 $$PRE = \frac{\text{protein fish gain [g]}}{\text{protein intake [g]}} \bullet 100$$

189 $$DPRE = \frac{\text{protein fish gain [g]}}{\text{protein digested [g]}} \bullet 100$$

190 Amino acid retention efficiency (AARE) or digestible amino acid retention efficiency
191 (DAARE) [%].

192 $$AARE = \frac{\text{AA fish gain [g]}}{\text{AA ingested [g]}} \bullet 100$$

193 $$DAARE = \frac{\text{AA fish gain [g]}}{\text{AA digested [g]}} \bullet 100$$

194

195 ***2.5 Haematological analysis***

196 At the end of the experiment and due to total FM replacement, a haematological
197 analysis was carried out (protein, haematocrit and haemoglobin) in some of the sea bream
198 slaughtered to determine their health status. Blood samples from the caudal vein of nine
199 fish per experimental treatment were taken with heparinised syringes (1 ml). Haematocrit
200 (Hct) was measured using double heparinised 75 mm capillary tubes. Capillary tubes were
201 centrifuged at 13,000 g for 10 min and read against a percentage scale. Results were
202 expressed as percentage Hct. For subsequent haemoglobin analyses, the same sample was
203 stored at 4 ºC, while another portion was separated by centrifugation at 3,000 g. Then,
204 plasma was pooled, immediately frozen on liquid N and thereafter stored at −80 °C.
205 Subsequently, this sample was used for total protein analyses.

206 The haemoglobin concentration (Hb) was determined by a miniaturised method
207 using Drabkin's reagent (Drabkin and Austin 1935). In brief, 2 µl of blood were added to
208 500 µl of Drabkin's reagent (Sigma) and 200 µl were transferred to duplicate wells of a
209 transparent 96-flat bottomed well microplate (Greiner). The Hb concentration of the blood
210 sample was calculated from a standard curve prepared from bovine haemoglobin (Fluka).
211 Absorbance was determined at 540 nm by a GeniosPro luminometer (TECAN, Austria).
212 Results were expressed as g/dl blood. Plasma protein was quantified according to Bradford
213 with the Roti-Quant kit and a BSA standard dilution (CARL ROTH, Karlsruhe, Germany)
214 series.

215 ***2. 6. Enzymatic analysis***

For enzymatic determination, the assays were performed in the stomachs and total intestines of three fish collected from three tanks for each diet. The samples were homogenised in 3-5 volumes (w/v) of ice-cold distilled water, followed by centrifugation (16,000 g, 30 min, 4ºC). Pepsin activity was analysed according to the method detailed by Díaz et al. (1998) using haemoglobin as substrate; one unit of activity being defined as 1 µg of tyrosine released per minute. Assays were carried out both at the pH previously measured in each stomach.

Specific trypsin- and chymotrypsin-like activities were analysed with 0.5 mM BAPNA (Na-benzoyl-DL-arginine 4-nitroanilidehydrochloride) and 0.2 mM SAPNA (N-succinyl-Ala–Ala-Pro_phe p-nitroanilide) substrates, respectively, in 50 mM Tris–HCl (pH 9.0), using 96-well plates. The slope until saturation curve was recorded at 410 nm by a Tecan Sunrise spectrophotometer (Tecan, Salzburg, Austria) using Magellan V.1.12

2.7. Amino acid analysis

Following the method previously described by Bosch et al. (2006), the amino acid contents of the fish carcasses, diets and faeces were determined using a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard before hydrolysation. Amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. Amino acids

were separated with a C-17 reverse-phase column Waters Acc. Tag (150 mm x 3.9 mm) and then converted to methionine and cysteine.

Digestible amino acids were determined by faeces analysis: ADCAA = 100 • [1- (Cr_2O_{3diet}/ $Cr_2O_{3faeces}$) • (AA_{faeces}/ AA_{diet})].

2.8 Histological analysis

At the end of the experiment, foregut samples from three fish from each tank were collected and dissected into small pieces and preserved in phosphate buffered formalin (4%, pH 7.4). All of the formalin-fixed tissues were routinely dehydrated in ethanol, equilibrated in ultraclean and embedded in paraffin according to standard histological techniques. Transverse sections were cut with a Microtomo Shandom Hypercut having a thickness of 5µm and stained with haematoxylin and eosin for examination. Eighteen sections per treatment were made for the gut and examined under a light microscope (Nikon, Phase Contrast Dry JAPAN 0.90).

2.8.2.Histological measurements and observations

For the measurements and observations of the distal intestine, a combination of criteria reported by several authors was followed (Sitjà-Bobadilla et al. 2005; Santigosa et al. 2008). The following parameters were measured: serous layer (SL), muscular layer (ML), villi length (VL), villi thickness (VT), intravilli space (IE) and lamina propria length and thickness (LP) (six measurements of each parameter, and the average values were

calculated). The observed measurements are shown in Figure 3. In addition, a quantification of the Goblet cells (GCs) was performed by counting the number of GC present in each villus. Per section, six villi were used.

2.9. Ethical statement

The *Sparus aurata* study complied with European Union Council Directive 2010/63/UE which lays down minimum standards for the protection of animals, and was also in accordance with Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes.

Fish in the tanks were checked on a daily basis. Every four weeks, fish were weighed individually and their health status was assessed by observation, after sedation with clove oil dissolved in water (1 mg / 100 ml of water) to minimize animal suffering.

Animals were euthanized by an excess of clove oil (150 mg / l) and then dissected.

2.10. Statistical analysis

Growth data, feed utilisation and the data obtained from the measurements of the blood parameters were evaluated using one-way analysis of variance (ANOVA), with the initial live weight as covariate (Snedecor and Cochran 1971). The Newman-Keuls test was used to assess specific differences among diets at a level of $p = 0.05$ (Statgraphics, Statistical Graphics System, Version Plus 5.1, Herndon, Virginia, USA).

3. Results

3.1. Main performances and biometric parameters

At the end of the growth period (Table 3), fish fed the FM100 diet presented the lowest final body weight and specific growth rate (SGR) (361 g and 0.64%/day, respectively). The fish survival rates exceeded 91% in all tanks and did not differ significantly among treatments. Significant differences were not found in the feed intake ratio (FI), which was close to0.91 to 1.04 kg/100 kg fish•d and in the feed conversion ratio (FCR), close to 1.54 to 1.80 kg/kg.

[Table 3. Main performances of gilthead sea bream fed experimental diets.]

Statistical differences were observed in biometric parameters on the viscerosomatic index between the control diet and experimental diets (Table 4). The condition factor, hepatosomatic index, mesenteric fat index and dress-out percentage were similar in all treatments. Whole-body composition (Table 4) was not affected by FM substitution at the end of the growth trial. The retention of ingested (PIR) and digested (PDR) protein was similar in all three diets (Table 4). The apparent digestibility coefficient of protein ($ADC_{protein}$) were similar, namely96.6, 97.3 and 95.3% for the FM 100, FM 25 and FM0 diets respectively (Table 1). Likewise, the ADC of the amino acids (ADC_{AA}) was similar in all diets (Table 2).

[Table 4. Biometric parameters, proximate composition and retention efficiency (expressed as percentage of wet weight, w.w.) of gilthead sea bream fed experimental diets.]

No significant differences were observed in the whole-body amino acid content of the fish (Table 5).

The retention efficiency of digestible essential amino acids (DAARE) was calculated using the values of amino acid digestibility and whole-body fish amino acid composition (Figure 1). No significant differences were found among the diets with regard to retention efficiencies for Arg, His, Lys, Met, Phe and Thr. The DAARE values for Ile, Leu and Val were lowest (28, 29.5 and 28.1%) in the fish fed the FM100 diet.

[Table 5. Effects of diet on whole-body amino acid composition at the end of the trial.]

[Figure 1. Retention efficiency of digestible essential amino acids in Gilthead sea bream fed with the experimental diets at the end of the experiment.]

3.2. Enzymatic analysis.

pH increased from 6.1 to 6.6 in the control diet vs the fishmeal-free diet. However, in the FM-free diet (Table 1) the buffering capacity decreased from 1.2mEq/g to 0.8 mEq/g in the diet based in fishmeal.

With regard to gastric pH, significant differences between sea bream fed the three treatments were obtained; the pH of fish fed the FM 100 diet was lower with respect to fish fed the FM0 diet (5.48, 5.79 and 5.82 for FM100, FM 25 and FM0, respectively).

The pepsin and trypsin activity in fish fed the FM100 diet was higher than in fish fed the FM0 diet. Chymotrypsin activity did not present significant differences (Figure 2).

[Figure 2. Enzyme activities of pepsin, trypsin and chymotrypsin (UA/ g fish) obtained from the stomach and intestine of sea bream fed plant protein–substituted. Bars indicate

standard deviations of the mean, and letters show significant differences among the diets (ANOVA, $p< 0.05$).]

3.3. Haematological analysis.

Haematocrit values (45%) as well as blood serum values (Hb 7.5 g/dL and total protein 4.5 g/dL) did not show significant differences between treatment groups and did not correlate with dietary FM substitution (Table 7).

[Table 7. Blood parameter (hematocrit, hemoglobin and protein) of sea bream fed the experimental diets.]

3.4. Histological measurements.

The results of the measurements of the evaluated parameters in the distal intestine are displayed in Table 6. The serous layer, muscular layer, villi and lamina propria thickness did not present any significant differences, whereas villi and lamina propria length and intravilli space showed significant differences between fish fed with the control diet and the FM25 and FM0 diets, being longer with these latter diets (Figure 4).

Concerning the number of goblet cells (GCs), significant differences were also found, especially between the FM100 diet and the FM25 diet (Figure 4), being higher in the FM25 diet.

[Table 6. Effects of diet on distal intestine parameters.]

[Figure 3. Detail of the different measurements of the gut.]

[Figure 4. Photomicrograph of 5-µm villi intestinal sections stained with haematoxylin-eosin showing intestinal morphology in *Sparus aurata.]*

4. Discussion

The most common alternatives for FM as the main protein source are different vegetal protein meals that vary in available nutrient contents, the best results can be obtained by using a mixture of different ingredients (Burr et al. 2012; Slawski et al. 2012) and supplementing the diet with indispensable amino acids (Rodehutscord et al. 1995; Kaushik et al. 2004; Espe et al. 2007), resulting in the formulation of nutritionally complete diets with an inclusion level of FM as low as 10%. In the present experiment, the vegetable mixture consisting of SBM, WG and RM (including 47 g/kg krill meal) supplemented by essential amino acids may totally replace FM protein without negatively affecting sea bream performance. As a result, fish growth with the FM25 and FM0 diets was higher than with the FM100 diet. Results are interesting from a nutritional point of view, but several EAAs had to be added in the mixture, as they were not commercially available, which is costly and of doubtful practicability. Even so, it is an important step in the total replacement of fishmeal in feed for on-growing sea bream.

The extruded diet without FM seemed, not only to be harmless to sea bream, but also efficient in growth promotion and performance (higher final weight and SGR). Supplementation of EAA in the non-FM diets to match the amino acid profiles proved beneficial.

The addiction of taurine has been positive in this study because it helped to improve fish growth. Previous studies found that taurine has a wide range of roles, and growth depressions is the most commonly observed sign in taurine deficient feeds (Saltze 2015)

These findings support previous studies in rainbow trout (Yamamoto et al. 2002, 2004, 2012) maintaining that supplementation of EAAs is essential to improve the nutritional value of FM-free diets based on a mix of vegetal and animal ingredients. In addition, the present results also indicate that this supplementation is suitable to improve the growth and feed efficiency in sea bream. However, in numerous other studies, the results indicated the opposite. For instance, rainbow trout fed diets in which FM was completely replaced by vegetal protein presented a significantly lower growth rate and reduced feed intake (Gomes et al. 1995; Adelizi et al. 1998; De Francesco et al. 2004; Burr et al. 2012).

The lower growth rates and reduced feed conversion in fish fed FM-free diets is caused, in most cases, by a reduced feed intake. Therefore, the use of feed attractants seems recommendable. Fish protein hydrolysate, blood meal, squid hydrolysate, stick water or krill meal, at dietary levels from 30 to 50 g/kg, have turned out to be effective feed attractants (potentially contributing to maintaining appetence), as well as sources of amino acids and minerals when FM-low diets were fed to carnivorous fish (Espe et al. 2006; Torstensen et al. 2008). In the present study, a minor amount of krill meal was included in all replacement diets to increase acceptability of the plant protein diets, and no significant difference was found in nutrient utilisation when sea bream were fed vegetal diets.

When new sources are being tested, the EAA content of the dietary proteins and digestibility of the protein or each EAA should be considered to assess the adequacy of the EAA profile of the diet. In the present trial, the ADC protein was not affected ($ADC_{protein}$ was 97, 97 and 95% for the FM 100, FM 25 and FM0 diets respectively). Fish fed the plant protein diets digested all indispensable amino acids just as well, or even better, than those fed the FM control diet. However, several authors concluded that, due to the fast absorption

of crystalline amino acids, a reduced utilisation of dietary amino acids used for anabolic purposes took place (Espe et al. 1994). Data of the present study does not confirm this fact, as protein retention efficiency values (PRE and DPRE) were almost identical, suggesting a similar effectiveness of dietary protein regardless of the supplementation of free amino acids. This is in agreement with earlier studies on Atlantic salmon, in which inclusion of large amounts of plant protein did not affect protein utilisation, as long as the amino acid composition in the diet was balanced (Espe et al. 2006, 2007).

The retention efficiency values for Ile, Leu and Val were lowest in the fish fed the FM100 diet, possibly caused by a lower amount of these amino acids in the FM25 and FM0 diets.

The improvement of protein digestibility and, thus, of the digestive enzyme activity are crucial in the promotion of fish growth. Protein digestion takes place first in the stomach through the action of pepsin, its activity being greatly affected by the pH. Therefore, it is necessary to maintain a low pH in the stomach to induce the maximum conversion of pepsinogen to pepsin and reach an efficient digestion of diets based on vegetal protein sources. According to Munilla-Moran and Saborido-Rey (1996), the pH optimum for pepsin activity in the stomach of *S. aurata* is 2.0. However, in the present work, stomach pH ranged from 5.5 to 5.8, being far above the optimum pepsin value for any diet. Furthermore, and despite the fact that fishmeal has a 15-fold higher buffering capacity compared to cereals, the lowest buffering capacity observed in a vegetal diet was not sufficient to reduce the pH of the stomach. Nonetheless, gastric pH depends on numerous factors, such as feeding regimes (Yufera et al. 2012), and is ahigher in a single regime, as is the case of the present experiment. It also depends on the time passed between ingestion and pH measuring (Bucking and Wood 2009), which was 8 h in the present trial.

The higher the vegetable protein level, the higher the decrease in trypsin activity, which is possibly related to the presence of plant inhibitors (Krogdahl et al. 1994), as the extracts for the enzyme assay were prepared from fish in the process of digestion; therefore, it is possible that samples contain certain inhibitors.

Rapeseed meal presents a high content of this compound, which, in turn, may reduce the activity of pepsin and trypsin, but not the chymotrypsin activity in trout (Morales et al. 2011), similarly to the results achieved in the present study. This lower enzymatic activity, could be related to the fact that the ADC_{AA} in the vegetable diets is lower in the fish fed with FM0 diet than in the control diet. This is due to the fact that trypsin plays a very important part as a key enzyme for feed utilisation and growth in its role in the protein digestion processes (Rungruangsak-Torrissen and Male 2000) because it actives quimotrypsine and other digestive enzymes such as carbopeptidases.

Blood serum parameters were not significantly affected by treatments, and no correlation with the FM-free diet was found. Haematological parameters displayed normal values, and the absence of significant differences in mortality confirms an equal nutritional status among feeding groups. The results of the present study indicate that fish did not suffer any malnutrition and that dietary nutrient supply was sufficient to support growth in all feeding groups.

The values of the different intestinal parameters may be indicative of the overall health of the animals. Previous studies showed that the inclusion of plant protein at a high rate in the diet can lead to problems in the intestinal morphology, a consequence of the presence of antinutrients in these vegetal sources. Overall, fish fed the FM 25 and FM0

diets showed significant differences in the length of villi, LP, intravilli space and the goblet cell number with respect to the control diet.

In relation to goblet cell count (GC), the group fed fishmeal (FM100) had fewer goblet cells per villi. These cells are involved in the secretion of mucus, with functions related to the immune system, acting as a lining, avoiding injuries and keeping it moist in order to prevent drying (Martínez-Llorens 2012). An increase number of goblet cells in the fish with the vegetal dietary inclusion in the present study can be interpreted as a defence mechanism against the gastrointestinal system damage produced, resulting in greater amounts of mucin and a higher intestinal protection.

Length (VL) and villi thickness (VT) are correlated with absorption surface, so that longer villis, entail a facilitation of the absorption of nutrients. Some authors have observed a mechanism of adaptation to plant diets,in wich the size of villi increases in order to help the digestion (Aslaksen et al. 2007). Thus, the increase of villi length, together, with an increase in vegetal substitutions, could lead to a better absorption area in the intestine.

In conclusion, the present results show that on-growing sea bream fed diets containing alternative protein blends in place of fishmeal grow better than fish fed a fishmeal-based diet (including 47 g/kg krill meal). A possible explanation for the results obtained in this study on fishmeal-free diets, when compared to the results of other published research, is the fact that the diets were supplemented with essential amino acids and taurine.

5. Acknowledgements

This work was supported by the Vicerrectorat d'Investigació, Innovació i Transferència – Universitat Politècnica de València, Project Name: Aquaculture feed without fishmeal (SP20120603). URLs of funder: http://www.upv.es/entidades/VIIT/info/indexnormalc.html

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors are grateful to Ralph Wilk, TEFL Cambridge University RSA, Dip Translator and Dip ATh for revising the language of this paper.

Ethical statement: The protocol was approved by the Committee on the Ethics of the Polytechnic University of Valencia (UPV). All information entered here was also included in the Methods section of the manuscript

6. References

Adelizi, P.D., Rosati, R.R., Warner, K., Wu, Y.V., Muench, T.R., White, M.R., Brown, P.B. 1998. Evaluation of fishmeal free diets for rainbow trout, *Oncorhynchus mykiss*. Aquacult. Nutr. 4:255–262.

A.O.A.C., 1995. Official methods of analysis. Washington (DC): Ass Off Anal Chem.

Aslaksen M.A., Kraugerud O.F., Penn M., Svihus B., Denstadli V., Jorgensen H.Y., Hillestad M., Krogdahl A., Storebakken T. 2007. Screening of nutrient digestibilities and intestinal pathologies in Atlantic salmon, *Salmo salar*, fed diets with legumes oilseeds, or cereals. Aquaculture 272, 541–555.

488 Baeza-Ariño, R., Martínez-Llorens, S., Nogales-Mérida, S., Jover-Cerda, M., Tomás-Vidal,
489 A. 2014. Study of liver and gut alterations in sea bream, *Sparus aurata* L., fed a
490 mixture of vegetable protein concentrates. Aquacult. Res. 1-12.
491 Bosch, L., Alegria, A., Farré, R. 2006. Application of the 6-aminoquinolyl-N-
492 hydroxysuccinimidyl carbamate (AQC), reagent to the RP-HPLC determination of
493 amino acids in infant foods. Journal of Chromatography B: J Chromatogr B Analyt
494 Technol Biomed Life Sci. 831:176–178.

495 Bucking, C., Wood, C. W. 2009. The effect of postprandial changes in pH along the
496 gastrointestinal tract on the distribution of ions between the solid and fluid phases of
497 chyme in rainbow trout. Aquacult. Nutr. 15:282 -296.

498 Burr, G.S., Wolters, W.R., Barrows, F.T., Hardy, R.W. 2012. Replacing fishmeal with
499 blends of alternative proteins on growth performance of rainbow trout
500 (*Oncorhynchus mykiss)*, and early or late stage juvenile Atlantic salmon (*Salmo*
501 *salar*). Aquaculture. 334-337:110-116.

502 Cho, C. Y., Kaushik, S. J. 1990. Nutritional energetics in fish: Energy and protein
503 utilization in rainbow trout (*Salmo gairdneri*). World Rev Nutr Diet. 61:132-172.

504 Cho, C.Y., Slinger, S.J., Bayley, H.S. 1982. Bionergetics of salmonids fishes: energy
505 intake, expenditure and productivity. Comp. Biochem. and Physiol. 73:25–41.

506 De Francesco, M., Parisi, G., Medale, F., Lupi, P., Kaushik, S.J., Poli, B.M. 2004. Effect of
507 long-term feeding with a plant protein mixture based diet on growth and body/fillet
508 quality traits of large rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 236:413–
509 429.

510 De Francesco, M., Parisi, G., Perez-Sánchez, J., Gómez-Requeni, F., Medale, F., Kaushik,
511 S., Mecatti, M., Poli, B.M. 2007. Effect of high-level fishmeal replacement by plant
512 proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality
513 traits. Aquacult. Nut. 13:361–372.
514 Dias, J., Conceição, L.E.C., Ramalho Ribeiro, A., Borges, P., Valente, L.M.P., Dinis, M.T.
515 2009. Practical diet with low fish-derived protein is able to sustain growth
516 performance in gilthead sea bream (*Sparus aurata*) during the grow-out phase.
517 Aquaculture. 293:255–262.
518 Díaz, M., Moyano, F.J., Alarcón, F.J., García-Carreño, F.L., Navarrete, M.A. 1998.
519 Characterization if fish acid proteases by substrate-gel electrophoresis. Comp.
520 Biochem. and Physiol 121:369–377
521 Drabkin, D.L., Austin, J.H. 1935. Spectrophtometric studies. II. Preparations from washed
522 blood cells; nitric oxide haemoglobin and sulfhemoglobin. J. Biol. Chem.112:51.
523 Espe, M., Lied, E. 1994. Do Atlantic salmon (*Salmo salar)* utilize mixtures of free amino
524 acids to the same extent as intact protein sources for synthetic purposes? Comp.
525 Biochem. Physiol. 107:249–254.
526 Espe, M., Lemme, A., Petri, A., El-Mowafi, A. 2006. Can Atlantic salmon (*Salmo salar*)
527 grow on diets devoid of fishmeal? Aquaculture. 255:255–262.
528 Espe, M., Lemme, A., Petri, A., El-Mowafi, A. 2007. Assessment of lysine requirement for
529 maximal protein accretion in Atlantic salmon using plant protein diets. Aquaculture.
530 263:168–178.
531 Gomes, E.F., Rema, P., Kaushik, S.J. 1995. Replacement of fishmeal by plant proteins in
532 the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth
533 performance. Aquaculture. 130:177–186.

Kaushik, S.J., Covès, D., Dutto, G., Blanc, D. 2004. Almost total replacement of fishmeal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchux labrax*. Aquaculture. 230:391–404.

Kissil, G. W., Lupatsch, I. 2004. Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. Israeli J. Aquacult.-Bamidgeh. 56:188-199.

Krogdahl, A., Lea, T.B., Olli, J.J. 1994. Soybean protease inhibitors affect intestinal trypsin activities and amino acids digestibilities in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. and Physiol. 107:215–219.

Márquez, L., Øverland, M., Martínez-Llorens, S., Morken, T., Moyano, F.J. 2013. Use of a gastrointestinal model to assess potential amino acid bioavailability in diets for rainbow trout (*Oncorrhynchus mykiss*). Aquaculture. 384–387:46-55.

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

Morales, G.A., Moyano, F.J., Márquez, L. 2011. In vitro assessment of the effects of phytate and phytase on nitrogen and phosphorus bioacessibility within fish digestive tract. Animal Feed Sci. Technol. 170:209-221.

Munilla-Moran, R., Saborido-Rey, F. 1996. Digestive enzymes in marine species. Proteinase activities in gut from redfish (*Sebastes mentella*), seabream (*Sparus*

aurata) and turbot (*Scophthalmus maximus*). Comp. Biochem. and Physiol. 113B:395–402.

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

Rodehutscord, M., Mandel, S., Pack, M., Jacobs, S., Pfeffer, E. 1995. Free amino acids can replace protein-bound amino acids in test diets for studies in rainbow trout (*Oncorhynchus mykiss*). J. Nutr. 125:956–963.

Rungruangsak Torrissen, K., Male, R. 2000. Trypsin isozymes: development, digestion and structure. In: Seafood Enzymes, Utilization and Influence on Postharvest Seafood Quality. pp. 215–269. Edited by N.F. Haard and B.K. Simpson. Marcel Dekker, Inc., New York.

Salze, G.P., Davis, A. 2015. Taurine: a critical nutrient for future fish feeds. Aquaculture. 437: 215-229.Sánchez-Lozano, N., Martínez-Llorens, S., Tomás-Vidal, A., Jover-Cerdá, M. 2009. Effect of high-level fishmeal replacement by pea and rice concentrate protein on growth, nutrient utilization and fillet quality in gilthead seabream (*Sparus aurata*, L.). Aquaculture. 298:83–89.

Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M.A. 2008. Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (Sparus aurata) in response to dietary fishmeal replacement by plant protein sources. Aquaculture. 282:68-74.

Sitja-Bobadilla, A., Peña-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., Perez-Sánchez, J. 2005. Effect of fishmeal replacement by plant protein sources on non-

579 specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus*
580 *aurata*). Aquaculture. 249:387–400.
581 Slawski, H., Adem, H., Tressel, R.P., Wysujack, K., Koops, U., Kotzamanis, Y., Wuertz,
582 S., Schulz, C. 2012. Total fishmeal replacement with rapeseed protein concentrate
583 in diets fed to rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquaculture Int.
584 20.3: 443-453.
585 Snedecor, G., Cochran , W. 1971. Statistical methods. The Iowa State University Press
586 Ames. lowa, USA. 593pp.
587 Tacon, A.G.J., Metian, M. 2009. Fishing for Feed or Fishing for Food: Increasing Global
588 Competition for Small Pelagic Forage Fish. Ambio. 38:294-302.
589 Torstensen, B.E., Espe, M., Sanden, M., Stubhaug, I., Waagbø, R., Hemre, G.I.,
590 Fontanillas, R., Nordgarden, U., Hevrøy, E.M., Olsvik, P., Berntssen, M.H.G. 2008.
591 Novel production of Atlantic salmon (*Salmo salar*) protein based on combined
592 replacement of fishmeal and fish oil with plant meal and vegetable oil blends.
593 Aquaculture. 285:193–200.
594 Yamamoto, T., Shima, T., Furuita, H., Suzuki, N. 2002. Influence of feeding diets with and
595 without fishmeal by hand and by selffeeders on feed intake, growth and nutrient
596 utilization of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 214:289–
597 305.
598 Yamamoto, T., Shima, T., Furuita, H., Suzuki, N. 2004. Antagonistic effects of branched-
599 chain amino acids induced by excess proteinbound leucine in diets for rainbow trout
600 (*Oncorhynchus mykiss*). Aquaculture. 232:539–550.
601 Yamamoto, T., Matsunari, H., Sugita, T., Furuita, H., Masumoto, T., Iwashita, Y., Amano,
602 S., Suzuki, N. 2012. Optimization of the supplemental essential amino acids to a

603 fishmeal-free diet based on fermented soybean meal for rainbow trout
604 *Oncorhynchus mykiss*. Fish. Sci. 78:359–366.
605 Yúfera, M., Moyano, F.J., Astola, A., Pousão-Ferreira, P., Martínez-Rodríguez, G. 2012.
606 Acidic digestion in a teleost: Postprandial and circadian pattern of gastric pH,
607 pepsin activity, and pepsinogen and proton pump mRNAs expression. PLOS ONE.
608 7(3):e33687. doi:10.1371/journal.pone.0033687
609

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

	Diets		
Ingredients [g/ kg]			
	FM100	FM 25	FM 0
Fishmeal herring	589	150	
Wheat	260	143	
Wheat gluten		177	190
Soybean meal		100	226
Rapeseed meal		105	226
Krill meal		47	47
Soybean lecithin	10	10	10
Soybean oil	66	66	65
Fish oil	65	91	102
Mono calcium phosphate		27.5	38.5
Taurine		20	20
DL-Methionine		6.4	8.6
Lysine		18.4	22
Arginine		13	16
Histidine		7.7	9.6
Threonine		8	9.3
Multivitamin and minerals mix [*]	10	10	10
Proximate composition [g/ kg dry weight matter.]			
Dry matter DM	833	841	829
Crude protein CP	450	450	451
Ether extract EE	200	201	201
Ash	103	77	80
Crude fibre CF	5	25	47
Calculated values			
N-free extract [g/ kg] [#]	232	236	211
Digestible protein DP [g/ kg] [†]	434	438	430
$ACD_{protein}$ [%]	96.6	97.3	95.3
Diet pH	6.10	6.20	6.60
Buffering capacity [mEq/ g] [‡]	1.2	0.9	0.8

611 [*]Vitamin and mineral mix (values are g/kg except those in parenthesis): Premix: 25;
612 Choline, 10; DL-a-tocopherol, 5; ascorbicacid, 5; $(PO_4)_2Ca_3$, 5. Premix composition:
613 retinol acetate, 1 000 000 IU kg^{-1}; calcipherol, 500 IU/kg; DL-a-tocopherol, 10;menadione
614 sodium bisulphite, 0.8; thiamine hydrochloride, 2.3;riboflavin, 2.3; pyridoxine
615 hydrochloride, 15; cyanocobalamine, 25;nicotinamide, 15; pantothenic acid, 6; folic acid,
616 0.65; biotin, 0.07;ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.
617 [#] Nitrogen free-extract, NFE (%) =100-%CP-%CL-%Ash-%CF
618 [†] Digestible protein [DP] was calculated based on the respective values of apparent
619 digestibility coefficients
620 [‡]mEq/ g needed to reach pH 3.0.

Table 2. Amino acid composition of experimental diets in dry matter.

	Experimental diets					
	Gross amino acids			Digestible amino acids		
	FM 100	FM 25	FM 0	FM 100	FM 25	FM 0
EAA [g/100g]						
Arginine	2.63	2.88	3.43	2.56	2.84	3.36
Histidine	1.17	1.25	1.28	1.13	1.22	1.24
Isoleucine	1.72	1.35	1.11	1.65	1.30	1.04
Leucine	3.00	2.48	2.18	2.89	2.40	2.05
Lysine	3.10	2.83	2.52	3.04	2.78	2.45
Methionine	1.11	1.22	1.25	1.08	1.19	1.23
Phenylalanine	1.62	1.45	1.30	1.53	1.40	1.24
Threonine	1.76	1.97	1.91	1.70	1.92	1.81
Valine	2.02	1.55	1.31	1.94	1.50	1.21
NEAA [g/100g]						
Alanine	2.39	1.58	1.23	2.30	1.52	1.14
Aspartate	3.50	2.16	1.58	3.37	2.08	1.46
Cystine	0.32	0.39	0.44	0.30	0.37	0.42
Glutamine	5.58	7.18	7.66	5.42	7.06	7.48
Glycine	2.15	1.45	1.16	2.01	1.36	1.04
Proline	1.91	2.55	3.10	1.84	2.49	2.99
Serine	1.52	1.34	1.30	1.45	1.29	1.22
Tyrosine	1.07	0.89	0.69	1.02	0.86	0.66

EAA: Essential amino acids.

NEAA: Non-essential amino acids.

ADC_{AA} expressed as % as follows:

Diets	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
FM 100	96.2	97.1	96.4	94.1	97.2	93.7	96.9	96.0	96.1	97.9	96.6	94.7	96.3	95.1	97.0	95.7	96.0
FM 25	96.1	98.9	96.1	95.1	98.3	94.0	97.9	96.5	96.7	98.3	97.6	96.3	97.8	95.9	97.4	96.9	96.2
FM 0	92.6	98.7	92.3	94.5	97.6	90.1	97.0	93.5	94.2	97.2	97.1	94.6	96.7	93.5	95.3	95.0	92.8

654 Table 3. Main performances of gilthead sea bream fed experimental diets.

	FM 100	FM 25	FM 0	Mean	SEM	*p*-value
Initial weight [g]	125.6	134.8	125.6	131.4	5.456	0.471
Final weight [g]	360.7[b]	395.9[a]	390.3[a]	382.3	5.300	0.017
Survival [%]	97.3	94.7	90.7	94.2	2.667	0.280
SGR [%/ d]	0.637[b]	0.700[a]	0.689[a]	0.675	0.009	0.015
FI [g/100 g fish•d]	0.946	0.967	1.04	0.98	0.048	0.487
FCR	1.57	1.58	1.79	1.65	0.077	0.157

655 Means of triplicate groups. Data in the same row with different superscripts differ at $p <$
656 0.05. SME: pooled standard error of the mean. Initial weight was considered as covariable
657 for final weight (P-value: 0.0089) and SGR (P-value: 0.0213).

658 Table 4. Biometric parameters proximate composition and retention efficiency (expressed
659 as percentage of wet weight, w.w.) of gilthead sea bream fed experimental diets.

	FM 100	FM 25	FM 0	Mean	SEM	*p*-value
CF	1.50	1.32	1.59	1.47	0.104	0.182
VSI [%]	4.86[b]	6.66[a]	5.18[b]	5.56	0.295	0.000
HSI [%]	0.89	0.90	0.91	0.90	0.088	0.986
MFI [%]	1.10	1.11	0.70	0.97	0.153	0.117
DP [%]	75.5	73.0	72.3	73.6	0.894	0.053
Moisture [%]	66.5	65.6	65.7	65.9	0.809	0.693
Crude protein [%*w.w.*]	17.2	17.8	19.2	18.1	0.639	0.151
Ether extract [%*w.w.]*	12.8	13.0	11.8	12.5	0.603	0.369
Ash [%*w. w.*]	2.4	3.1	3.3	2.9	0.324	0.188
PIR [%]	29.6	30.7	31.2	30.5	0.639	0.904
PDR [%]	30.6	31.5	32.8	31.6	2.750	0.860

660 Means of nine replicates in biometric indices and means of triplicate groups in proximate
661 composition and retention efficiency. Data in the same row with different superscripts
662 differ at $P < 0.05$. SME: pooled standard error of the mean.
663

664 Table 5. Effects of diet on whole-body amino acid composition at the end of the trial

	FM 100	FM 25	FM 0	Mean	SEM	*p*-value
EAA [g/k^{g} wet weight]						
Arginine	16.05	17.03	17.61	16.89	1.059	0.602
Histidine	5.08	5.72	5.64	5.47	0.350	0.422
Isoleucine	6.78	7.09	7.26	7.05	0.253	0.446
Leucine	12.45	13.20	13.56	13.07	0.384	0.196
Lysine	12.52	12.50	13.51	12.84	0.712	0.552
Methionine	4.77	6.22	6.10	5.70	0.611	0.256
Phenylalanine	7.44	8.03	7.83	7.77	0.537	0.741
Threonine	7.66	7.36	7.88	7.63	0.854	0.912
Valine	7.91	8.24	8.54	8.23	0.250	0.283
NEAA [g/kg wet weight]						
Alanine	10.83	10.69	12.05	11.20	0.585	0.272
Aspartate	14.90	14.78	16.11	15.27	0.634	0.334
Cystine	1.03	1.46	1.46	1.32	0.177	0.224
Glutamine	22.46	22.25	23.75	22.82	1.021	0.563
Glycine	15.11	14.75	15.45	15.11	1.591	0.950
Proline	8.17	7.38	8.08	7.83	0.446	0.443
Serine	6.96	7.40	7.69	7.35	0.330	0.374
Tyrosine	5.68	6.47	5.88	6.01	0.485	0.527

665 Means of triplicate groups. Data on the same row not sharing a common superscript letter
666 are significantly different ($P<0.05$). SEM: pooled standard error of the mean.
667 EAA: Essential amino acids.
668 NEAA: Non-essential amino acids.

669 Table 6. Effects of diet on distal intestine parameters

	FM 100	FM 25	FM 0	Mean	SEM	*p*-value
Serous Layer [µm] (SL)	43	42	50	45	5.202	0.576
Muscular Layer [µm] (ML)	36	47	55	46	3.501	0.077
Villi length [µm] (VL)	332^{b}	542^{a}	572^{a}	482	50.317	0.011
Villi thickness [µm] (VT)	75	79	83	79	4.704	0.552
Goblet cells (GC)	10^{b}	19^{a}	14^{ab}	14.3	1.899	0.026
Lamina propria thickness[µm] (LPT)	13	14	17	14.7	1.852	0.184
Lamina propria length[µm] (LPL)	280^{b}	498^{a}	511^{a}	429.7	45.711	0.006
Intravilli space	19^{b}	47^{a}	52^{a}	39.3	3.296	0.000

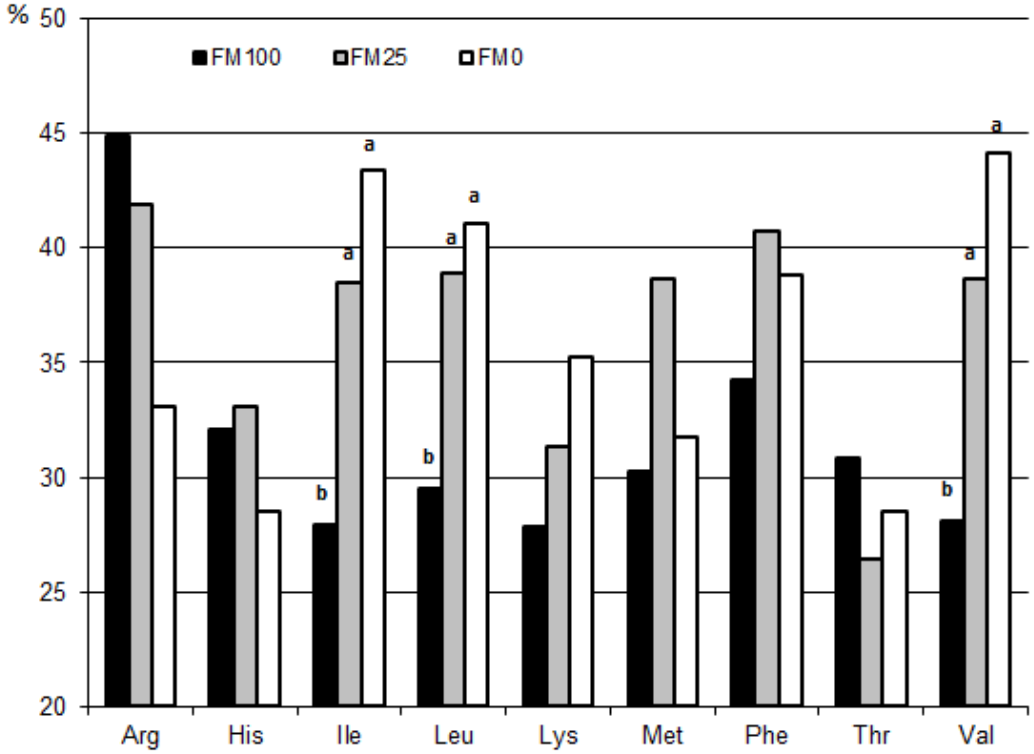
670 Values are the mean ±.S.E.M (n=6). The same letters are not significantly different
671 (p<0.05). Newman- Keuls test. SEM: pooled standard error of the mean.
672

673 Table 7. Blood parameter (hematocrit, hemoglobin and protein) of sea bream fed the
674 experimental diets

	FM 100	FM 25	FM 0	Mean	SEM	*p*-value
Hematocrit [%]	45.4	42.2	47.0	44.9	1.743	0.158
Haemoglobin [g/dl]	7.3	7.6	7.7	7.5	0.514	0.829
Protein [g/dl]	4.4	4.3	4.8	4.5	0.189	0.207

675 Values (mean±SD, n=9) with the same superscript are not significantly different ($p<0.05$).
676 SEM: pooled standard error of the mean.
677

678 Figure 1. Retention efficiency of digestible essential amino acids in Gilthead sea bream fed
679 with the experimental diets at the end of the experiment (Data are presented as means , n=3;
680 Significant differences ($p< 0.05$) are indicated with different letters).



681

682

683

684 Figure 2. Enzyme activities of pepsin, trypsin and chymotrypsin (UA/ g fish) obtained from
685 the stomach and intestine of sea bream fed plant protein–substituted. Bars indicate
686 standard deviations of the mean, and letters show significant differences among the
687 diets (ANOVA, $p< 0.05$).
688

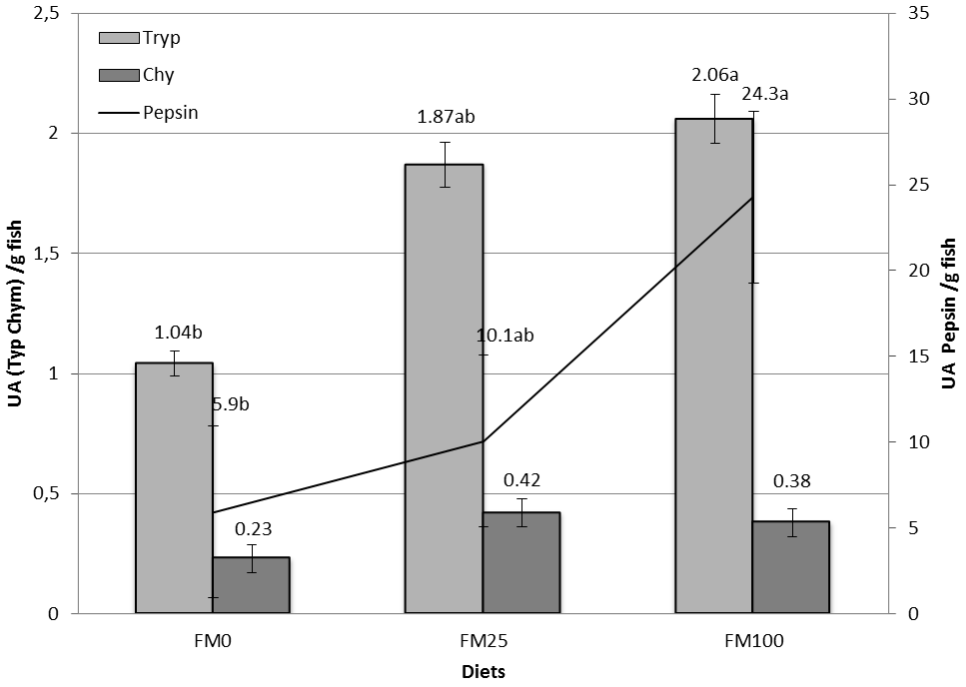


Figure 3. Detail of the different measurements of the gut. (a) Detail of the intestine [20x] with measurements of the serous layer (SL) and muscular layer (ML). villi length (VL), villi thickness (VT), intravilli space (IS), Goblet cells (GC) and lamina propria (LP) length and thickness.

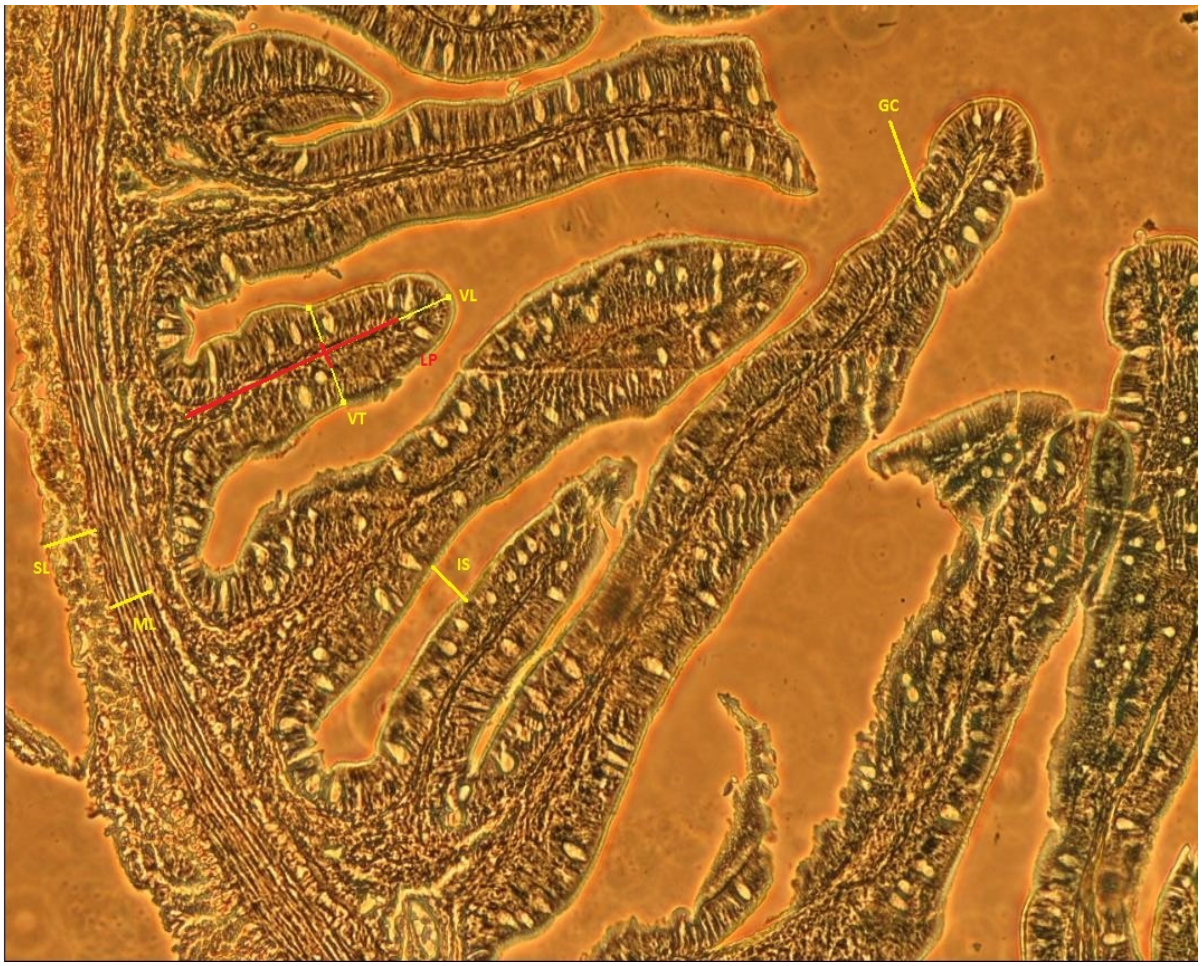


Figure 4. Photomicrograph of 5-µm villi intestinal sections stained with haematoxylin-eosin showing intestinal morphology in *Sparus aurata* (a) Detail of villi length and goblet cells from the distal intestine of fish fed with the FM100 diet [10x]; (b) Detail of villi length and

goblet cells from the distal intestine of fish fed with the FM 25 diet [10x]; (c) Detail of villi thickness and goblet cells of distal intestine from fish fed with the FM0 diet [10x].