Almost total replacement of fish meal by a mixture of plant proteins in gilthead sea bream diets *(Sparus aurata)*

Abstract:

Evaluation of total substitution of fish meal (FM) by plant protein (PP) mixtures were studied. A group of gilthead sea bream (average initial body weight 127 ± 4 g) in the on-growing phase were fed four (FM 100, FM 50, FM 25, FM 0) isonitrogenous (45% crude Protein, CP) and isolipidic (20% crude Lipid, CL) diets with inclusion level of either 100, 50, 25 and 0 percent of FM. Amino acid profile of experimental diets with different PP inclusions percentages were adjusted by synthetic amino acids (AA). Fish were fed to satiation twice daily for 154 days and each diet was tested in triplicate. At the end of the trial, fish reached weights 391, 402, 391 and 361g in the FM 100, FM 50, FM 25, and FM 0 respectively. No statistical differences were observed in specific growth rate (SGR), feed intake ratio (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER), where they were in the average of (0.71% day^{-1}) , (1.37g 100 g $fish^{-1}$ day^{-1}), 2.23 and 1.01 respectively. Biometric indices didn't show any significant differences except in condition factor (CF) and hepato-somatic index (HSI) as they were significantly decreasing by increasing FM replacement percent. Moreover, body composition and retention of ingestible protein were not affected by different experimental diets. Regarding the retention efficiency of essential amino acids (EAA) no statistical differences were found between different diets. AA supplemented to different experimental diets were successfully utilized by fish, where retention efficiency of supplemented lysine of FM 25 (27.05%) and FM 0 (26.64%) were higher than FM 50 (24.60%) and FM 100 (19.02%). The results of the current study clearly demonstrate that total replacement of fish meal by diet containing a blend of plant protein sources balanced with synthetic amino acids is possible in gilthead sea bream in the on-growing phase.

Key words: sea bream, vegetable mixture, fish meal, amino acids, feed utilization.

1.Introduction:

Gilthead sea bream (*Sparus aurata*), a carnivorous fish, needs high protein levels in its diet. Up until a few years ago this nutrient was supplied by fish meal. Thanks to collaboration of scientific research with aquaculture industry, now inclusion level of fish meal lowered to 30 %, but total elimination is not possible with actual diets.

In the last few years, there has been an increase in the demand for aquaculture production as an important source of high biological value animal protein in order to fulfill the demand for animal protein supplement (FAO, 2012).

The largest user of fish meal and fish oil is aquaculture in which total world production reached 79 million tons in 2010 (FAO, 2010; APROMAR, 2012). According to Tacon and Metian (2008) aquaculture sector consumed 68.2% from total global fish meal and 88.5% from total global fish oil production in 2006. Incorporation of fish meal in commercial diet is of high importance in function of the species (Tacon and Metian, 2009) as it is considered a balanced meal that contains high biological value protein (balanced amino acid composition), energy, minerals (e.g. calcium and phosphorous), vitamins (such as choline, biotin and vitamin B_{12}, A, D and E) with respect to trace elements (like selenium and iodine), and the content of highly unsaturated fatty acids omega-3 (Cho and Kim, 2011).

A question of cost and sustainability as the global fish meal and fish oil production is decreasing at annual average rate of 1.7 and 2.6 percent respectively (FAO, 2012). Consequently, nutritionists searched for different protein alternatives, also the fact that fish in their natural habitat fed on small crustaceans, pelagic fish and algae found in the sea.

The possibility of plant feedstuffs use, which are suitable environmentally, friendly and economically alternatives, were studied to fulfill the increase in the need to aqua feed production (Gatlin et al., 2007).

Single protein constituents and their by-products such as soybean meal (SBM) (Kissil et al., 2000; Martínez-Llorens et al., 2007, 2009; Tomas et al., 2009), pea meal (PM) (Gouveia and Davies, 2000; Pereira and Oliva-Teles, 2002; Sánchez-Lozano et al., 2011), sunflower meal (SFM) (Ahmed et al., 2004; Sánchez Lozano et al., 2007; Nogales-Mérida et al., 2011), carob

seed meal (Martínez-Llorens et al., 2012), lupin seed (Pereira and Oliva-Teles, 2004) and faba bean meal (FBM) (Azaza et al., 2009). These feed stuffs successfully substituted fish meal from 12% to 40% in different species.

Plant protein sources were not only the suggested solution but also animal protein alternatives were proposed. Animal protein alternatives sources tried until this moment are meat and bone meal, poultry meal, related poultry by product and blood and hemoglobin meal in gilthead sea bream (Robaina et al., 1997; Nengas et al., 1999; Martínez-Llorens et al., 2008). A few years ago, the use of some of these alternatives was banned by the European Union (EU), but now due to the recent changes in the regulations they might have an interesting future.

Previously mentioned candidates suffered from some drawbacks. As for plant constituents, they are deficient in some limiting EAA content also they contain several anti-nutritional factors such as indigestible carbohydrate (non starch polysaccharides, NSP), unavailability of phosphorus and cationic minerals due to their binding with phytic acid (Gatlin et al., 2007) and high content of fiber (Nogales-Mérida et al., 2011). As for the animal by-product, palatability and digestibility were the main problems (Fasakin et al., 2005). Another successful alternatives were used such as freeze-dried krill (Tibbetts et al., 2011) and fish protein hydrolysate (Zhang et al., 2012), but cost and availability remained unsolved problems. All pre-discussed constrains limited their inclusion with higher percents.

In order to overcome these constrains and maximize their inclusion levels, the use of combinations of different protein sources was proved to compensate PP deficiencies in some nutrients, especially essential amino acids. As described by Kaushik et al. (1998) precise data on amino acids requirements is required before formulating an alternative diet, even more important than total protein content and digestibility.

In this order, De Francesco et al. (2007), Sánchez-Lozano et al. (2009) and Dias et al. (2009) succeeded to include 75%, 60% and 87% of PP mixture in gilthead sea bream diets respectively. In rainbow trout (*Oncorhynchus mykiss)* Zhang et al. (2012) and Burr et al. (2012) reached 95% and 87% inclusion level respectively and 80% in juvenile turbot (*Psetta maxima*) by Fournier et al. (2004).

Total replacement of FM was achieved with good results when supplemented with adequate balance of crystalline amino acids (CAA) to simulate the FM amino acid profile in gilthead sea bream (Kissil and Lupatsch, 2004; Tomas et al., 2011), Atlantic salmon *(Salmo salar)* (Espe et al., 2006; Burr et al., 2012), European sea bass *(Dicentrarchus labrax)* (Kaushik et al., 2004) while in rainbow trout diet was supplemented with CAA in addition to taurine (Gaylord et al., 2006).

Crystalline amino acids content were kept below 10% because higher inclusions render the growth (Espe and Lied, 1994; Espe et al., 2006). However, previous studies were carried out with different mixtures for short periods (Gomez-Requeni et al., 2004) and on young stages of fish from 39 g until 127 g (Kissil and Lupatsch, 2004) as it is very important to study the effect of replacement in different stages in fish life as described by Burr et al. (2012). In other trial, experiment diets were supplemented with attractant as in the trial of Tomas et al. (2011).

The objectives of the present work were to evaluate the effect of high and complete replacement of fish meal by vegetable protein mixture supplemented with crystalline amino acids on growth performance, feed parameters, amino acid retention and nutrient utilization of gilthead sea bream on the on-growing phase.

2. Materials and Methods:

2.1. *Experimental setup and fish sampling*

Gilthead sea bream *(S. aurata)*, 240 fish with initial average weight of 127 ± 4 g (mean ± SD), were obtained from PISCIMAR company (Burriana, Castellon, Spain) and transported alive to the Fish Nutrition Laboratory of the Polytechnic University of Valencia, Spain. They were randomly distributed (20 fish per tank) in 12 cylindrical fiberglass experimental tanks with capacity (1750 L). Prior to feeding trial they were acclimatized for 2 week fed with a standard sea bream diet (48% crude protein, CP, 23% crude lipid, CL, 11% ash, 2.2% crude fiber, CF and 14% nitrogen free extract, NFE).

The tanks were set up in a marine water recirculation system (65 m^3capacity) with a rotary mechanic filter and a gravity biofilter (approximate 6 m^3). The duration of the trial was 154 days from November to April.

Average temperature during the trial was 23 ± 1.5 °C, dissolved oxygen was over 6 ± 0.9 mg L^{-1}, ammonium value was undetectable, salinity 38 ± 1.8 g L^{-1} and PH was 7.2 ± 0.3. All tank was equipped with aeration and the water was heated by a heat pump installed in the system. The photoperiod was natural throughout the experiment with similar lightening conditions for all tanks. All stocked fish were weighted every 30 days approximately. Fish were anaesthetized previously with 30 mg l^{-1} clove oil (Guinama®, Valencia, Spain), containing 87% eugenol. At the beginning and end of the experimental trial, five fish per tank were randomly sampled and stored at - 30 °C to determine the whole–body composition.

2.2. Experimental diets

Formulation of four isolipidic 20% CL and isonitrogenous 45% CP diets with different levels of FM inclusion percent (FM 100, FM 50, FM 25, FM 0) in which FM 100 serves as a control diet.

Previously mentioned diet's formulation and proximate composition are shown in (Table 1). They were formulated based on the proximate analysis of the six protein feed ingredients wheat meal (WM), wheat gluten (WG), soybean meal (SBM), pea meal (PM), sunflower meal (SFM), faba bean meal (FBM) and fish meal (FM). The FM substitution by vegetable protein mixture was carried respectively in the following percentages 25%, 50% and 100%.

The plant protein mix was chosen due to its high protein content supplemented with crystalline amino acids in diets FM 0, FM 25 and FM 50 to fully meet the amino acids requirements of gilthead sea bream fingerlings that were described by Peres and Oliva- Teles (2009). All diets except the control diet were equally supplemented with taurine (20 $g.kg^{-1}$) due to its great importance in osmoregulation, gut development, retinal development (li et al., 2009), membrane stabilization, antioxidation, detoxification and finally conjugation with bile acids (Yun et al., 2012). Additionally, soybean oil was included at 80 ± 10 $g.kg^{-1}$ in all diets according to the results obtained in previous study by Martínez-Llorens et al. (2007).

Diets were prepared using a semi-industrial twin-screw extruder (CLEXTRAL BC 45, St. Etienne, France). The processing conditions were as follows: a screw speed of 100 rpm, a temperature of 110 ^{o}C, and a pressure of 40-50 atm. The experimental diets were assayed in triplicate groups. The fish were fed by hand twice a day (9.00 and 16.00) until apparent satiation. The pellets were distributed slowly to allow all fish to eat. The uneaten diet was collected and dried to determine FI.

2.3. Chemical analysis

Experimental diets and fish carcass were analyzed, following AOAC (1990) procedures: dry matter (105°C to constant weight), ash (incinerated at 550°C to constant weight), CP (N x 6.25) was determined by the kijeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser; Tecator, Hoanas, Sweden) and CL was extracted with diethyl ether (Soxtec 1043 extraction unit; Tecator). All analyses were performed in triplicate except amino acids samples that were done only once.

Following the method previously described by Bosch et al. (2006), amino acids of diets, raw materials fish and carcass were analyzed in 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. The amino acids were derivatized with AQC(6-aminoquinolyl-N-hydroxysuccinimidylcarbamate). Methionine and cystine were determined separately as methionine sulphone and cystine 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 transformed to methionine and cystine.

2.4. *Protein and amino acid retention estimation*

Retention efficiency of protein intake %

$$\text{P IR} = \frac{\text{protein fish gain}\,(\text{g})}{\text{protein intake}\,(\text{g})} \times 100$$

Retention efficiency of amino acid intake %

$$\text{AARE} = \frac{\text{AA fish gain}\,(\text{g})}{\text{AA intake}\,(\text{g})} \times 100$$

2.5. Statistical analysis

Growth data parameters, feed utilization, nutrient efficiency, amino acid composition and retention were treated via multifactor analysis of variance (ANOVA), taking the initial live weight as covariate (Snedecor and Cochran, 1971). The Newman-Keuls test was used to assess specific differences among diets at the P = 0.05 level (Stargraphics, Statistical Graphic System, Version Plus 5.1, Herndon, Virginia, USA).

Table 1: Formulation and proximate composition of the experimental diets

Ingredients (g kg $^{-1}$)	**Diets**			
	FM 100	**FM 50**	**FM 25**	**FM 0**
Fish meal herring	589	295	150	
Wheat	260	66		
Wheat gluten		110	200	295
Soybean meal		130	160	182
Faba bean meal		25	42	41
Pea meal		25	42	41
Sun flower meal		130	160	158
Soybean lecithin	10	10	10	10
Soybean oil	92.88	91.22	91	90
Fish oil	38.12	63.78	77	90
Mono calcium phosphate		19	28	38
Taurine		20	20	20
L-Methionine		5	5	7
Lysine			5	10
Arginine				5
Threonine				3
Multivitamin and minerals mix [1]	10	10	10	10
Proximate composition (g kg $^{-1}$ dry weight matter)				
Dry matter	881	914	928	939
Crude protein	442	447	450	451
Crude lipid	185	193	210	198
Ash	100	98	90	75
Calculated values				
Carbohydrate [2]	273	262	250	276

[1]Vitamin and mineral mix (values are g kg^{-1} except those in brackets): Premix: 25; Choline, 10; DL-a-tocopherol, 5; 2.ascorbicacid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, (1 000 000 IU kg^{-1}); calcipherol, (500 IU kg^{-1}); DL-a-3.tocopherol, 10;menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3;riboflavin, 2.3; pyridoxine hydrochloride, 4.15; cyanocobalamine, 25;nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07;ascorbic acid, 75; inositol, 15; 5.betaine, 100; polypeptides 12.

[2]Carbohydrate calculated as 1000 - (CP +CL +Ash).

Table 2: Amino acid composition of raw materials and experimental diets in dry mater:

	Raw material							Experimental diets			
AA	**FM**	**Wheat**	**WG**	**FBM**	**SBM**	**PM**	**SFM**	**FM 100**	**FM 50**	**FM 25**	**FM 0**
[1]EAA (g 100g $^{-1}$)											
Arginine	5.84	0.38	2.57	1.99	3.66	0.96	1.30	3.39	3.87	3.16	3.3
Histidine	0.54	0.26	1.45	0.74	1.42	0.58	1.14	1	1.11	0.9	0.82
Isoleucine	3.40	0.36	3.01	1.03	2.33	0.98	1.56	1.47	1.3	1.26	1.17
Leucine	6.55	0.80	5.79	2.04	4.22	1.78	2.48	3.24	2.84	3.03	2.98
Lysine	6.01	0.37	1.21	1.92	3.45	1.92	1.39	3.68	2.6	2.12	2.26
Methionine	2.30	0.22	0.88	0.31	0.92	0.36	1.00	1.16	1.14	1.09	1.06
Phenylalanine	3.73	0.49	4.31	1.10	2.60	1.11	1.86	1.8	1.75	1.67	1.87
Threonine	3.55	0.08	2.29	0.47	1.41	0.86	1.52	1.98	1.66	1.45	1.44
Valine	3.88	0.47	3.26	1.13	2.30	1.06	1.73	2.01	1.67	1.57	1.47
[2]NEAA (g 100g $^{-1}$)											
Alanine	4.32	0.43	2.00	1.10	2.16	0.96	1.30	2.74	2.12	1.71	1.39
Aspartate	6.97	0.65	2.23	6.91	6.54	2.72	3.55	4.29	4.48	3.5	3.07
Cystine	0.56	0.20	1.12	0.24	0.47	0.24	0.65	0.61	1.02	0.72	0.58
Glutamine	10.00	3.40	31.98	4.65	10.67	4.23	7.51	7.64	9.82	12.14	12.77
Glycine	4.26	0.48	2.45	1.15	2.11	0.97	2.49	2.44	2.16	1.76	1.65
Proline	2.86	1.09	10.82	1.00	2.46	0.85	1.6	2.3	2.5	3.13	3.18
Serine	3.41	0.53	3.67	1.36	2.74	1.11	1.85	2.11	1.8	1.98	2.05
Tyrosine	2.67	0.08	2.29	0.47	1.41	0.40	0.74	1.5	1.16	0.99	1.07
EAA/NEAA	1.07	0.53	0.43	0.87	0.80	0.91	0.81	0.83	0.72	0.63	0.64

[1] EAA: Essential amino acids.
[2] NEAA: Non-essential amino acids

3.Results:

3.1. Fish Growth and nutritive efficiency

Evolution of mean weights during the experiment of all experimental diets were similar except in the final period of experiment where the FM 0 diet showed lowest evolution of weight as shown in (Fig. 1).

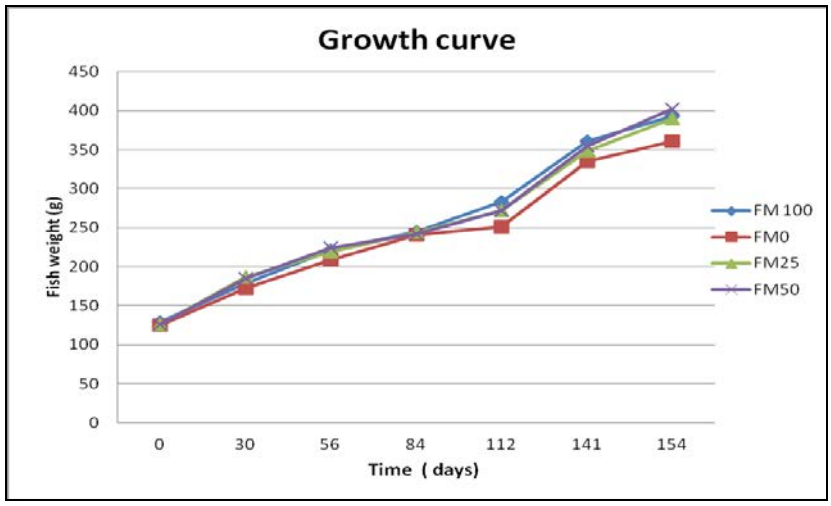

Fig 1. Evolution of gilthead sea bream average weight during 154 days.

No significant differences were found at final weight (Table 3) as it ranged from 361 g FM 0 to 402 g FM 50 which was higher than the control diet. Statistical differences were found concerning diet FM 0 that showed the lowest survival rate 60% among all the experimental diets. Regarding the rest of the parameters, no significant differences were found as SGR ranged from (0.68% day $^{-1}$) FM 0 to (0.73% day $^{-1}$) FM 100, FI ranged from (1.34 g100 g fish $^{-1}$ day $^{-1}$) FM 100 to (1.38 g 100 g fish $^{-1}$ day $^{-1}$) FM 0, FCR ranged from (2.1) FM 100 to (2.4) FM 0 and finally PER ranged from (0.93) FM 0 to (1.07) FM 100 (Table 3).

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

	FM 100	FM 50	FM 25	FM 0	SEM
Initial weight (g)	129.6	125.9	126.1	124.98	4.65
Final weight (g)	391.2	402.3	390.5	361.4	15.6
Survival (%)	88.3 [a]	85 [a]	78.3 [a]	60 [b]	5.53
SGR (% day^{-1}) [1]	0.73	0.7	0.73	0.68	0.02
FI (g 100 g $fish^{-1}day^{-1}$)[2]	1.34	1.38	1.40	1.38	0.02
FCR[3]	2.11	2.13	2.26	2.40	0.10
PER [4]	1.07	1.05	0.99	0.93	0.04

Means of triplicate groups. Data in the same row with different superscripts differ at ($P < 0.05$). SEM: pooled standard error of the mean. Initial weight was considered as co-variable for final weight and SGR.

[1] Specific growth rate (%day^{-1}) SGR=100×ln(final weight/initial weight)/days.

[2] Feed Intake ratio (g 100 g $fish^{-1}day^{-1}$) FI=100×feed consumption (g)/average biomass (g)×days.

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

[4] Protein Efficiency ratio PER= Weight gain (g)/ Protein intake (g)

3.2. Biometrics, body composition and nutrient retentions

Biometrics and body composition fish data are shown in (Table 4), statistical differences were observed only in two parameters; a gradual decrease of CF which was contributed by increasing level of replacement by PP mixture from (1.92%) FM 100 to (1.69%) FM 0 and the same was noticed regarding HSI with values ranged from (1.56%) FM 100 to (1.18%) FM 0. No statistical differences were observed regarding the viscero-somatic index (VSI) and mesenteric fat index (MF), where they varied from (9.16%) FM 100 to (8.73%) FM 0 and from (1.6%) FM 100 to (1.5%) FM 0 respectively.

Respecting body composition (Table 4), no significant differences were found in which CP values ranged from (17.7%) FM 100 to (17.9%) FM 0, CL values ranged from (16.1%) FM 100 to (14.1%) FM 0 and ash content varied from (2.7%) FM 100 to (3.2%) FM 0. Respecting retention of protein intake (PIR) parameter, no significant differences were found as it ranged from FM 100 (19.26%) to FM 0 (17.30%).

Table 4: Biometric indices, proximate composition and retention efficiency (data are expressed as percentage of wet weight, ww) of gilthead sea bream fed experimental diets.

	FM 100	**FM 50**	**FM 25**	**FM 0**	**SEM**
CF (%)[1]	1.92 [a]	1.87 [ab]	1.73 [bc]	1.69 [c]	0.05
VSI (%) [2]	9.16	9.98	8.95	8.73	0.45
HSI (%) [3]	1.56 [a]	1.45 [ab]	1.29 [bc]	1.18 [c]	0.05
MFI (%)[4]	1.6	1.25	1.29	1.54	0.18
Moisture (%)	62.3	64.4	64.2	64.4	0.66
Crude protein (%*wet weight*)	17.7	17.7	17.7	17.9	0.36
Crude lipid (%*wet weight*)	16.1	14.8	14.6	14.1	0.68
Ash (%*wet weight*)	2.7	2.9	3.2	3.2	0.26
PIR (%) [5]	19.26	19.02	18.05	17.30	0.74

Means of triplicate groups. Data in the same row with different superscripts differ at ($P < 0.05$). SEM: pooled standard error of the mean.
[1]Condition factor. CF=100×total weight (g)/total length^3 (cm^3).
[2]Viscero-somatic index (%), VSI = 100×Visceral weight (g) / Empty fish weight (g)
[3]Hepato-somatic index (%) HSI =100×liver weight (g)/fish weight (g).
[4]Mesenteric fat index (%) MFI = 100 x Visceral fat (g) / Empty fish weight (g)
[5] Efficiency of Protein Intake Retention (%) PIR =Fish Protein gain (g)/Protein intake (g)×100.

Amino acid composition of whole fish body expressed as percentage of wet weight is shown in (Table 5). Results showed no significant differences among different diets. Retention of ingested amino acids expressed by percentage were illustrated in (Table 6). Although diets FM 25 and FM 0 had higher retention values concerning arginine, histidine, isoleucine, lysine and valine, no significant differences were noticed among different treatments regarding retention of ingested EAA. Significant differences were found in the retention of three of the NEAA; where in alanine and tyrosine of fish fed FM 100 obtained the lowest values, on the contrary, glutamine retention lowest value was corresponding to fish fed FM 0 and FM 25 .

Table 5: Amino acid body composition (data are expressed as percentage of wet weight, ww) of gilthead sea bream fed experimental diets.

AA	Iniciales	FM 100	FM 50	FM 25	FM 0	SEM
[1] EAA (g/100 g ww)						
Arginine	1.61	1.47	1.37	1.55	1.54	0.098
Histidine	0.36	0.39	0.39	0.4	0.38	0.022
Isoleucine	0.50	0.66	0.63	0.65	0.66	0.033
Leucine	1.20	1.16	1.14	1.14	1.16	0.050
Lysine	1.27	1.42	1.33	1.29	1.37	0.072
Methionine	0.41	0.47	0.47	0.45	0.44	0.026
Phenylalanine	0.60	0.58	0.58	0.58	0.55	0.028
Threonine	0.72	0.66	0.79	0.66	0.65	0.065
Valine	0.67	0.81	0.8	0.81	0.83	0.033
[2] NEAA (g/100 g ww)						
Alanine	1.07	1.08	1.1	1.06	1.08	0.033
Aspartate	1.57	1.6	1.58	1.51	1.6	0.073
Cystine	0.07	0.13	0.16	0.13	0.11	0.019
Glutamine	2.33	2.41	2.34	2.34	2.43	0.091
Glycine	1.24	1.25	1.39	1.3	1.3	0.110
Proline	0.75	0.7	0.78	0.75	0.78	0.046
Serine	0.70	0.59	0.57	0.58	0.58	0.022
Tyrosine	0.44	0.42	0.41	0.45	0.41	0.021
EAA/NEAA	0.90	0.93	0.90	0.93	0.91	

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

Table 6: Retention percentage of ingested amino acids in each experimental diet.

AA	FM 100	FM 50	FM 25	FM 0	SEM
[1]EAA					
Arginine	19.35	14.93	21.54	19.07	2.06
Histidine	19.23	17.28	20.66	20.18	1.21
Isoleucine	23.20	25.07	25.49	27.39	1.78
Leucine	16.42	18.27	16.16	15.86	1.22
Lysine	19.02	24.60	27.05	26.64	1.92
Methionine	20.55	20.87	19.38	17.98	1.22
Phenylalanine	14.91	15.29	14.98	11.67	0.84
Threonine	14.98	23.67	19.10	17.64	3.45
Valine	20.73	24.48	25.15	26.25	1.36
[2]NEAA					
Alanine	18.43 ^{c}	24.62 ^{b}	27.41 ^{b}	32.71 ^{a}	1.54
Aspartate	17.76	16.56	18.78	22.10	1.24
Cystine	12.76	9.46	10.10	9.53	1.68
Glutamine	15.04 ^{a}	11.22 ^{b}	8.57 ^{c}	8.17 ^{c}	0.65
Glycine	24.04	31.88	33.90	34.01	4.61
Proline	13.69	15.04	10.55	10.51	1.14
Serine	11.91	13.22	11.60	10.29	0.80
Tyrosine	12.79 ^{c}	16.18 ^{ab}	19.75 ^{a}	14.82 ^{c}	1.16

Retention of ingested amino acids = fish amino acid gain (g)/ amino acid ingested (g) x100.
Means of triplicate groups. Data on the same row not sharing a common superscript letter are significantly different ($P<0.05$). SEM: pooled standard error of the mean.
[1]EAA: Essential amino acids.
[2]NEAA: Non-essential amino acids.

4.Discussion

Several studies showed the success of using synergistic combinations of plant protein mixtures to perform high or total substitution in gilthead sea bream *(S. aurata)*, such as (Kissil and Lupatsch, 2004; De Francesco et al., 2007; Dias et al., 2009; Sánchez-Lozano et al., 2009; Tomas et al., 2011). In addition to other species like rainbow trout (Gomes et al., 1995; Gaylord et al., 2006; Burr et al., 2012; Zhang et al., 2012), Atlantic Salmon (Espe et al., 2006; Burr et al., 2012), European sea bass (Kaushik et al., 2004) and juvenile turbot (Fournier et al., 2004) where the results varied accordingly.

In previous studies on gilthead sea bream, total levels of FM replacement by vegetable mixture (supplemented with adequate balance of synthetic amino acids) resulted in good growth levels as in the trial of Kissil and Lupatsch (2004) and Tomas et al. (2011). However, De Francesco et al. (2007), Dias et al. (2009) and Sánchez-Lozano et al. (2009) also achieved good growth levels with 75%, 87% and 60% PP mixture inclusion levels, respectively. In the latter study, 90% replacement negatively affected the growth, which was attributed to anti-nutritional factors present in diets or intestinal alteration that occurs with high levels of vegetable meal inclusion (Martínez-Llorens et al., 2009).

The results of the present trial show that substitution of FM with vegetable mixture supplemented with synthetic EAA doesn't not affect the daily rate of gilthead sea bream growth. SGR values were within the average obtained by Tomas et al. (2011) except in the current study, no significant differences were observed among different treatments but in the trial of Tomas et al. (2011) the SGR obtained values, were (0.69% day^{-1}) for fish fed diet containing 100% PP that were significantly higher than fish fed 100% FM with SGR (0.64% day^{-1}). Kissil and Lupatsch (2004) also obtained higher SGR (1.28% day^{-1}) with fish fed diet 100% PP than SGR (1.21% day^{-1}) with fish fed 100% FM diet. However, average SGR values obtained by Kissil and Lupatsch (2004) were higher than the current trial. This difference could be due to young stages of growth (average 41.2 g to 122 g) used in this study. In contrast, Gomez-Requeni et al. (2004) reported a gradual decrease in SGR with increasing PP inclusion percent, anyhow, that was a normal result for low FI occurred in that trial.

In the current study, the average SGR was (0.72% day^{-1}), which was lower than the average values obtained by Sánchez-Lozano et al. (2009), that was (0.9 % day^{-1}) within fish with the same average weight. However, this was attributed to lower FI coincidence with the subclinical infection occurred during 1the current study. In other species, literature shows that a decrease in SGR occurs with high PP inclusion levels (Gomes et al., 1995; Kaushik et al., 2004; Fournier et al., 2004; Espe et al., 2006) as rainbow trout, European sea bass, juvenile turbot and Atlantic salmon respectively.

Survival rates were lower than previous studies carried on gilthead sea bream (De Francesco et al., 2007; Sánchez-Lozano et al., 2009; Dias et al., 2009; Adamidou et al., 2011; Tomas et al., 2011) with significant differences in fish fed diet FM 0. These lower rates were caused by "red point disease" (*Pseudomonas anguiliseptica)* that was transmitted to gilthead sea bream through the water circulation system as the whole installation share the same system.

The infection was transported to fish used in current study from other hatchery specimens having winter syndrome brought from the sea to be used in another concurrent experiment. Gilthead sea bream were treated using Septrin® pills antibiotic from the sulfonamide family, its active principal trimethoprim and sulfamethoxazole together known as co-trimoxazole. They inhibit the synthesis of folic acid (folate). Without folate, bacteria cannot produce DNA thus, they are unable to increase in number. Co-trimoxazole therefore stops the spread of infection.

Mortalities occurred in all tanks, but the highest number was recorded in FM 0 tanks. It can be explained that the fish with this treatment were highly susceptible, thus, a decrease in immune defense mechanism takes place with high level of vegetable meal inclusion (Sitjà-Bobadilla et al., 2005).

Regarding nutritional parameters, there weren't differences in FI with the different levels of replacement by PP mixture. Same results were observed in previous studies on gilthead sea bream (Sánchez-Lozano et al., 2009; Dias et al., 2009; Adamidou et al., 2011; Tomas et al., 2011) and European sea bass (Kaushik et al., 2004). That was in contrast to De Francesco et al. (2007), that obtained low FI with 75 % PP mixture and Gomez-Requeni et al. (2004) who had a decrease in FI with 100%, 75% and 50% PP mixture in gilthead sea bream. Low FI due to high FM replacement was also observed in juvenile turbot (Fournier et al., 2004),

Atlantic Salmon (Espe et al., 2006) and rainbow trout (Burr et al., 2012).

However low FI was attributed to decreased palatability (Pereira and Oliva-Teles, 2002; Gomez-Requeni et al., 2004; De Francesco et al., 2007), level of anti-nutritional factors (Fournier et al., 2004; Gomez-Requeni et al., 2004; Martínez-Llorens et al., 2009) and inadequate EAA profile of diet (Gomez-Requeni et al., 2004).

FCR and PER were not affected by different PP inclusion levels in agreement with previous studies on gilthead sea bream (Kissil and Lupatsch, 2004; Sánchez-Lozano et al., 2009; Tomas et al., 2011) and on juvenile turbot (Fournier et al., 2004). On the contrary, Adamidou et al. (2011) had an increase in FCR with increasing level of replacement by mixtures containing mainly bean meal 350 g kg^{-1} or pea meal with the same inclusion level, although, the mixtures used in that trial were different in quantity and quality. De Francesco et al. (2007) obtained higher PER values with diet contains inclusion percent of 75 % PP than FM based diet suggesting better utilization of diet by fish. PER values decreased by increasing level of replacement in another trial in European sea bass (Kaushik et al., 2004) reflecting different pattern of diet utilization species dependent.

In the biometric indices, HSI was decreasing significantly by increasing level of PP percent contradicting most of literature (Gomez-Requeni et al., 2004; De Francesco et al., 2007; Sánchez-Lozano et al., 2009; Adamidou et al., 2011) in gilthead sea bream and other species such as European sea bass, juvenile turbot and Atlantic salmon (Kaushik et al., 2004; Fournier et al., 2004; Espe et al., 2006) respectively. However, Nogales-Mérida et al. (2011) obtained a decrease in HSI due to coincidence of reproduction period within the time of experiment and high inclusion level of crude fiber which was not the case in the current trial.

Two explanations would clarify this decrease in HSI. The first one as discussed by other authors in different species is that any increase in lysine and methionine content would affect liver size, since they play a role in carnitine biosynthesis which is involved in fatty acid transportation through the outer mitochondria membrane, where a reduction of whole lipid content will be noticed (Hansen, 2009). On the contrary, in rainbow trout an increase in HSI occurred due to reduction in lysine content (Walton et al., 1984). Marcouli et al. (2006) in gilthead sea bream found that an increase in dietary lysine affect positively protein content and negatively lipid content. Espe et al. (2008) obtained an increase in HSI due to low methionine content in Atlantic salmon. In the same study, Espe et al. (2008) mentioned

methionine as a potential hepatotoxic AA when in excess could cause cirrhosis and shrinkage of liver.

The other explanation, as pre-discussed an infection with red point caused by *Pseudomonas anguiliseptica* took place during current experiment. It is usually isolated from liver and other organs such as kidney and spleen (Doménech et al., 1997; Magi et al., 2009). In this sense, it would be possible that after treatment, cirrhosis and shrinkage occurred to the part infected of liver and it is increased by increase in susceptibility starting from FM 100 to FM 0 tank where FM 0 tanks coincidence with highest mortality.

Experimental diets didn't affect the whole-body proximate composition values that were nearly similar in agreement with Kissil and Lupatsch (2004), De Francesco et al. (2007) and Dias et al. (2009) in gilthead sea bream, Espe et al. (2006) in Atlantic salmon and Fournier et al. (2004) in juvenile turbot.

In the current study, no significant differences in PIR among different diets were observed, which ranged in the same interval of Sánchez-Lozano et al. (2009), on the contrary, these authors obtained higher PIR values with diet containing 60% PP mixture but these values significantly lowered in 90% replacement diet. PIR was negatively affected in diets containing 20% FM supplemented with crystalline amino acids in juvenile turbot (Fournier et al., 2004). Low PIR values in studies containing PP mixtures supplemented with crystalline amino acid could be explained due to their fast absorption (Espe and Lied, 1994). That was not the case in the current study, and the same was observed in Atlantic salmon and European sea bass (Espe et al., 2006, 2007, 2008; Kaushik et al., 2004). Current trial results are in agreement with the scientific hypothesis that was proposed in previous study on Atlantic Salmon stating that as long as dietary amino acid composition is balanced, high inclusion of PP mixture won't affect protein utilization (Espe et al., 2006, 2007, 2008).

Experimental diets were supplemented with taurine, which is absent in plant protein ingredients. Taurine plays an important role in several body functions such as osmoregulation, gut development and antioxidation (li et al., 2009; Yun et al., 2012). Although fish can partially synthesize it, it is not sufficient to meet fish requirement (Yokoyama et al., 1997; Gaylrod et al., 2006) as high replacement percents with PP mixtures could not be achieved without taurine supplement (Gaylrod et al., 2006). This fact was demonstrated in several species (Kim et al., 2005; Gaylrod et al., 2006; Lunger et al., 2007;

Takagi et al., 2008).

Although Kaushik et al. (1998) determined the optimum dietary needs of EAA by using the amino acid pattern in muscle protein of sea bass, sea bream and turbot, Peres and Olivia-Teles (2009) shaped a new system for evaluating EAA profile using deletion method, definitive data regarding each of the EAA are still unavailable. All experimental diets of this trial containing blend of PP include higher levels of EAA than Peres and Oliva-Teles (2009) recommendations for fingerlings. Except a decrease in the final quantity of lysine and methionine in analyzed feed respecting to the designed one, possibly due to Maillard reaction that occurred during extrusion process which didn't affect the retention efficiency and consequently the growth of fish in the current study.

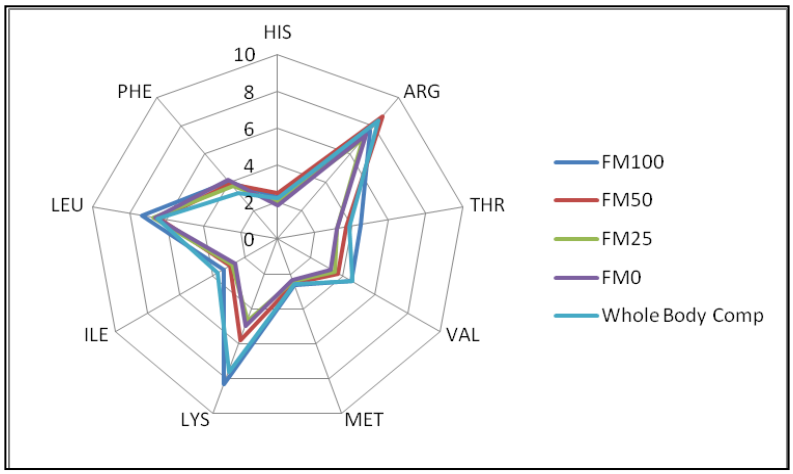

Fig.2. Essential amino acid profile of experimental diets and average of whole body fish expressed as g per 100 g^{-1} of protein

The average of whole-body composition was homogenous with the EAA profile (Fig. 2) in agreement with Sánchez-Lozano et al. (2009). The lysine content of PP diets appears to be inferior to its content in FM 100 diet and whole-body composition. In fact, if we reviewed the requirements stated by Peres and Oliva-Teles (2009) for lysine which is 5.13 ± 0.73 compared to amino acids composition of the experimental diets, it is clear that all PP based diets contain lysine levels higher than the former recommendations.

The retention efficiency of AA and the growth didn't show differences in fish fed FM diet and fish fed PP mixture supporting the hypothesis of Santigosa et al. (2011) who observed higher availability for free AA than protein-bound AA. Results of the current study were in

agreement with Kaushik et al. (2004), Espe et al. (2007) and Burr et al. (2012) in European sea bass and Atlantic salmon respectively, concluding that total substitution of FM by PP meal has no negative effect on growth if the amino acid profile was well balanced.

The use of synthetic AA to diets include different PP percentages are demonstrated by the current study to be successfully utilized by fish. Retention efficiency of lysine (Table 6) in PP based diets FM25 (27.05%) and FM 0 (26.64%) supplemented with synthetic lysine seemed to be (without statistical differences) higher than FM 50 (24.60%) and FM 100 (19.02%) diets, where no synthetic lysine was added. Synthetic arginine and threonine added to diet FM 0 were efficiently utilized by fish equally to the rest of the diets resulting in efficient retention with no significant differences between all the treatments.

It is well known that the relation between the dietary AA content and its retention is inversely proportional which means that higher retention of specific AA is contributed to its low content in diet and vice versa. This fact explains why FM 100 diet had the lowest retention efficiency of lysine among the treatments with no significant differences and that was due to it is high content of dietary lysine which was higher than the recommendations (Peres and Oliva-Teles, 2009) resulting in its lower retention.

Concerning the retention of NEAA, significant differences were only found in alanine, glutamine and tyrosine, these differences could be explained by the previous mentioned scientific fact in which diets contain higher amounts of a certain AA will result in its lower retention, thus comparing the retention results of alanine for example which were in FM 0 (32.71%) and FM 100 (18.43%) with the alanine content of the same diets FM 0 (1.39 g 100 g $^{-1}$) and FM 100 (2.74 g 100 g $^{-1}$), it is clearly demonstrated that the relation in inversely proportional.

Generally, AA retention results obtained in the current trial were slightly low compared to the previous studies (Sánchez-Lozano et al., 2009; Martínez-Llorens et al., 2012) on gilthead sea bream. As pre-discussed before, due to the infection that occurred for a period during the trial, FI was affected negatively resulting in low growth and low retention values compared to PP diet with inclusion level 60% (Sánchez-Lozano et al., 2009). In the study of Martínez-Llorens et al. (2012), high retention values of EAA obtained in that trial were logic because the trial was conducted on fingerlings in which their growing rate was higher than the on-growing phase studied in the current study.

In Conclusion, as has been shown in this work, diets of gilthead sea bream in the on-growing phase containing vegetable mixture supplemented with synthetic EAA can replace 100% of FM without negatively affecting sea bream performance. Likewise, these results clearly indicate that the use of synthetic amino acids to balance diet with high or total FM replacement is a successful approach that has no effect on growth performance and nutrient utilization by fish. In which it is clearly demonstrated, the ability of gilthead sea bream to utilize crystalline AA added to PP based diet is as efficiently as protein-bound AA found in FM.

Acknowledgment

I would like to thank Erasmus Mundus Welcome project (Action 2) for giving me the opportunity to study master in aquaculture in University Polytechnic Valencia. My deep thanks to my supervisors Prof. Miguel Jover Cerdá and Prof. Ana Tomás Vidal for their supervision. My appreciation to Raquel Ortiz for her supervision and guidance during working in the laboratory. Many thanks to Prof. Dr. Ramadan EL Banna, Prof. Dr. Maha Hady and Dr. Adel Tohamy (Cairo University, Egypt), Prof. Abdel Rahman EL Gammal (World Fish Center, Egypt), Dr. Hanan Abosetet (National Research Center, Egypt) for continuous encouragement and scientific support. Finally, I would like to thank my family and colleagues for their help and support during my stay in Spain.

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