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POULTRY BY-PRODUCT MEAL AS AN ALTERNATIVE TO FISH MEAL IN GILTHEAD SEABREAM (Sparus aurata) DIET

M.Sabbagh[1], R.Schiavone[1], G.Brizzi[2], B. Sicuro [3], L.Zilli[1] and S.Vilella[1]

[1]University of Salento Di.S.Te.B.A Laboratory of Comparative Physiology, [2]Chlamys Srl, [3] Department of Veterinary Sciences, University of Torino, ITALY

Abstract

The aim of the present study was to evaluate the actual possibility of fish meal substitution in the diet of farmed seabream by poultry by-product meal (PBM) to verify its sustainability in farming practice. Therefore, inclusion of PBM in seabream diet was investigated by a multidisciplinary analysis to evaluate its possible effects on fish growth performances, fish welfare and fillet quality. Thus, control diet (commercial diet) and two experimental diets Feed A and Feed B contained PBM with 50% and 100% of FM substitution respectively were formulated. All diets were isoproteic 45% and isolipidic 20%. The growth trial lasted 110 days including 2 weeks of fish acclimatization. Juveniles gilthead seabream with an initial average weight of 73,57 ± 10,47 g were allotted randomly in 9 tanks (3 replicates per diet), fed once a day by hand (feeding rate 1%). Sampling was performed monthly.

As results, average weight gain increased in all fish groups without any statistically significant difference (P>0.05). Measured zootechnical parameters were similar among fish groups, condition factor as an indicator of fish condition was about 2 (good to excellent condition) and survival rate was 100%. Investigations through hematological parameters, digestive enzymes and liver histology analyses demonstrated that no statistical difference was found among dietary treatments and this clear evidence suggest that PBM inclusion in seabream diets did not affect negatively fish welfare. Protein patterns obtained from fish fed with control diet and PBM diets, showed similar expression of structural proteins such as actin, tropomyosin, MLC1, MLC2, MLC3. Results concerning fish fillet compositions were comparable in all fish groups with some exception for fatty acids composition. Gross energy content of seabream muscle was, also, not affected by PBM and resulted value was about 148 Kcal/100g.

The present study, demonstrated that the total substitution of fish meal with poultry by product-meal in the commercial diet of gilthead seabream (*Sparus aurata*) is achievable without compromising fish growth performances, fish welfare and fillet quality and suggests that PBM could be considered as a good sustainable raw material for fish food.

Keywords: Fish nutrition, Fish meal substitution, Poultry by-product meal, Gilthead seabream (*Sparus aurata*), Growth performances, Fish welfare, Fillet quality.

INTRODUCTION

Aquaculture growth in the last decade and global consumption of farmed fish has been increasing. The food is one of the major costs in aquaculture, understanding the effect of nutrition strategy on quality and marketability of farmed fish fillet is very important (Baghaei Jezeh et *al*., 2014). Fish meal (FM) is currently the major source of proteins in fish feeds (Emre et *al*., 2003) but its limited availability affected by many factors such

as climate changes and specially el Nino phenomenon in Peru and Chile (about 2/3 of annual global production) has notably heightened the cost of this commodity (Tacon & Metian, 2008; Goda et *al*., 2007). Accordingly, the substitution of FM is the main modern challenge for fish feed suppliers and has stimulated nutritionists and researchers to find sustainable, effective and cheaper protein sources (Shepherd et al., 2005). Substitution of fish meal by less expensive plant proteins has progressively increased in modern aquafeed industry, but the use of these resources in fish diets is hampered by the presence of a variety of endogenous anti-nutritional factors (Ytrestoil et al, 2015; Tacon, 1992).

Poultry by-product meal (PBM) is considered as a suitable alternative protein of fish meal (Gaylors & Rawles 2005; Rawles et *al*., 2006; Thompson et *al*., 2007) in artificial diets for carnivorous and omnivorous aquaculture species, due to its high production volume, nutritional composition, price and supply advantages over fish meal (Hardy 2000; Yu, 2004). According to the Association Of American Feed Control Officials (AAFCO), Poultry by-product meals consists of the ground, rendered, clean parts of the carcass of slaughtered poultry such as necks, feet, undeveloped eggs and intestines, exclusive of feathers (Watson, 2006).

As any other ingredient obtained by animal by-product, the PBM nutrient content can be variable as its composition depends on the processing methods applied and by original composition (Dale et *al*., 1993; Lupatsch el *al*., 1997; Johnston et *al*., 1999; Shapawi et *al*., 2007). It is generally considered a palatable and high-quality feed ingredient in fish nutrition due to its optimal content in essential amino acids, fatty acids, vitamins and minerals (Bureau et *al*., 1999; 2000; Zhou et *al*., 2004; NRC, 2011). PBM is currently used in livestock nutrition, particularly in pig nutrition, its demand is increasing in aquafeed industry and several authors confirmed that PBM has considerable potential as feed ingredients in fish production systems (Bureau et *al*., 2000; Millamena, 2002; Fasakin et *al*., 2005). Some studies have shown that PBM could replace 75% or even 100% of fish meal without significant decrease in fish growth. De facto, PBM could replace 75% of FM in the diet of juvenils gilthead Seabream *Sparus aurata* without amino acid supplementation (Nengas et *al*., 1999) and up to 100% for red sea bream *Pagrus major* (Takagi et *al*., 2000). Cuneate drum, *Nibea miichthioides*, fed successfully with 50% of the fish meal replaced by PBM (Wang et al., 2006). In red drum, *Sciaenops ocelatus*, Kureshy et al., (2000) were able to raise juvenile fish on a diet with 66.7% of PBM. The inclusion of PBM in the diets of freshwater fish were also successful. Muzinic et *al*., (2006) replaced 100% of the fish meal protein with turkey meal without any negative effects on the growth of sunshine bass (*Morone chrysops x M. saxatilis*). Gibel carp (*Carassius auratus gibelio)* grew well with the substitution of 50% of fish meal by PBM protein (Yang et al., 2006).

Research on the digestibility of PBM, suggested that is well digested by many fish species (Yang et *al*., 2006). Studies on PBM substitution showed a high protein digestibility (>88 %) and energy (> 80 %) in the diet of rainbow trout, salmon, Japanese

seabass and striped bass. These digestibility values suggest that PBM could be used in aquafeeds to a level similar to FM. Yu, (2004) affirmed that these animal proteins, has a nutritional composition and feeding value similar to that of fish meal for tilapia and trout. In the diet of Gilthead seabream the digestibility in protein for PBM was about 81.8%, 84.3% in lipids and 80.3% in energy (Nengas et *al*., 1999), for seabass protein digestibility was 84.5% (Alexis et *al*., 1997).

The aim of this study is to investigate the PBM inclusion in gilthead sea bream (*Sparus aurata*) nutrition and its effects on fish growth, physiology of digestive system and quality of final product.

MATERIAL AND METHODS

EXPERIMENTAL DIETS

Two different diets Feed A and Feed B were formulated with increasing levels of BPM inclusion (50% and 100% of fish meal substitution respectively). These diets were tested against a control diet without PBM. Fish feeds were prepared on request by a commercial company Veronesi Group (Verona, Italy) as extruded pellets (diameter 3 mm). All the diets were isoproteic (45% of crude protein) and isolipidic (20% crude fat) (Tab. 2).

In addition to fish meal and poultry-by-product meal, feed composition contain other ingredients in order to reach a balanced amino acid profile complying with the nutritional requirements of gilthead seabream (*Sparus aurata*), (Table 1).

Table 1: Fish feed composition

CONTROL DIET	FEED A (50%)	FEED B (100%)
Fish meal	Fish meal	-
-	Poultry-by-product meal	Poultry-by-product meal
Flour de-hulled soya beans	Flour de-hulled soya beans	Flour de-hulled soya beans
Wheat	Wheat	Wheat
Soybean oil	Fish oil	Vital wheat gluten
Peas protein	Soybean oil	Fish oil
Fish oil	Canola oil	Soybean oil
Vital wheat gluten	Vital wheat gluten	Canola oil

The percentages of feed components are confidential and were not disclosed by the feed manufacturer.

Table 2 : Analytical component of the control and experimental fish feeds

%	CONTROL DIET	FEED A	FEED B
Crude protein	45	45	45
Crude fat	20	20	20
Cellulose	1.80	1.60	1.60
Ash	7.50	9.60	8.50
Calcium	1.22	2.30	2.15
Phosphorus	1.04	1.48	1.35

Sodium	0.45	0.45	0.18

The analysis of the analytical constituents (Table 2) of the three diets were performed and certified by the feed manufacturer.

EXPERIMENTAL DESIGN AND GROWTH TRIAL

110 days of growth trial was carried out in an industrial aquaculture facility, belonging to a commercial company Panittica Pugliese S.r.l (Torre canne, Brindisi, Italy). 1818 juveniles' seabream (*Sparus aurata*), with an initial mean body weight of 73.57 ± 10.47g, were randomly allotted to 9 tanks, each one containing 202 fish.

Before starting the growth trial, fish were acclimatized to experimental tanks for two weeks (from July 15, to August 1, 2014) and fed only with the control diet. Growth trial was held from August 1 to November 1, 2014. The experimental design was monofactorial, balanced with three replicates per diet (3 x 3) and the experimental factor was the diet. Fish were weighted every 15 days in order to check fish biomass gain and to adjust the feeding rate that was 1% of biomass. The three diets were supplied seven days at week, once a day, by hand.

Water temperature was stable at 19.4 ± 0.16 °C, and dissolved oxygen was added to water input and was > 100% of saturation. These parameters were measured every two days using an oximeter. Fish samplings were conducted monthly (T_0, T_{30}, T_{60} and T_{90}).

SAMPLING PROCEDURE

After two weeks of adaptation, an initial sampling was carried out and 30 fish were sacrificed, in iced water for 40 min as the normal practice in Italian fish farming. Total weight and standard length were measured to calculate the morphometric parameter (initial mean body weight, initial mean length). 24 fishes were monthly sacrificed from each experimental group (8 x 3 x 3), then transported immediately to the Laboratory of Comparative Physiology (D.i.S.Te.B.A.) at the University of Salento, in a cool bag with ice within one hour of sample collection.

GROWTH PERFORMANCE AND SOMATIC INDEXES

Upon arrival at the laboratory, fish were individually weighted using a technical balance and standard length was measured with a metric ruler. At each sampling, the following mean individual growth performance indexes were calculated per treatment: Average weight gain (AWG), condition factor (K), Specific growth rate (SGR), Food conversion rate (FCR), viscerosomatic index (VSI) and hepatosomatic index (HSI), and survival rate (SR). 24 fish were used to determine productive parameters and 15 were used for somatic index determination.

DISSECTION PROCEDURE

The peritoneal cavity of fish was opened along a ventral midline incision. The entire visceral package was extracted and weighted to determine viscero-somatic index (VSI). Then liver was removed and weighted to calculate the hepato-somatic index (HSI). Individual liver and intestine were isolated, washed with distilled water and conserved at -80°C for further analysis (digestive enzymes activities). One part from each liver was conserved immediately after dissection into formaldehyde (10%) to be used in histological analysis.

Fish muscle dissection was performed as follows: a piece of muscle was taken from the fish, the skin was removed, and the central core of the muscle was used for muscle quality analysis (Proteomic analysis, amino acid profile, fatty acid profile, lipid peroxidation etc.). Muscle samples were extracted from the loin muscle, under the dorsal fin and above the lateral line and then conserved at -80°C until further analysis

BLOOD PLASMA ANALYSIS

From all sampled fish, before dissection procedure, blood (1 ml) was collected from caudal vasculature into heparinized syringes and plasma was separated by centrifugation at 2500 g for 20 min and stored at -20°C until use. Plasma protein concentration was measured by Bradford Method (Bradford, 1976) and plasma osmolality was measured using an automatic osmometer (5520 VAPRO, Delcon).

Cortisol determination was performed with a commercially available immunoassay Kit (EIA cortisol Kit; Cayman Chemical, Ann Arbor, MI). Commercial kits (Sigma-Aldrich) were used for Alanine Aminotransferase (ALT) and Aspartate aminotransferase (AST) activities measures.

DIGESTIVE ENZYMES ASSAYS

Nine fish per treatment (3 fish per tank) were used for enzymes analysis. 0,3 g of each organ (liver and intestine) were homogenized in 3 ml of 1.1 % NaCl, 0.5 mM Phenylmethanesulphonylfluoride (PMSF; 100 µl) buffer using a mechanical homogenizer a polytron (Kinematica, GmbH). All analysess were carried out immediately after homogenate preparation at 4°C. Enzyme activities were measured by spectrophotometric method.

All the enzyme analyses were conducted in three replicates. In order to establish the specific activities of the enzymes, protein concentrations were determined in the enzyme extracts by Bradford method (Bradford, 1976), with bovine albumin as standard.

Alkaline phosphatase activity was measured, both in the gut and liver following Bessey et *al*. (1946) method. Lipase determination was adapted from Albro et *al*. (1985) method.

HISTOLOGICAL EXAMINATION OF LIVER

For liver histology analysis, five fish per tank were collected at each sampling. Liver samples were preserved in formalin (10%) within one week. Samples were routinely dehydrated in ethanol and embedded in paraffin according to standard histological techniques. Transverse sections were cut to a thickness of 5 µm and stained with haematoxylin and eosin for examination with light microscopy. The morphology of the liver was evaluated on a scale of 1 to 3 using the criteria of McFadzen et *al*. (1997).

PROTEOMIC ANALYSIS

Proteomic analysis was performed following Schiavone et *al*. (2008) protocol. Analysis was performed by protein extraction, Isoelectric Focusing (IEF) using IPGphor (Amersham Biosciences) and SDS-PAGE separation. The acquisition and the analysis of 2D gels was done using Imagemaster 2D Elite software 3.1 (Amersham Biosciences).

AMINO ACID ANALYSIS

Amino acids profile in fish muscle (edible part) was performed following Bosch et *al*. (2006) protocol. Acid hydrolysis was used for all amino acids except cysteine and methionine for which performic acid oxidation followed by acid hydrolysis was used. The amino acid contents was determined using an HPLC system (Agilent 1100 series) consisting of two pumps, manual sampler and a fluorescence detector. Aminobutyric acid was added as an internal standard before hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Chromatographic separation was carried out using a C18 Column (Gemini® 5 µm 150 x 4.6 mm). The column was thermostated at 37 °C and the flow rate was 1.0 ml/min. The injection volume was 10 µl and injection was carried out manually. Mobile phase A consisted of AccQ.Tag eluent A (100 ml AccQ.Tag A concentrate + 1l Milli-Q water). Mobile phase B consisted of eluent B acetonitrile-water solution (60:40, v/v). Gradient conditions shown in table 3 were selected in a previous study. Beginning the gradient, the column was equilibrated in 100% A for 10 min. Detection was carried out by fluorescence (λ excitation 250 nm and λ emission 395 nm).

Table 3 : Gradient profile for mobile phases A and B

Time (min)	*A (%)*	*B (%)*
Initial	100	0
0.5	98	2
15	93	7
19	90	10
32	67	33
35	67	33
36	0	100
41	0	100

FATTY ACID ANALYSIS

Fillet lipids extraction was performed using gas chromatography (6890 Series GC System, Agilent Technologies) with a capillary column DB - 5MS (60 m x 0.32 mm) and a manual sampler. The GC unit is connected directly to a mass spectrometer detector (MSD), (5973 Network Mass Selective Detector). The MSD provides structural information on the separate components that help the chemical identification of unknown materials. Pure helium (82 kPa flow) was used for the analysis. The helium had an air flow of 50 kPa and a hydrogen flow of 60 kPa under the following temperature conditions: first, the initial temperature was at 150 °C for 5 min. Second, the temperature was increased by 5 °C/min until 170 °C and then maintained for 10 min. Third, the temperature was increased at a rate of 5 °C/min up to 220 °C and maintained for 20 min.

The data are reported as mean values ± standard deviation of mean. (n= 3 from each tank for all the sampling at t0, t30 days, t60 days and t90 days).

Total lipid, cholesterol, phospholipids and gross energy content in muscle

Fillet total lipid content was determined using the commercial kit “Cell Biolabs lipid quantification kit”. Three fish per tank and treatment (3 x 3 x 3) were used for total lipid

analyses. Cholesterol quantification was performed using a commercial Kit "Sigma-Aldrich Cholesterol Quantification Kit".

Phospholipids analysis was performed using a commercial Kit (Sigma-Aldrich Phospholipid Assay Kit), in order to assess the effect of diets in fish muscle phospholipids content.

The gross energy content of fish muscle and diets was measured by combustion in a Parr bomb calorimeter 1241 (Parr, Instrument Company, Moline, IL, USA), (Figure 41) using benzoic acid as the standard.

LIPID PEROXIDATION: TBARS METHOD

Commercial lipid peroxidation (MDA) Assay Kit (Sigma-Aldrich) was used in order to evaluate the effects of the experimental diets on lipid peroxidation in fish fillet. Lipid peroxidation was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product, proportional to the MDA. Thiobarbituric acid-reactive substances (TBARS) were expressed as nanomoles MDA per µl sample volume.

STATISTICAL ANALYSIS

Chemical analysis

Fish diet was the experimental factor tested. The experimental design was balanced, monofactorial, with three replicates (3 x 3). All data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (IBM SPSS Statistics 23). After ANOVA test, the differences among means were determined by a multiple comparisons test, Tukey test using the significance level of $P<0.05$. Results are presented as means ± SD.

Proteomic analysis

The amount of protein in a spot was taken as the area of the spot multiplied by the density and referred to as the volume. Following removal of background, the spot volumes were normalized to the total protein detected for each gel by dividing the individual spot volume by the sum of all spot volumes and multiplying by 100. The normalized spot volume is referred to as abundance. Normalized volume of each spot considered in this study was evaluated as mean ± SD of spot normalized volumes measured in all gels. Considering that these data did not show normal distribution, non-parametric statistic was used in the statistical elaboration (*i.e.* Mann - Whitney test).

RESULTS

1. GROWTH PERFORMANCE AND SOMATIC INDEX

Productive parameters and somatic indexes were monthly measured (30, 60 and 90 days). All diets were well accepted by fish and the survival rate was 100% for all fish groups. The initial average body weight was 73,57 ± 10,47 g and has steadily increased in all the experimental groups (figure 1). At the end of the trial all the fish showed a similar (not statistically different) weight, at 150,46 ± 22,07g in fish fed control diet, 138,71 ± 20,87g and 145,19 ± 22,2g in fish fed by Feed A and Feed B respectively.

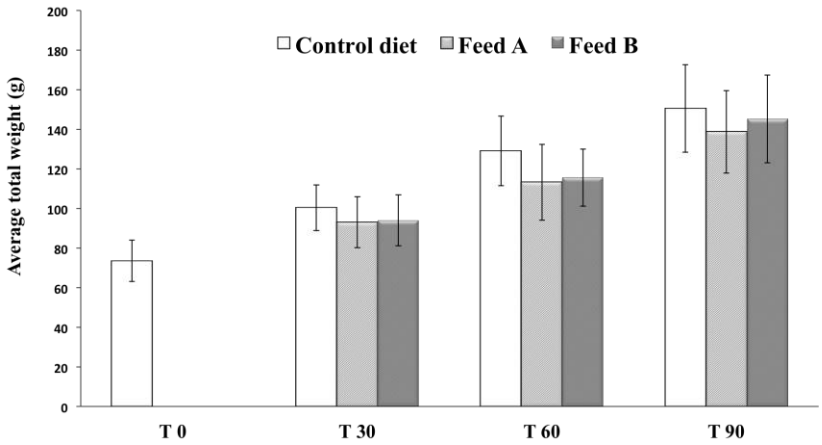

Figure 1: Weight growth of fish during the experimental period. (mean ± SD, n=24).

The initial average length was 13,76 ± 0,58 cm and has showed a length gain in all fish groups (figure 2). At the end of the trial the average length was 17,65 ± 0,85 cm in fish fed by control diet, 17,25 ± 0,82 cm and 17,52 ± 0,83 cm in fish fed Feed A and Feed B respectively. No statistical difference was found on final fish length among all fish groups.

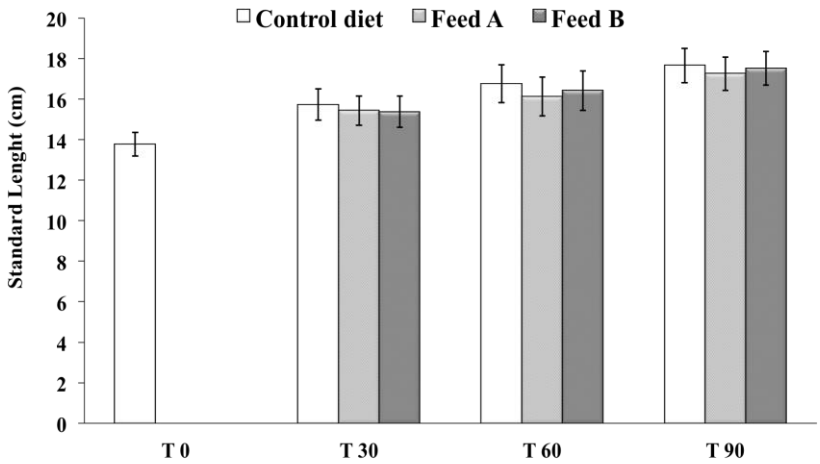

Figure 2: Fish length during the experimental period. (mean ± SD, n= 24)

The reported results, clearly suggest that the replacement of fish meal by poultry by-product meal in juveniles gilthead seabream diets does not affect significantly fish growth in terms of weight and length.

Productive parameters of fish experimental groups, either fed with control and experimental diets, were similar, (Table 4). Only at 60 days the AWGs of fish fed A and B showed lower ($P<0.05$) values with respect to that of fish fed control diet, but at the end of the trial no statistical difference was found.

The Condition Factor K allows to compare quantitatively the condition of individual fish within a population, individual fish from different populations, and two or more populations. The value of K is influenced by age of fish, sex, season, stage of maturation, fullness of gut, type of food consumed, amount of fat reserve and degree of

muscular development (Barnham and Baxter, 1998). In our trial, condition factor (K) was similar in all groups and ranged from 2,54-2,75.

Table 4: Productive parameters and somatic indexes.

	T_0	T_{30}		
		CONTROL	**FEED A**	**FEED B**
SGR[a]	-	1.03 ± 0.20	0.79 ± 0.04	0.81 ± 0.30
AWG[b]	-	26.73 ± 6.19	19.63 ± 1.11	20.54 ± 8.16
K[c]	2.82 ± 0.43	2.58 ± 0.17	2.54 ± 0.05	2.58 ± 0.04
HSI[d]	1.92 ± 0.18	2.42 ± 0.23	2.64 ± 0.31	2.56 ± 0.30
VSI[e]	7.82 ± 0.95	8.80 ± 1.12	9.99 ± 1.42	9.10 ± 1.15

	T_{60}			T_{90}		
	CONTROL	**FEED A**	**FEED B**	**CONTROL**	**FEED A**	**FEED B**
SGR	0.84 ± 0.09	0.72 ± 0.09	0.75 ± 0.03	0.79 ± 0.05	0.71 ± 0.04	0.76 ± 0.06
AWG	55.48 ± 6.92	42.67 ± 5.91*	43.98 ± 1.75*	76.88 ± 6.15	70.24 ± 3.58	71.62 ± 1.91
K	2.74 ± 0.05	2.70 ± 0.07	2.57 ± 0.12	2.75 ± 0.07	2.73 ± 0.07	2.72 ± 0.04
HSI	2.68 ± 0.32	2.78 ± 0.29	2.67 ± 0.27	2.83 ± 0.28	2.23 ± 0.37	2.79 ± 0.35
VSI	8.65 ± 1.06	9.06 ± 0.82	8.77 ± 0.96	8.29 ± 1.21	8.16 ± 0.47	7.40 ± 0.79

[a]Specific Growth Rate (SGR) = 100* (ln final weight – ln initial weight)/time (days)
[b] Average Weight Gain (AWG) = Final weight (g) – Initial weight (g)
[c] Condition factor (K) = [weight of the fish (g) / standard length of fish^3(cm)] *100
[d] Hepato-somatic Index (HSI) = (Liver weight/body weight) *100
[e] Viscero-somatic Index (VSI) = (Visceral weight/body weight) *100
All Values are given as the mean ± standard deviation (SD), n= 15
*: Significant difference *P< 0.05*

2. FISH WELFARE

2.1. Blood biochemical assays

Table 5 : Protein concentration (n=9), osmolality (n=9), cortisol level (n=5), ALT (n=9) and AST (n=9) in gilthead seabream plasma.

	T_0	T_{90}		
		Control	**Feed A**	**Feed B**
Protein concentration (mg/ml)	7.33 ± 1.33	10.99 ± 0.99	10.59 ± 1.71	10.78 ± 0.93
Osmolality (mmol/kg)	634.8 ± 18.34	615 ± 18.67	601.4 ± 8.99	618.4 ± 42
Cortisol (pg/ml)	1476 ± 400	918.86 ± 329	582.44 ± 243	1137.97 ± 330
AST (mU/ml)	21 ± 2.53	24.66 ± 1.65	24.20 ± 1.42	23.96 ± 1.09
ALT (mU/ml)	6.27 ± 0.89	9.56 ± 1.23	10.01 ± 0.97	9.45± 1.72

The replacement of fishmeal by poultry by-product meal in the diet does not significantly (*P>0.05*) affect cortisol, proteins levels, osmolality, ALT and AST activities in fish plasma (Table 5).

2.2. Digestive enzyme activities

No statistically significant difference was found among experimental groups concerning liver alkaline phosphatase. The intestinal enzymatic activities (alkaline phosphatase, lipase and leucine-amino peptidase) at the end of the trial showed similar values in all experimental groups (Table 6).

Table 6: Liver and Intestine enzymes specific activities of gilthead seabream fed with control and experiment diets (n= 9).

Enzymes activities (mU/mg)	T_0	T_{90}		
		Control	Feed A	Feed B
Liver Alkaline phosphatase	158,72 ± 14,90	89,24 ± 18,41	89,66 ± 18,06	88,02 ± 19,72
Intestine Alkaline phosphatase	489,49 ± 75,97	71,95 ± 20	110 ± 22,67	100 ± 19
Lipase	3,14 ± 0,93	1,76 ± 0,68	2,52 ± 0,97	2,35 ± 0,57
Leucine-amino peptidase	31,29 ± 6,95	21,16 ± 3,21	27,65 ± 6	18,54 ± 4,92

All values are given as the mean ± standard deviation (SD)

2.3. Liver histological analysis

As regard as liver, 336 histological analyses were analyzed, corresponding to 28 sections per diet per sampling, were analyzed to assess liver condition.

At the beginning of the trial, fish showed a healthy liver characterized by normal irregular shaped hepatocytes with very prominent circular nuclei and conspicuous centrally located circular nuclei. At the end of the trial the most of examined liver showed healthy hepatocytes (Figure 3).

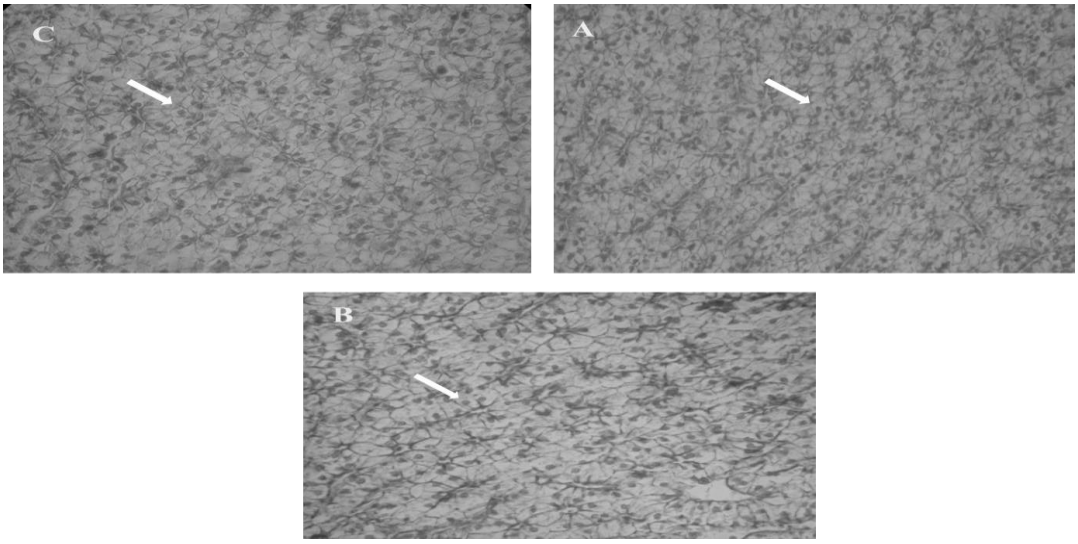

Figure 3: Livers microscopic structure at the end of growth trial. (C) Control diet, (A) Feed A, (B) Feed B. Normal hepatocytes with circular conspicuous and centrally located nuclei (arrows) (H and E staining).

However, 7% of fish fed control diet showed slight alterations (characterized by hepatocytes with nuclei dislocated to the cells border) and the liver samples were classified at the intermediate grade. The frequency of histological alterations increased to 9 % in fish fed A and to 10% in fish fed B.

3. FILLET QUALITY

3.1. Fillet protein profile: proteomic analysis

Proteomic analysis of fish fillet was carried out to evaluate the effect of the different experimental diets on the proteoma. At each sampling date, 3 fish were randomly sub-sampled in order to carry out the analysis. All analyses were performed in triplicate runs (90 gels).

Image analysis using "Image Master 2D Platinum" in fish muscle revealed 700-800 spots. No statistical difference was found on total spots number between fish groups at the end of the trial (743 ± 22, 751 ± 14 and 756 ± 18 in the control, Feed A and Feed B group respectively). Considering that the resolution of spots corresponding to higher molecular weight (>60 kDa) was scarce, the successive analysis was focused on the partial proteome with the optimal resolution (figure 4)

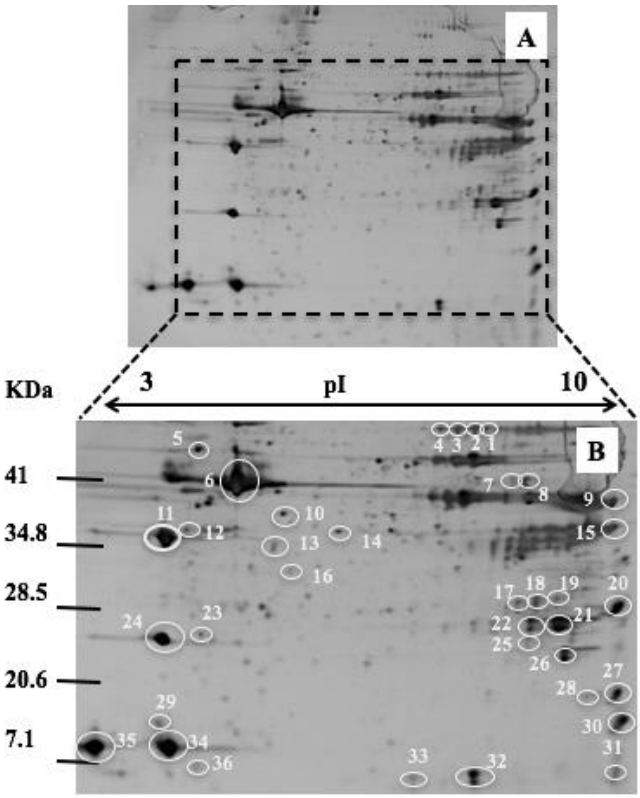

Figure 4: 2-D PAGE of sea bream muscle proteins at the end of acclimatization period. The *dotted line square* specifies the area in the gels used for further proteome analysis. The marked spots were investigated.

Spots differences were initially examined by visual inspection and subsequently by using image analysis software. Spots of interest were selected according to the criteria

used in Schiavone et al. (2008) study. Following these criteria, 36 spots were selected to be investigated during the experimental trial. Within these 36 selected spots, only few (named 2, 4, 5, 6, 10, 11, 12) showed changes in the normalized volume (Figure 5).

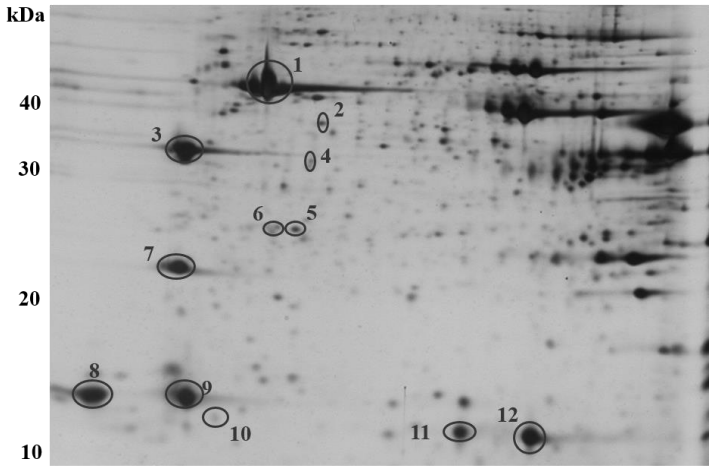

Figure 5: 2-D Page of gilthead seabream muscle proteins selected in control fish group at 30 days of growth trials.

Table 7: Molecular weight (MW) and isoelectric point (pI) of selected spots

Spots number	MW kDa	pI
1	43	5.1
2	38	5.4
3	33	4.6
4	32	5.3
5	26	5.4
6	26	5.2
7	21	4.6
8	19	3.5
9	19	4.7
10	18.5	4.8
11	18	6.1
12	17.5	6.6

The results (Tab. 7) demonstrated that the normalized volume of spots 2,4,5,6, reached a maximum of increase at 30-days and kept constant up to 90-days both in fish fed A and B. Visual inspection of gels also suggest that the spots expression increased with the increase of poultry by-product meal content in diets (Figure 7). In addition, our results demonstrated that the normalized volume of spot number 10 increased only in muscle of fish fed B.

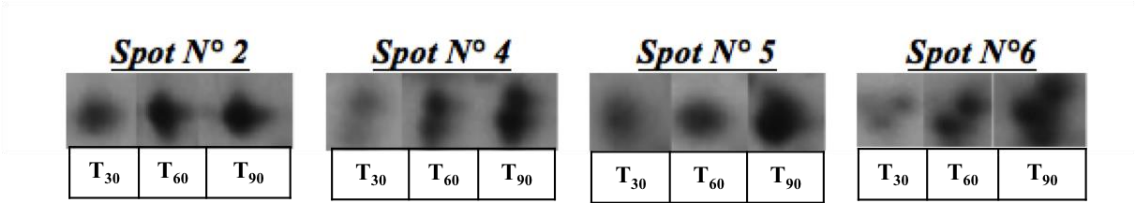

Figure 7: Spots with increased normalized volume during growth trial

A different pattern of volume variation was observed for the spots 11 and 12. With respect to control group, these spots in fish fed A and B, showed a decrease of the normalized volume after 30 days of trial, but at the end of the trial no significant difference was observed (among fish groups) between them and with respect to the control (Figure 8 and 9).

Figure 8: Normalized volume of spots N° 11 and N° 12 at 30 days of trial (C: Control group, A: Fish fed A; B: Fish fed B)

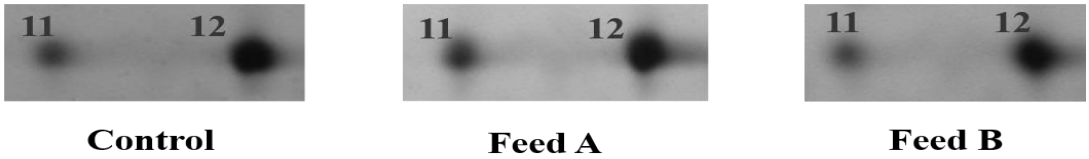

Figure 9: Normalized volume of spots N°11 and N° 12 at the end of growth trial

In addition, our results demonstrated that the normalized volume of spot 10 increased in muscle of fish fed B after 30 days but at the end of trial it showed a normalized volume similar to the control.

The normalized volume of spots 1, 3, 7, 8, 9, did not show any change during all the trials (Figure 52). Based on previous studies (Schiavone et *al*., 2008; Addis et al., 2013) these spots were identified as: Spot N° 1 Actin, alpha skeletal muscle; Spot N° 3: Tropomyosin 1, alpha chain; Spot N° 7: Myosin light chain 1 (MLC 1); Spot N° 8: Myosin light chain 2 (MLC 2); Spot N° 9: Fast skeletal myosin light chain 3 (MLC 3).

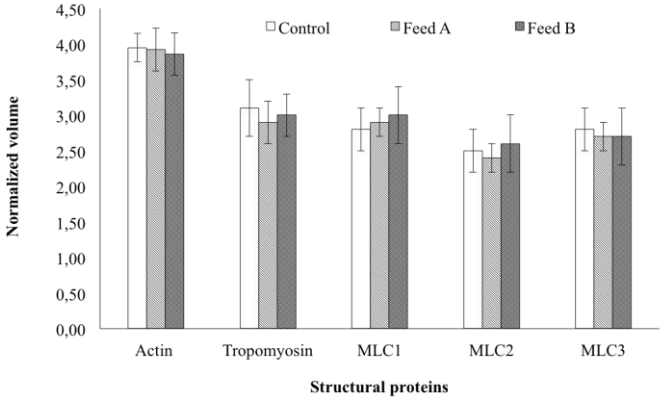

Figure 6: Expression profiles of structural proteins. Spot intensity is expressed as mean ± SD of normalized spot volume (n=9, 3 fish per experimental treatment condition in three replicates).

3.2. Muscle amino acid profile

Amino acids profile in the fillet was performed by AQC-derivatization method and using High Performance Liquid Chromatography.

Table 8: Amino acids profile in fish fillet (data are expressed as g 100 g^{-1} wet weight, mean ± DS, n=3).

	T_0	T_{90}		
		Control	**Feed A**	**Feed B**
Arginine	2,93 ± 0,81	3,31 ± 0,45	3,38 ± 0,17	3,45 ± 0,21
Histidine	0,70 ± 0,12	0,81 ± 0,11	0,79 ± 0,08	0,77 ± 0,10
Isoleucine	0,52 ± 0,07	0,61 ± 0,07	0,66 ± 0,05	0,61 ± 0,03
Leucine	1.03 ± 0,09	1,39 ± 0,21	1,35 ± 0,11	1,41 ± 0,16
Lysine	1,08 ± 0,13	0,90 ± 0,07	0,84 ± 0,13	0,83 ± 0,09
Methionine	0,53 ± 0,14	0,78 ± 0,14	0,70 ± 0,10	0,68 ± 0,17
Phenylalanine	0,62 ± 0,08	0,71 ± 0,06	0,70 ± 0,04	0,68 ± 0,08
Threonine	1,29 ± 0,04	1,75 ± 0,10a	1,46 ± 0,15b	1,46 ± 0,12b
Valine	0.65 ± 0,12	1,15 ± 0,21	0,97 ± 0,09	0,93 ± 0,10
∑ EAA	7,67 ± 0,54	11,40 ± 1,32	10,85 ± 1,10	10,80 ± 1,52
Alanine	1,22 ± 0,11	1,67 ± 0,21a	1,49 ± 0,13b	1,45 ± 0,11b
Aspartate	2,19 ± 0,20	2,81 ± 0,40	2,31 ± 0,12	2,18 ± 0,76
Cystine	0,12 ± 0,03	0,18 ± 0,04	0,14 ± 0,06	0,16 ± 0,03
Glutamine	3,05 ± 0,30	3,69 ± 0,53	3,20 ± 0,32	3,10 ± 0,82
Glysine	0,20 ± 0,06	0,33 ± 0,04	0,30 ± 0,04	0,30 ± 0,06
Proline	0,87 ± 0,13	1,00 ± 0,08	0,87 ± 0,18	0,88 ± 0,18
Serine	1,35 ± 0,72	1,14 ± 0,16	1,00 ± 0,08	0,95 ± 0,09
Tyrosine	1,89 ± 0,16	0,98 ± 0,06a	0,72 ± 0,15b	0,71 ± 0,08b
∑ NEAA	10,91 ± 1,01	12,01 ± 2,34	10,04 ± 1,07	9,74 ± 1,96
EAA/NEAA	0,70 ± 0,04	0,95 ± 0,07	1,08 ± 1,01	1,11 ± 0,09

At the end of the experiment, the results show that the amino acid profile in the muscle of seabream is similar in fish fed feed A, feed B and in those fed with the control diet, with the exception of alanine, tyrosine and threonine, (Table 8).

3.3. Muscle total lipid, total phospholipids and total cholesterol content

Lipid concentration increased at the end of growth trial in all the experimental groups and no statistical difference was detected in the fillet fat content (Table 9). The analysis of phospholipids did not reveal any differences between experimental groups.

Table 9: Total lipid (n=3), Total phospholipids (n=5), Total cholesterol (n=3) and Lipid peroxidation (n=5) in fish muscle.

T 0	T 90

		Control	Feed A	Feed B
Total lipid (mg/dL)	0.21 ± 0.06	0.38 ± 0.07	0.48 ± 0.08	0.53 ± 0.15
Total phospholipids (µM)	312 ± 72	437 ± 25	425 ± 36	428 ± 18
Total cholesterol (pg/µl)	1.79 ± 0.5	3.85 ± 1.2	4.18 ± 1.2	3.14 ± 1.0
Lipid peroxidation MDA(nmole/µl)	0.30 ± 0.01	0.28 ± 0.04	0.30 ± 0.03	0.32 ± 0.01

All values are given as the mean ± SD.

Total cholesterol in seabream muscle increased almost linearly during the growth trial. However, no statistical differences were found between fish groups (Table 9). Moreover, free cholesterol and cholesterol esters measured in the muscle of all fish groups showed no statistical difference (data not reported). Lipid peroxidation was investigated in order to evaluate the possible effects of fish meal substitution with PBM on fillet quality and resulted similar in all fish groups (Table 9) at the end of trials.

3.4. Fillet fatty acids profile

Fillet fatty acid analysis was performed by lipids extraction, transesterification and fatty acids separation using GC-MS. Qualitative analysis was determined by mass spectrometry and retention time corresponding to the standard mixture, while quantitative analysis was determined as the percentage of identified fatty acid of total fatty acids in each sample.

The most abundant fatty acid in gilthead seabream fillet is oleic acid (C18:1n-9) (figures 7; 8) that after 30 days was measured at range of 34-36 % of total fatty acids, and 33-38% after 90 days of experimentation. Oleic acid content in the muscle of fish fed with experimental diets was higher ($P<0.05$) than that found in muscle of control fish group.

Linoleic acid (C18:2n-6) content in the muscle of all fish groups increased at 60 days of trial and remained almost stable at the end of the experiment. As for oleic acid, the linoleic acid content in fish muscle of experimental groups is higher than that found in control fish muscle. The values of linoleic acid measured at 90 days-trial, were 18,93%; (control) 19,95 % (Feed A) and 20,44% (Feed B).

At 30-days of the trial, the palmitic acid content was higher ($P<0.05$) in the muscle of control fish group than those fed with experimental diets. At the end the percentage of C16:0 was about 16% of total fatty acids in the muscle of all fish groups ($P>0.05$). The content of the remaining fatty acids identified, (C14:0; C16:1; C18:0; C20:1n-9; C20:5n-3 and C22:6n-3) was less than 10% of total fatty acids in fish fillet.

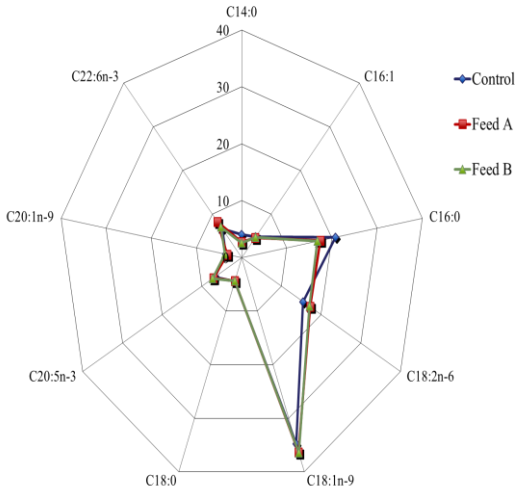

Figure 7: Fatty acid profile of gilthead seabream muscle at 30 days of growth trial. (Data are expressed as % of individual fatty acid from of total fatty acids in samples), n=3.

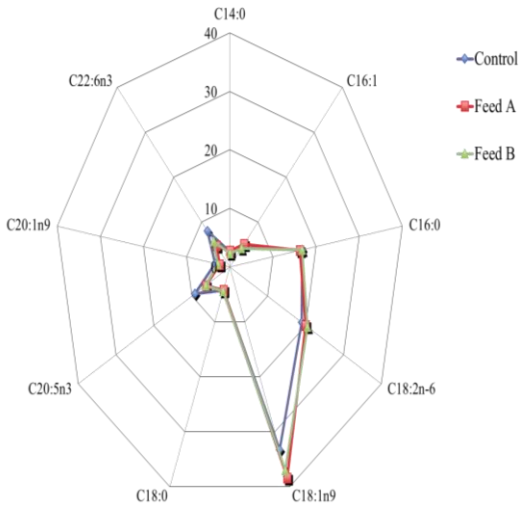

Figure 8: Fatty acid profile of gilthead seabream muscle at 90 days of growth trial. (Data are expressed as % of individual fatty acid from total fatty acids in samples), n=3.

Eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) levels decreased in the muscle of the Feed A and Feed B fish groups in comparison with control group at 60 and 90 days of trial, but no statistical difference (*P>0.05*) was found, except for DHA at 90 days (*P<0.05*) (Table 10).

Table 10: Eicosapentaenoic acid and docosahexaenoic acid content (% of total fatty acids) in fish muscle (Mean ± SD, n=3).

	T 0	T 90		
		Control	Feed A	Feed B
EPA (%)	8.22 ± 1.41	9.22 ± 1.86	6.32 ± 1.23	6.36 ± 1.07
DHA (%)	7.45 ± 1.72	8.01 ± 1.47 [a]	4.73 ± 1.11 [b]	5.72 ± 1.23

The total amount of monounsaturated fatty acids (MUFA) is higher (*P<0.05*) in muscle of seabream fed with poultry by-product meal compared to those fed with control diet, while the opposite occurs for the quantity of polyunsaturated fatty acids (PUFA), especially, at the end of the experimental trial (*P<0.05*), (Table 11).

n-3/n-6 ratio resulted significantly lower in experimental fish group than the control one only at 60 days (*P<0.05*). Concerning EPA/DHA ratio, no significant difference (*P>0.05*) was detected among treatments.

Table 11: Fatty acid composition of fish muscle fed control and two experimental diets (Feed A and Feed B) at the end of the trial, mean ± SD, n=3 (in triplicate)

	Control	Feed A	Feed B
SFA	23,61 ± 1,05	23,21 ± 0,92	23,52 ± 0,57
MUFA	40,92 ± 0,73 [a]	45,79 ± 0,70 [b]	43,96 ± 0,30 [b]
PUFA	36,16 ± 0,90 [a]	31,00 ± 0,57 [b]	32,52 ± 0,62 [b]
n-3	17,23 ± 0,08 [a]	11,05 ± 0,11 [b]	12,08 ± 0,22 [b]

n-6	21,67 ± 1,08	19,95 ± 0,30	20,44 ± 0,39
n-9	36,65 ± 0,28 [a]	40,73 ± 0,41 [b]	39,79 ± 0,16 [b]
EPA/DHA	1,15 ± 0,29	1,34 ± 0,18	1,11 ± 0,22
n-3/n-6	0,80 ± 0,35	0,55 ± 0,09	0,59 ± 0,07

All values are given as the mean ± standard deviation (SD)
Means in the same row followed by different letters are significantly different *P<0.05*

3.5. Muscle gross energy content

Gross energy content of fish muscle was similar in all fish groups and no statistical difference (*P>0.05*) was detected. At the end of trials gross energy content values were 145 ± 8; 148 ± 8 and 149 ±10 Kcal/100g in control, feed A and feed B fish groups respectively.

4 DISCUSSION

Poultry by-product meal is considered as a suitable alternative to fish meal especially because has some nutritional characteristics comparable to those of fish meal. Poultry by-product meal has been investigated in the diet of several fish species in aquaculture (Nengas et al., 1999; Kureshy et al., 2000; Emre et al., 2003; Sevgili and Erturk, 2004; Yu, 2004; Rawles et al., 2006; Subhadra et al., 2006; Cruz-Suarez et al., 2007; Guimaraes 2008; Hu et al., 2008; Zhou et al., 2016; Booth et al., 2012; Tabinda and Butt, 2012; Baboli et al., 2013; Fuertes et al., 2013; Gunben et al., 2014; Hernandez et al., 2014; Mohamadsalehi and Baboli, 2015). Some researchers have been focused on the nutritional value of this ingredient (proximate composition, amino acids and fatty acids content) and on its effect on fish growth. Neither studies on the effect of poultry by-product meal, nor on the physiological parameters (such as enzyme activities) nor on fillet quality such as protein patterns, and lipid peroxidation are available for gilthead seabream (*Sparus aurata*).

Gilthead seabream is among the most important marine finfish species in the Mediterranean (Gómez-Requeni et *al*., 2004) and its production is still expanding rapidly. In the Mediterranean, the *S.aurata* production in 2013 was about 173 062 tonnes and about 6184 tonnes in Italy (According to FAO- Fisheries and Aquaculture Information and Statistics Service). Few studies tested the substitution of fish meal by PBM in the diet of *S.aurata* (Alexis, 1997; Lupatsch et *al*., 1997; Nengas et *al*., 1999). Alexis, (1997) reported that good quality of PBM could replace 100% of fish meal without a significant loss in fish performance.

4.1 Growth performances

Previous research on artificial feed digestibility suggested that PBM-containing feed is suitable by several fish species (Bureau et al., 1999; Lupatsch et al., 1997; Yu, 2004; Yang et al., 2006). The present results confirmed that feeds containing PBM (50% and 100%) used during this trial did not show any negative effect on fish growth. These findings confirm those of Alexis, (1997) which demonstrated that the inclusion of 75% and 100% in the diet of *S. aurata* fingerlings gave productive results close to that of fish fed fish meal.

The ability of poultry by-product meal to substitute fish meal was also supported by the observation that all the productive parameters measured in this experimentation (Specific Growth, Average Weight Gain, Condition factor, Viscerosomatic index, and hepatosomatic index) were similar in all the experimental groups. These results are in agreement with a previous study carried out in seabream by Nengas et al. (1999) and are similar to those obtained in other marine fish species fed PBM such as red seabream (Takagi et al., 2000), sunshine bass (Rawles et al., 2009) and grouper (Shapawi et al., 2007).

No significant differences were revealed on productive parameters and somatic indexes among different fish groups at the end of the experimental period. In the present experiment, the SGR of all fish groups ranged from 0.8-1.0 (without differences between groups). This value is similar to that obtained in previous study (Sicuro et al., 2010) but lower with respect to that measured by Nengas et al. (1999) in gilthead seabream fry fed with PBM. However, this difference could be explained by the difference on development stage of fish under experiment.

In addition, the AWG at the end of trials did not differ between experimental fish groups. Several studies have demonstrated that FM replacement with PBM did not affect neither SGR nor AWG in several species such as juvenile Black seabass (60% PBM; Sullivan, 2008), juvenile Spotted rose snapper (75% PBM; Hernandéz et al., 2014), Tilapia (60% PBM; Fasakin et al., 2005 and 100% PBM, Yones and Metwalli, 2015), Humpback grouper (100% PBM, Shapawi et al., 2007), Cobia (60% PBM, Zhou et al., 2011), Golden pompano (100% PBM, Ma et al., 2014).

Condition factor (K) was not affected by our experimental diets and the obtained results were positive, in fact a K value > 1.4 in seabream and seabass indicates a good or excellent condition (Pésic et al. 2015). Previous study demonstrated that farmed gilthead seabream fed with 54 % protein and 17% lipid showed a K value of 2.2 (Valente et al., 2011). Our results demonstrated that, although the protein content of the PBM diets used during our trial was lower (45%) with respect of that used by Valente et al. (2011) we obtained a higher K value. In addition, the K value resulted higher with respect to that in the experiment performed by of Nogueira et al. (2012) using a diet containing the 51% of proteins (feather and blood meal).

The analysis of the somatic indexes, also suggested that PBM did not affect neither HSI nor VSI. Similar results have been obtained in seabream reared in intensive and semi-intensive system (Valente et al, 2011) and in other species fed PBM (Ma et al., 2014; Zhou et al., 2016).

4.2 Fish welfare

Hematic cortisol is a parameter commonly used to evaluate fish welfare (Sadler et al., 2000; Campbell, 2004; Haukenes et al., 2008; Sicuro et al., 2009) and provides a good indication of intensity and duration of stress in gilthead seabream (Jentoft et al., 2005; Syriou et al., 2011). Our result clearly suggests that PBM meal based diet did not determine, *per se*, stress in this fish species. No data was found in literature about plasma cortisol level in gilthead seabream fed with PBM, but plasma cortisol concentrations in all fish groups are consistent with those measured previously (Rotllant et al., 2000, 2001; Yildiz et al., 2008; Piccinno et al., 2013) and lower than those found in red porgy (*Pagrus pagrus*) cultured under normal farming conditions (Rotllant et al., 2003).

Changes in plasma protein have been used as indicators of stress response and hepatic efficiency in fish (Sala-Rabanal et al., 2003). In our experimentation, plasma protein content increased at 90-days in all the fish, independently from fish diet. A similar result was found in tilapia fed with 100% PBM, and in cobia juveniles fed 60% PBM (Yones and Metwalli, 2015; Zhou et al., 2011).

The plasma osmolality, a useful marker of osmoregulatory regulation (Zou et al., 2016), resulted similar among all experimental treatments during growth trial.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are aminotransferases present in liver and spleen. Their plasma levels increase when fish are stressed or when tissue necrosis occurs (Wells et al., 1986; Jung et al., 2003) indicating liver damage. Our results demonstrated that ALT and AST were not affected by the substitution of FM with PBM and both enzyme activities were similar in all fish groups with slight increase during growth trial. Similar results were found in Tilapia fed with PBM (Yones and Metwalli, 2015).

In the present study, the enzymes activities changes showed the same pattern for all fish groups during the whole experimental period. To date, no information exists concerning the effect of PBM on digestive enzymes neither in gilthead seabream nor in other fish species. Our results demonstrated, that no significant difference ($P>0.05$) was found in AP activity (hepatic and intestinal AP) between fish groups and that at the end of the trial values were similar to those found by several other studies (Del Coco et al., 2009; Sicuro et al., 2010; Piccino et al., 2013) in farmed and wild gilthead seabream.

Intestinal lipase activity was not affected by the replacement of FM with high levels of PBM (50 and 100%) and its activity was similar to that measured in gilthead seabream fed with Holoturian meal (Piccino et al., 2013), and in seabream fed with diets containing olive mill vegetation water (Sicuro et al., 2010).

Concerning the activity of leucine aminopeptidase (LAP), no differences among groups were observed during and at the end of growth trial. Similar LAP activity was measured in farmed and wild sea bream in previous studies (Sicuro et al, 2010; Del Coco et al, 2009).

In order to investigate eventual sub pathological alterations induced by PBM inclusion in fish feeds, histological analysis was performed on fish liver. Previous studies compared the liver morpho-physiological condition with feeding (Caballero et al., 2003; Ostaszewska et al., 2005). The most common changes observed in the liver are hepatocytes vacuolization, changes in metabolic activity, changes in parenchyma structure and necrosis (Takashima and Hibiya, 1982; Roberts, 1989).

In the present study about 90 % of liver samples were classified as healthy liver (Grade 1). Only few liver samples showed nuclear displacement (independently from experimental treatments), while hepatocyte vacuolization, steatosis as well as degradation were not observed. At the best of our knowledge no information exists about liver histological analysis in seabream fed with PBM. In previous studies, healthy liver conditions were found in seabream fed with diet containing 15% lipid (Caballero et al, 1999), probiotics (Gultepe et al, 2014) and Carob germ meal up to 34 % of inclusion (Martinéz-Llorens et al., 2012a). At the contrary, hepatocyte vacuolization and liver steatosis occurred in gilthead seabream fed with Carob meal at 52% of FM replacement (Martinez-Llorens et al., 2012b) and in seabream fed with bioprocessed soybean at 60 % of FM replacement (Kokou et al., 2015).

4.3 FILLET QUALITY

4.3.1 Fillet protein pattern

Characterization of fish tissue proteomes is key to many aspects of farmed fish, encompassing physiology; growth; food safety; seafood authentication and quality; traceability and shelf-life (Piñeiro et al., 2003; Vilhelmsson et al. 2004; Forné et al., 2010).

In order to accomplish systematic characterization of the gilthead seabream muscle proteome and to investigate its modification eventually induced by PBM inclusion in diets, a 2-DE study was performed aimed to monitor fillet quality. In literature no information was available on protein pattern in the fillet of seabream fed with PBM. Concerning quality and shelf-life, preliminary data on postmortem changes in seabream muscle were generated by Schiavone et al. (2008). Addis et al. (2013) reported also fillet quality of seabream farmed in off-shore floating cages system.

Muscle protein maps of gilthead seabream fed with control diet, feed A and Feed B displayed the same protein spots and resemble to farmed seabream maps investigated by Addis et al. (2013). The obtained protein patterns in all fish groups showed a similar expression of the structural proteins such as actin, tropomyosin, MLC1, MLC2 and MLC3 and result was comparable to that reported previously (Schiavone et al., 2008; Addis et al., 2013). Actin protein spot was the most predominant proteins among the cytoskeletal proteins. The inclusion of PBM in seabream diet determine the change of the normalized volume (small but significant) of only 7 over 800 total spots, therefore suggesting a very small effect of poultry-by-product meal on protein muscle composition.

4.3.2 Fillet amino acids content

Essential amino acid (EAA) deficiency is one of the most important issues regarding FM substitution with alternative ingredients (Kaushik and Seiliez, 2010) and unbalanced EAA levels in the diets have been reported as one of the main causes for growth depression in fish fed animal by-products based diets (Garcia-Gallego et al., 1998; Millamena, 2002; Xavier et al., 2014).

In the present study, experimental diets were formulated to have a balanced amino acid profile in order to satisfy the nutritional requirements of fish. According to NRC. (2011), protein level and amino acid profile of poultry by-product meal are relatively similar to fish meal making this ingredient a valuable protein source for many species. Dietary impacts on muscle amino acids, as well as on muscle free amino acids, which are major taste and flavour contributors, have been mentioned for salmonids (Mente et al., 2003; Sunde et al., 2004; Yamamoto et al., 2004, 2005).

For gilthead seabream, the study of Gomez-Requeni et al. (2004), referred to juvenile fish, indicated that muscle free amino acid pool increase by more dietary plant protein supply. The anti-nutritional factors of plant protein source in fish diet reduce the availability of amino acids (Francis et al., 2001). Animal protein source, such as PBM used in the present study, is known to be free of anti-nutritional factors (Bureau et al., 2000, 2002; NRC 1993). Amino acid composition values in the muscle of fish fed PBM-based diets at 50 % and 100% of FM substitution exceed those found in whole-body of gilthead seabream fed meat and bone meal-based diet at 75% of FM replacement (Moutinho et al., 2017).

In literature, no information was found concerning muscle amino acid profile variation by the use of PBM in the diet of farmed seabream. By this result, it is clearly evident that PBM did not affect negatively the fillet composition of gilthead seabream

in term of amino acids profile. Similar results were found in the fillet of juvenile spotted rose snapper (*Lutjanus guttatus*) fed with PBM diets (until 75% of FM substitution) where amino acid profile does not change compared to fish fed with control diet (Hernandéz et al., 2014).

In this study, ∑EAA was lower than ∑NEAA and ∑EAA/∑NEAA ratio was similar among fish groups at 90-days of trials. These finding are in accordance with previous results (Valente et al., 2011) reporting amino acids profile in the fillet of seabream reared in different farming systems (Intensive, integrated, semi-intensive and extensive) in southern Europe.

4.3.3 Fillet lipid composition

Lipids in the edible part of fish are important because they affect the taste and the aspect of cooked flesh for the costumer (Grigorakis, 2007).

In this study, steady increase of muscle total lipid was observed during growth trial without any significant difference among fish groups. The increase of lipid content in the muscle of farmed seabream is expected (Company et al., 1999; Anedda et al., 2013).

However, the obtained values at the end of the trial were lower than those found in market-size farmed seabream (Ballester-Lozano et al., 2011) and in the muscle of seabream (final body weight about 450g) fed with vegetable oil for long-term period (Menoyo et al., 2004; Izquierdo et al., 2005). Previous research reported for the same farmed species a higher lipid content in the muscle than those found in this study (Alasalvar et al., 2002; Grigorakis 2007; Yildiz et al, 2008). The change of lipid content in muscle is function of fish growth muscle fattening, muscular activity within farm facilities (Davison, 1997) and use of high-energy diets (Company et al., 1999; Grigorakis & Alexis, 2005).

No data was found on lipid content in seabream muscle fed with PBM, and the only information was that whole body fat (%) of seabream fed with PBM (until 75% of FM replacement), decreased compared to seabream fed with fish meal (Nengas et al., 1999).

PBM inclusion in fish diet did not affect fillet energy content and measured values in this experimentation did not change between experimental treatments. These values resulted higher than those reported in scientific literature and may be explained by diet energy content.

4.3.4 Fillet Fatty acid profile

Several studies have shown that fatty acids profile of fish tissue reflects the dietary fatty acids composition (Maina et al., 2003; Francis et al., 2006; Xue et al., 2006; Bahurmiz and Neg, 2007; Gümüş and Erdogan, 2010; Hu et al., 2013). Marine fish cannot produce highly unsaturated fatty acid (HUFA). The dietary history of farmed fish can also influence their lipid composition (Benedito-Palos et al. 2009).

In the present study, considerable differences were, nevertheless, observed in fatty acid profiles of muscle of fish fed PBM diets with respect to fish fed control diet.

Our results showed a high level of monounsaturated (40-45%) fatty acid and a relatively low level (31-36%) of polyunsaturated fatty acids (PUFA). Oleic acid (C18:1n-9), Palmitic acid (C16:0), and linoleic acid (C18:2n-6) represent the main fatty acids. Similar results were found previously in farmed gilthead seabream (Valente et al., 2011).

ΣMUFA increased significantly with the increased of dietary PBM and this is mainly due to the abundance of C18:1n-9 in fish muscle that is a consequence of the high oleic acid content of poultry by-product meal (Sullivan, 2008).

However, high PBM levels in gilthead seabream diets resulted in low ΣPUFA levels. This was due to the decrease of eicsopentoenoic acid (EPA, C20:5n-3) and decosohexoenoic acid (DHA, C22:6n-3) in muscle of fish fed PBM diets. Poultry by-product meal is free of EPA and DHA (Bureau et al., 2000), which explain the low values of EPA and DHA found in the fillet in fish fed PBM-based diet.

No data was found on fillet fatty acid profile of gilthead seabream fed with PBM but similar results were found in the muscle of fry carp (Gumus and Aydin, 2013) and in juvenils black seabass (Sullivan, 2008) fed with PBM.

According to Lenas et al. (2011), EPA and DHA % in the muscle of farmed gilthead seabream are about 4,49 % and 9,19% of total fatty acids and about 0,28% and 9,54% respectively in the muscle of wild seabream. Another study, demonstrated that EPA % in the muscle of farmed gilthead seabream grow-on a commercial diet ranged from 5,7-7% of total fatty acid while DHA % was about 4,9-7% (Ballester-Lozano et al., 2011). In the present study, the lowest values of EPA and DHA found in the muscle of gilthead seabream were 6 ± 0,77% (Feed B at 60 days) and 4,73 ± 1,23% (Feed A at 90 days) respectively and were comparable to those found in Ballester-Lozano et al. (2011) study.

Low value of EPA and DHA were found in the fillet of seabream fed by a mixture of plant protein compared to seabream fed FM (De Francesco et al., 2007).

Our results show that Σn-3 fatty acid decreased in the muscle of fish fed Feed A and Feed B. Consequently, Σn-3/Σn-6 ratio decreased and this may be related to the low content on Σn-3 fatty acid in PBM (Bureau et al., 2000). Σn-3 PUFA in the fillet of seabream fed with a mixture of plant protein resulted lower than Σn-3 PUFA in seabream fed FM diet (De Francesco et al., 2007).

In comparison to red meat and poultry, the health benefit of fish consumption is based on levels of n-3 fatty acids (FA) and in particular a high ratio of n-3/n-6 FAs and high levels of EPA/DHA (Din et al., 2004; Keli et al. 1994; Kris-Etherton et al. 2000; Sargent 1997; Torstensen et al. 2004).

At the end of growth trial EPA/DHA ratio did not reveal any significant difference among fish groups and the found value was higher than that found in the study of Lenas et al. (2011), where EPA/DHA was about 0,03% and 0,49% in wild and farmed *Sparus aurata* respectively. Ibeas et al. (1997) reported a similar value of EPA/DHA ratio in the muscle of juveniles seabream and confirmed that EPA/DHA ratio is dependent on the stage of fish development and may be influenced by EPA/DHA ratio of the diet.

4.3.5 Fillet phospholipid content

The three principal lipids present in cell membranes are phospholipids, glycolipids and cholesterol, being phospholipids the most abundant. Phospholipids are the dominant lipid fraction in fish muscle tissue (Mukhopadhyay et al., 2004; Keriko et al., 2010). Its content can be influenced by diet; environment factors such as temperature (Anedda et al., 2013); and may vary between wild and cultured species (Rodriguez- Serna, et al., 1996).

Within the recent literature, only a few reports analyze the effect of diet on structural phospholipids in adult gilthead sea bream and other marine fish, while several studies

confirm the importance of dietary phospholipids in larvae and juveniles fish (Benedito-Palos et al., 2008; Liu et al., 2002; Olsen et al., 1999; Tocher et al., 2008). Fish in these stages of development are not able to synthetize PL at a sufficient rate to meet their requirements (Izquierdo and Koven, 2011). Due to this suggestion, several fish larvae receive an abundance of phospholipids in their diets (Tocher et al., 2008). Phospholipid consists mainly of polyunsaturated fatty acids (PUFA) (Moriya et al., 2007), considered as beneficial to human nutrition (Exler and Weihrauch, 1976).

In the present study, phospholipids content in the muscle of seabream were not affected by diets composition in all fish groups. PL represents the fatty acid substrate for the formation of eicosanoids such as eicosapentaenoic acid (EPA, 20:5n-3), (Tocher et al., 2008). At 90 days of trial, the % EPA of total fatty acids in the muscle of seabream was not affected by PBM among fish groups as well as phospholipids.

4.3.6 Fillet cholesterol content

Fish are universally considered as a valuable source of EPA, DHA and vitamins while containing a low level of cholesterol (Harlioglu et al., 2016).

Cholesterol as well as lipid content depends on many factors including fish species, size and diets (Izquierdo et al., 2003). In the present study, cholesterol content in seabream fed with PBM increased steadily without significant difference among fish groups, which suggest the absence of negative effect of PBM based-diets.

Cholesterol content was lower than that found in the muscle of commercial-size farmed seabream and seabass (Harlioglu et al., 2016). This difference could be explained by fish size differences. Benedito-Palos et al. (2013), demonstrated that cholesterol content in the muscle of gilthead seabream decreased with decreasing of feeding ration size.

4.3.7 Fillet lipid oxidation

Lipid peroxidation is of great commercial importance (because of its negative impact on the flavor, color and nutritional characteristics) (Monahan, 2000), given the concern of the consumer regarding farm products.

Therefore, TBARS as secondary lipid oxidation compound was measured through MDA quantification to evaluate the possible effects of PBM. TBARS method has been utilized in some studies on fish flesh quality (Menoyo et al., 2002; Sicuro et al., 2010) and the greatest peroxidation levels are closely related to high PUFA concentrations in tissues (Lopez-Bote et al., 2001; Menoyo et al., 2002).

In the present study, The obtained MDA concentration was comparable to results found in the muscle of gilthead seabream at the beginning of post mortem process (Aubourg et al., 2010; Sicuro et al., 2010). Menoyo et al. (2004), demonstrated a significant decrease on lipid peroxidation in the muscle of seabream fed with diets containing vegetable oil (60-80% linseed oil and soybean oil) and results were higher than MDA concentrations found in the present study.

CONCLUSION

This study clearly demonstrates that PBM can be used in the artificial feeding of gilthead seabream, thus showing that there are not negative effect nor physiological or sub pathological and in the welfare conditions of farmed fish. This result has an immediate effect in aquafeed industry, showing the possibility of utilization of this feedstuff in the artificial feeding of the main fish species of Mediterranean aquaculture.

In general terms this research also confirms that an aquafeed industry based on locally available feedstuffs will benefit the entire sector of aquaculture , thus showing that the re-utilization of by products, as the case of PBM, is a good example of circular economy, a concept that is familiar to modern consumers. The positive experience of Atlantic salmon in Norway that is the icon of carnivorous fish farming in North Europe, should be transferred on vicariant carnivorous species in the Mediterranean region as gilthead seabream and a future massive replacement of fish meal will greatly reduce the environmental impact and consequently the social impact of this sector, considering the this species will likely dominate the fish farming in all the Mediterranean region.

In order to promote a really sustainable future for aquaculture, it is of prioritary importance to demonstrate that research and aquafeed industry can share same target of sustainability, as shown in this study in which "in field" experimentation" has been carried out completely in a private fish farm company, but more importantly, sharing a common clear objective of improving sustainability of gilthead seabream farming.

FAO estimates clearly show that carnivorous fish farming will increase in Europe and in the Mediterranean region. Carnivorous fish species are those traditionally more criticized by public opinion and mass media for their indirect impact on natural resources, by the consumption of fish meal. Results as those obtained in this study clearly show that modern aquaculture takes immediately advantage of opportunities as this case of utilization of BPM in Italy, that circular economy concept has a central role in the modern aquaculture and a different future is possible of the most important fish farming activity of Mediterranean region.

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