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Meat and bone meal as partial replacement for fish meal in diets for gilthead seabream (*Sparus aurata*) juveniles: growth, feed efficiency, amino acid utilization, and economic efficiency

Sara Moutinho[1,2], Silvia Martínez-Llorens[3], Ana Tomás-Vidal[3], Miguel Jover-Cerdá[3],
Aires Oliva-Teles[1,2], Helena Peres[1,2].

[1]Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, Edifício FC4, 4169-007 Porto, Portugal;

[2]CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal;

[3]Institute of Animal Science and Technology, Group of Aquaculture and Biodiversity, Polytechnic University of Valencia, Camino de Vera, 14. 46071- Valencia, Spain.

Abstract

A trial was conducted to evaluate fish meal (FM) replacement with meat and bone meal (MBM; 53% CP, 15% CL, 27% Ash) in diets for gilthead seabream (*Sparus aurata*) juveniles. Three extruded experimental diets were formulated (45% CP; 20% CL) to include 0, 50 and 75% of protein from MBM (diets MBM0; MBM50; MBM75). Triplicate groups of seabream (IBW=25g) were fed these diets to satiety for 12 weeks. Growth performance and feed efficiency were similar with the diets MBM0 and MBM50, but were lower with diet MBM75, while the opposite was true for feed intake. Whole-body composition was not affected by diets composition except for crude lipid and energy content, which were lower with the diet MBM75. Protein and essential amino acids retention were unaffected by diet composition, while energy retention was lower with the diet MBM75. In terms of economic efficiency, diets with MBM resulted in a lower production costs, with the lowest economic conversion ratio (€ kg^{-1} fish produced) being obtained for the MBM diets while the maximum economic profit (€ kg $fish^{-1}$) was obtained for diet MBM50. Overall, up to 50% of FM protein can be replaced by MBM protein in diets for gilthead seabream juveniles, without compromising growth performance, feed utilization, and nutrient retention.

Key-words

alternative feedstuffs; fish meal replacement; meat and bone meal; amino acids; growth performance; economic profit

1. Introduction

Fish meal (FM) has been the preferred protein source for commercial aquafeeds, in particular for carnivorous species, being one of the most nutritionally well-balanced ingredient and so ensuring high production efficiency (Glencross *et al.*, 2007; Kokou *et al.*, 2012). However, prices of this commodity have significantly risen both for terrestrial and aquatic production, due to increased demand and environmental constrains associated with stagnating capture fisheries (Martínez-Llorens *et al.*, 2012). Thus, further growth of the aquaculture industry will depend on the availability of more cost-effective and sustainable feed resources.

Great efforts have been made to develop low-fish meal diets, mainly using plant based protein ingredients. However, despite the observed progresses, plant-protein based diets are often associated to reduced growth performance, feed intake and impaired intestinal health and function (Hardy, 2010; Krogdahl *et al.*, 2010; Oliva-Teles, 2012). Indeed, plant protein ingredients have some characteristics, such as high carbohydrate content, deficiency in certain essential amino acids (e.g. methionine, lysine, and tryptophan, threonine and arginine), low palatability, and presence of anti-nutritional factors (Barrows et al. 2008; Gatlin et al. 2007; Oliva-Teles *et al*., 2015) that limit its utilization in carnivorous fish diets. Furthermore, the relative high prices on the global market, and the competition among the aquaculture sector, animal husbandry sector, biofuel production, and direct use for human consumption, represent additional constrains to the use of plant protein ingredients (Karapanagiotidis, 2014). Under this scenery, the underutilized protein sources from terrestrial animals appear to be a more practical and cost-effective alternative to FM than plant ingredients.

The use of processed animal proteins (PAP) in aquafeeds is highly variable depending on the region. In the European Union (EU), its use was prohibited in 1990-2000, by the EU Commission Regulation (EC No. 999/2001) due to the arising of bovine spongiform encephalopathy in ruminants of Western Europe in the 1980-1990's. In 2013, however, this prohibition was partially lifted allowing the use of PAP derived from non-ruminant animals (Category 3) for feeding of aquaculture animals, yet maintaining the prohibition of intra-species recycling of protein (EU Commission Regulation, EC No. 56/2013). This opened the doors to a whole new range of ingredients that can be used in aquafeeds inside the EU. However, the technological process of PAP production was revised (EC No. 94/449; temperature over 133ºC, pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm), which may compromise its nutritional quality. Therefore, it is necessary to thoroughly evaluate these new ingredients.

One of these PAPs, manufactured and permitted for use in aquafeeds in Europe, is non-ruminant meat and bone meal (MBM). This is an animal by-product that derives from slaughterhouses leftovers, being manufactured worldwide with a steady availability, averaging a production of 3.5 million tons per year in the EU (Coutand *et al*., 2008). Relatively to plant ingredients, MBM holds several advantages, including a high protein content, with well-balanced amino acid profile; good source of digestible minerals, namely phosphorous and calcium; and lack of known anti-nutritional factors (Suloma *et al.*, 2013). MBM has also good digestibility values, but great variability among fish species has been shown (Bureau *et al.*, 1999). However, the high ash content, due to the presence of bone and other inorganic matter, is considered to be one of its major drawbacks and may limit its use in fish diets (Bureau et al., 1999). Also, the nutritive value of MBM is highly dependent of the freshness and quality of the raw materials and of the processing technologies used (Kureshy *et al.*, 2000), resulting in an inconsistent product. Moreover, the harmful effect of excessive heat applied to MBM may be even more pronounced in the EU due to the legislation of technological processing of PAP (EC No 1069/2009), further compromising the bioavailability of MBM's protein and amino acids.

Earlier studies have shown that the magnitude of FM replacement by MBM greatly differs among species. Some authors reported moderate FM protein replacement levels, from 20 to 45% for olive flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*) or large yellow croaker (*Pseudosciaena crocea*) (Ai *et al.*, 2006; Bureau *et al.*, 2000; Lee *et al.*, 2012), while higher replacement levels were achieved for other species, namely of 75% for African catfish (*Clarias gariepinus*) (Goda *et al.*, 2007) or 100% for Nile tilapia (*Oreohromis niloticus*) (El-Sayed, 1998). This discrepancy may be attributed to fish species specificities, fish feeding habits, as well as inconsistencies in the MBM nutritive quality. Nevertheless, animal by-product ingredients, in particular MBM, seem to have high potential to be included in fish feeds, reducing the supply constraints imposed by the high costs and competitiveness of FM and plant protein concentrates, thus reducing the long-term dependency on these commodities. Still, to optimize MBM use in aquafeeds, it is essential to accurately characterize its nutritional value for a particular fish species.

Gilthead seabream (*Sparus aurata*) is a species of great economic importance in Mediterranean aquaculture (Basurco *et al.*, 2011; Oliva-Teles *et al.*, 2011) but overproduction in the last decade has had a negative impact on the main European markets (Flos *et al.*, 2002), forcing farmers to improve feeding strategies to increase profitability. Since feeding can account for 45% or more of the overall variable costs in Mediterranean intensive aquaculture (Martinez-Llorens *et al.*, 2008; 2009; Tomás *et al.*, 2009), replacing FM with more cost-effective protein sources without compromising growth, quality, and welfare of farmed fish, would greatly increase profitability by reducing feeding costs (Martínez-Llorens *et al.*, 2012).

In line with that, the replacement of FM by plant protein sources in diets for gilthead seabream has been extensively studied (Emre *et al.*, 2008; Kissil and Lupatsch, 2004; Kissil *et al.*, 2000; Kokou *et al.*, 2012; Lozano *et al.*, 2007; Martínez-Llorens *et al.*, 2007, 2012; Monge-Ortiz *et al.*, 2016; Pereira and Oliva-Teles, 2002, 2003, 2004; Robaina *et al.*, 1995; 1997). However, the selection of plant ingredients is relatively limited due to the high protein requirements of seabream (N.R.C., 2011; Oliva-Teles, 2000; Oliva-Teles *et al.*, 2011). Since the EU lifted the restrictions on use of PAP, published studies on the use of these ingredients in gilthead seabream diets are limited to the one of Martínez-Llorens *et al.* (2008), which showed that blood meal could replace 15% of dietary FM protein in juveniles and on-growing gilthead seabream. Thus, the aim of the present study was to evaluate the potential of MBM as FM substitute in diets for gilthead seabream juveniles.

2. Materials and methods

2.1 Experimental diets

Target ingredient - meat and bone meal - was obtained from VALGRA S.A., Beniparrell, Valencia, Spain. It was produced from category 3 rendering non-ruminant animal by-products (70% swine, 20% poultry and 10% of other non-ruminant species) following the standard processing methods established in the European Regulations EC 1069/09 and 142/11 (temperature over 133ºC, pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm). Meat and bone meal proximate composition averaged (dry matter basis) 97.0% dry matter; 53.1% crude protein, 15.3% crude lipids and 26.9% ash and energy content of 17.7 kJ^{-1}.

Three extruded diets were formulated to be isoproteic (45% CP) and isolipid (20% CL) and with MBM replacing FM protein at increased levels: 0% (control diet, MBM0), 50% (MBM50), and 75% (MBM75). Diets were prepared using a cooking-extrusion processing with a semi-industrial twin-screw extruder (CLEXTRAL BC-45; Firmity, St. Etienne, France), at 100 rpm speed screw, 110 ºC temperature, and 40-50 atm pressure to form

2-3 mm diameter pellets. Ingredients and chemical composition of the experimental diets are presented in **Table 1** and the amino acid composition in **Table 2**.

2.2 Growth trial

Gilthead seabream (*Sparus aurata*) juveniles were provided by a local fish farm (Piscimar, S.L., Castellón, Spain) and transported to the Fish Nutrition Laboratory of the Polytechnic University of Valencia. Fish were then acclimatized to the indoor rearing conditions for 2 weeks while fed a standard seabream diet (48% CP; 23% CL; 11% Ash; 2.2% CF; 14% NFE). The growth trial was performed in a thermo-regulated recirculation seawater system (65 m^3 capacity), with a rotary mechanical filter and a gravity biofilter (approximately 6 m^3), equipped with 9 cylindrical fiberglass tanks of 1,750 L capacity, each one with aeration. During the growth trial, water temperature averaged 22.5 ± 1.3 °C, salinity 35.7 ± 0.8 ‰, dissolved oxygen 6.7 ± 0.4 mg L^{-1}, pH ranged from 6.5 to 7.5, and nitrogenous compounds were kept at levels within limits recommended for marine species.

After the acclimatization period, 405 gilthead seabream juveniles (IBW of 25 g) were randomly distributed to each tank (45 fish/tank). Each experimental diet was randomly assigned to triplicates of these groups. Fish were fed by hand, two times a day (9h and 16h), six days a week, to apparent visual satiation. Feed consumption was recorded daily. The trial lasted 12 weeks and during that period fish were bulk weighed every 4 weeks, under anesthesia (30 mg L^{-1} clove oil (Guinama®, Valencia, Spain) containing 87% of eugenol), after one day of feed deprivation, and their health status was assessed by direct observation.

Five fish from the initial stock and 5 fish from each tank at the end of the trial were randomly sacrificed by a lethal bath of clove oil (150 mg L^{-1}), and pooled for whole-body composition analysis. Fish length and wet weight, and liver, viscera, and visceral fat weights were recorded for determination of condition factor, hepatossomatic, visceral, and visceral fat indices.

2.3 Chemical analyses

Chemical analyses of the dietary ingredients were performed prior to diet formulation. Diets, ingredients, and whole fish were analyzed according to AOAC (1990) procedures: dry matter (105 °C to constant weight), ash (incinerated at 550 °C for 5h), crude protein (N x 6.25) by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyzer, Tecator Höganas, Marineeden), crude lipid extracted with methyl-ether ($ANKOM^{XT10}$ Extractor), and crude fiber by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator). Energy was calculated according to Brouwer (1965), from the C (g) and N (g) balance (GE = 51.8 x C – 19.4 x N). Carbon and nitrogen were analyzed by the Dumas principle (TruSpec CN; Leco Corporation, St. Joseph, MI, USA). All analyses were performed in triplicate. Total amino acid composition of ingredients, diets, and carcass was determined by 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. The amount of sample used was calculated to contain approximately 25 mg of crude protein that was hydrolyzed with 50 mL of 6 N HCl with 0.5% phenol at 115 ºC for 24 h. Aminobutyric acid was added as an internal standard before hydrolysis. Amino acids were derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. Amino acids were separated by HPLC with a C-18 reverse-phase column Waters Acc. Tag (150 mm x 3.9 mm).

2.4 Statistical analyses

Results were analyzed using IBM SPSS 23 software package for Windows (SPSS® Inc., Chicago, IL, USA). Normality and homogeneity of variances were tested (Shapiro-Wilk and Levene tests, respectively) and normalized when appropriate. Statistical analysis of data was done by one-way analysis of variance (ANOVA) with 0.05 as probability level for rejection of the null-hypothesis. Tukey test was used to assess significant differences among means.

2.5 Ethics statement

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

2.6 Economic analysis

The currency type for economic evaluations is the euro (€). The Economic Conversion Ratio (ECR) was calculated using the following equation:

ECR (€ kg of fish^{-1}) = FCR (kg diet kg of fish^{-1}) x diet price (€ kg of diet^{-1})

The price of each diet was determined by multiplying the respective contributions of each feed ingredient by their respective cost per kg and summing the values obtained for all the ingredients in each of the formulated diets. The price (per kg) of each ingredient (2015 average) was as follows: fish meal = 1.51 €; wheat meal = 0.15 €; meat and bone meal = 0.35 €; fish oil = 1.80 €; soybean oil = 0.63 €; vitamins and mineral mix = 2.75 €.

The Economic profit index (EPI) was calculated using the equation (Martinez-Llorens *et al.* 2007):

EPI (€ $fish^{-1}$) = [weight gain (kg) x selling price (4.5 € kg^{-1})] – [weight gain (kg) x diet price (€ kg of $diet^{-1}$)]

Gilthead seabream sale price was calculated at 4.5 € kg^{-1}.

3. Results

No differences in sinking rate of the pellets were observed and fish promptly accepted all diets. No pathological signs were observed during the trial, and mortality was very low and unaffected ($p>0.05$) by the dietary treatment (**Table 3**). Final body weight, weight gain, and daily growth index of fish fed diet MBM50 were similar ($p>0.05$) to those fed the control MBM0 diet (**Table 3**). Likewise, similar ($p>0.05$) feed efficiency and protein efficiency ratio were observed for control (MBM0) and MBM50 groups. Despite the highest voluntary feed intake, fish fed diet MBM75 obtained the lowest ($p<0.05$) growth performance and feed efficiency. Nitrogen retention (%NI) was similar ($p>0.05$) among groups while energy retention (%EI) of fish fed diet MBM50 was similar ($p>0.05$) to that of control MBM0 diet and higher ($p<0.05$) than that of fish fed diet MBM75.

At the end of the trial, whole-body composition and the measured biometric indices (condition factor, visceral index, hepatossomatic index, visceral fat index) were unaffected ($p>0.05$) by diet composition, except for crude lipid and whole-body energy content, which were lower ($p<0.05$) for fish fed diet MBM75 (**Table 4**). Also, no differences ($p>0.05$) were found in whole-body amino acid composition (**Table 5**)

There were no differences ($p>0.05$) in essential amino acid (EAA) retention (mg kg^{-1} day^{-1}; % intake) of gilthead seabream fed the different experimental diets (**Figure 1**). Except for methionine in group fed diet MBM75, the ratios between the EAA of the experimental diets and that of whole-fish were all higher than 0.7 ($\%EAA_{diet}$ / $\%EAA_{fish}$; **Figure 2).**

Regarding economic analyses, the different dietary levels of MBM affected ($p<0.05$) diet cost and economic parameters, ECR and EPI (**Table 6**). MBM was 77% cheaper (€ kg^{-1}) than FM and diet price was reduced as the inclusion of MBM increased. The Economic Conversion Ratio (ECR) of the control MBM0 diet was the highest (1.67 € kg^{-1}) whereas it was the lowest for the MBM diets (1.24 € kg^{-1} for MBM50 and 1.14 € kg^{-1} for MBM75). The Economic Profit Index (EPI) was higher for diet MBM50 (0.36 € $fish^{-1}$) and lower for the control MBM0 (0.33 € $fish^{-1}$) and MBM75 (0.32 € $fish^{-1}$) diets.

4. Discussion

A significant number of studies have been carried out to evaluate the potential use of PAP, including MBM, in diets for aquaculture species worldwide. However, as the EU prohibited its use in aquafeeds from 2001 to 2013, the most recent research regarding the potential use of these commodities was conducted with aquaculture species not produced in the EU.

The results of the present study indicate that up to 50% of FM protein can be replaced by MBM in diets for gilthead seabream juveniles without negative effects on growth performance and feed utilization. Contrarily to present results, also in this species only low to moderate levels of FM substitution with MBM were previously achieved (20%, Robaina *et al.* 1997; 40%; Alexis *et al.* 1997; Davies *et al.* 1991). This wide range of FM replacement by MBM may be attributed to differences in the nutritional value of raw materials used and/or processing technology. Indeed, more advanced technology and quality control in recently produced MBM in the EU may have contributed to its higher inclusion potential in diets for seabream than what was possible to achieve with the 90's MBM.

Previous studies in other species showed that up to 40-60% of FM could be replaced by MBM and/or meat meal (MM) in diets for large yellow croaker (*Pseudosciaena crocea*), Australian silver perch (*Bidyanus bidyanus*), yellowtail (*Seriola quinqueradiata*), and Japanese flounder (*Paralichthys olivaceus*), without negatively affecting fish performance (Ai *et al.*, 2006; Hunter *et al.*, 2000; Sato and Kikuchi, 1997; Shimeno *et al.*, 1993; Stone *et al.*, 2000). Likewise, for sutchi catfish (*Pangasius hypophthalmus*) and African catfish (*Clarias gariepinus*), the replacement level may be increased up to 67 and 75%, respectively (Goda *et al.*, 2007; Kader *et al.*, 2011), and even higher levels, up to 80%, can be used in diets for grouper (*Epinephelus coioides*) using a blend of MM and BM (Millamena, 2002). On the contrary, lower replacement levels of FM by MBM or MM were recommended in other studies in large yellow croaker (up to 30%; Li *et al.*, 2010), rainbow trout (*Oncorhynchus mykiss*) (up to 30%; Bureau *et al.*, 2000), Australian short-finned eel (*Anguilla australis australis*) (up to 23%; Engin and Carter, 2005), olive flounder (*Paralichthys olivaceus*) (up to 20%; Lee et al. 2012), gibel carp (*Carassius auratus gibelio*) (up to 20%; Zhang *et al.*, 2006) and Japanese flounder (up to 20%; Kikuchi *et al.*, 1997), red drum (*Sciaenops ocellatus*) (up to 16%; Kureshy et al., 2000) and yellowtail (up to 10%; Shimeno et al. 1993).

In this study, whole-body composition was unaffected by the dietary MBM inclusion level, except for crude lipid which were lower for fish fed the MBM75 diet than the other diets.

Energy content of whole body followed the same trend observed for lipid content. Similar results were also obtained by Ai *et al*. (2006) in large yellow croaker, where diets with more than 45% MBM caused a decrease in whole-body lipid content. On the contrary, juvenile snapper whole-body lipid content slightly increased with the increase in dietary MBM (Booth *et al.*, 2012). Other studies showed no significant differences in whole-body composition of fish fed diets with different levels of animal by-products (Bharadwaj *et al.*, 2002; Bureau *et al.*, 2000; Goda *et al.*, 2007; Jamil *et al.*, 2007).

Diet MBM75 lead to the highest voluntary feed intake, which suggests that palatability was not compromised by the inclusion of MBM, and may reflect an attempt of fish to adjust digestible energy intake. Indeed, it is accepted that, up to a certain level, animals adjust feed intake to meet digestible energy needs (Boujard and Medale, 1994; Cho and Kaushik, 1985; Peres and Oliva-Teles, 1999; Yamamoto *et al.*, 2000). Although fish increased feed intake when fed the high MBM diet, they were unable to maintain the same growth of the other groups. This suggest lower digestibility or metabolic utilization of diets with high MBM incorporation. Although not determined in this study, others authors have reported low to moderate lipid digestibility for MM/MBM in different species (Bureau *et al.*, 1999; Mabrouk and Nour, 2011). Indeed, the major fraction of MBM lipids are saturated fatty acids (Millamena, 2002; Robaina *et al*., 1997) and its digestibility may be lower than that of fish oil (Bureau *et al.*, 2002; Olsen and Ringo, 1997). This may have also contributed to the reduction of lipid deposition in the whole-body. These results are also according to the adipostatic model of feed intake regulation, which relates a lower body lipid deposition with a higher ingestion (Jobling and Johansen, 1999; Johansen *et al.*, 2003; Saravanan *et al.*, 2012). Contrarily, in a previous study with gilthead seabream, no correlation was observed between body lipid and feed intake in fish subjected to different feed deprivation periods that induced different body lipid contents (Peres *et al.*, 2011).

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). Although regulation of feed intake by dietary amino acid is still poorly studied (Kaushik and Seiliez, 2010), it was already reported that single EAA deficiency lead to a reduction of feed intake in gilthead seabream (Peres and Oliva-Teles, 2009; Tibaldi and Kaushik, 2005). In the present study, the EAA level of the experimental diets exceeded the estimated EAA requirements of gilthead seabream (Peres and Oliva-Teles, 2009), except for methionine and phenylalanine + tyrosine. Nonetheless, whole-body crude protein and EAA retention (g kg^{-1} day^{-1} or % intake) were not affected by the experimental diets.

The high ash content of MBM can also limit its use in fish feeds. High levels of indigestible inorganic matter (i.e. bones) may increase intestinal transit, leading to a higher feed intake but decreased feed efficiency and growth performance (Goda *et al*., 2007; Xavier *et al*. 2014), as it was observed in fish fed diet MBM75. In present study, although the ash content of the MBM diets were almost double the control MBM0 diet, protein utilization was little affected by the increasing ash content of MBM diets. Besides ash content, rendering process can reduce the utilization efficiency of MBM by damaging protein and amino acid structure (Booth *et al.*, 2005; Xavier *et al.*, 2014). Lysine, one of the first limiting amino acids in alternative protein sources, is particularly heat-sensitive (Nengas *et al.*, 1999; Zhang *et al.*, 2006) and its availability may greatly differ among different batches of MBMs (Parsons *et al.*, 1997). Tidwell *et al.* (2005) reported that when FM was replaced by 50% MBM, growth reduction of largemouth bass was attributed not to the dietary EAA composition but to EAA availability. In this trial, however, the retention efficiency of lysine was not affected, suggesting that lysine availability, as well as that of the other EAA, was not compromised by the rendering process.

Replacement of FM with MBM appears to be economically feasible. The cost of formulating present diets for gilthead seabream was reduced as MBM levels increased and, compared to previous studies, prices were lower than those obtained using sunflower meal (Lozano *et al.* 2007) but higher when using soybean meal (Martínez-Llorens *et al*. 2007). Of course, costs cannot be directly compared as there is a big time lapse between studies and this influences costs. Still, in the case of Lozano *et al.* (2007), the lower diet price did not compensate for the reduced growth, resulting in a lower ECR and EPI. On the contrary, in the present study the economic parameters evaluated improved with the dietary inclusion of MBM, resulting in lower ECR (i.e. the feed cost to produce 1 kg of fish) for the MBM diets, with a higher EPI at 50% inclusion of MBM. Since EPI is a more suitable parameter to evaluate economic profitability, as it considers production, feed costs, and selling price, our results suggest that there is a greater economic return when replacing 50% FM protein with MBM, at least during the on-growing phase of gilthead seabream.

In conclusion, MBM protein may replace up to 50% FM protein in feeds for gilthead seabream juveniles without compromising growth and feed efficiency, with a positive outcome in economic efficiency. Still, further studies are required aiming to improve MBM incorporation in the diets, either by adjusting dietary digestible EAA levels and reducing saturated lipids content.

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