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Potential use of poultry meal in diets for gilthead seabream (*sparus aurata*): effect on growth performance, feed utilization and digestibility

Pedro Miguel Azevedo Reis Moreira Campos

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Dissertação de Mestrado apresentada à
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Pedro Miguel Azevedo Reis Moreira Campos
Mestrado em Recursos Biológicos Aquáticos
Departamento de Biologia
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Orientador
Doutora Helena Peres, Investigadora Auxiliar, CIIMAR
Coorientador
Tiago Aires, Aquasoja, Sorgal, SA.

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Abstract

One of the biggest challenges in aquaculture is the formulation of nutritionally balanced diets, at a reduced cost but still maintain fish as valuable food for human consumption. Fishmeal has been an essential ingredient used in aquaculture feeds, due to its nutritional profile, being the primary choice as protein source in diets for carnivorous fish species. However, the price of fishmeal is expected to increase dramatically over the next few years as aquaculture and livestock production increase, which will certainly affect the profitability and economic sustainability of the aquaculture industry. Sustainable aquaculture industry will necessarily involve the dietary fishmeal replacement by alternative ingredients, ideally with an equivalent nutritional value to fishmeal. Poultry by-product meal (PBM) is a rendered animal by-product that has a relatively high protein content and a balanced amino acid profile. It was banned from inclusion in aquafeeds in the European Community since the bovine spongiform encephalopathy (BSE) crisis. Recently, with the re-authorization of non-ruminant processed animal proteins in European aquafeeds, it is expected that research insights will greatly contribute for its use and so for the sustainable development of European aquaculture. Gilthead seabream (*Sparus aurata*) is a highly important commercial valued specie in Europe, particularly in Mediterranean Sea, but to maintain its production it will be needed to consider new strategies, as the development of environmental sound diets at reduced cost. Therefore, the present study aims to evaluate the effect of gradual replacement of fishmeal by a local animal by-product, poultry by-product meal (PBM), in growth, feed efficiency, digestibility and economic efficiency of gilthead seabream juveniles. Six isoproteic and isolipidic diets were formulated (45% CP; 18% CL): a control diet (PBM0), with fishmeal as the main protein source, and PBM7.5; PBM15; PBM22.5; PBM30; PBM37.5 where fishmeal was replaced by inclusion of PBM at 7.5%, 15%, 22.5%, 30%, 37.5%, respectively. Triplicate groups of juvenile gilthead seabream, with an average body weight of 63 g, were fed for 70 days with each experimental diets. At the end of the feeding trial, growth rate, weight gain, voluntary feed intake, feed and protein utilization, were not affected by the dietary treatment. Similarly, whole-body composition was unaffected by the experimental diets. Apparent digestibility coefficients of dry matter, protein, energy and phosphorus were also not affected by the dietary replacement of fishmeal by PBM. Additionally, no effect of diets on the digestive protease, trypsin, chymotrypsin, lipase and amylase activities were observed, in both the anterior and posterior intestine. However, posterior intestine showed higher enzyme activity than that of anterior intestine. Economic efficiency ratio decreased with the increase of fishmeal replacement by PBM. Overall, results shown that for gilthead

seabream, up to 37.5% of PBM can be incorporated in practical diets, reducing the dietary fishmeal level to 7.5%, without affecting growth performance, digestibility, feed and protein utilization. Economic analysis revealed that PBM diets were less expensive and have the same economic profit index than fishmeal basis diets. Further investigations are required to determine the modulation effect of the dietary inclusion of PBM on other important aspects, such as the immunity and health status of the animal as well as on the fish/fillet quality traits.

Keywords: Aquaculture; gilthead seabream; alternative protein sources; poultry by-product meal; growth; digestibility; digestive enzymes; economic efficiency

Resumo

Um dos maiores desafios da aquacultura é a formulação de dietas que sejam nutricionalmente equilibradas, a um preço bastante reduzido, mas que mantenham o peixe como alimento valioso para o consumo humano. A Farinha de Peixe tem sido um ingrediente essencial usado em rações em aquacultura, devido ao seu perfil nutricional, sendo a principal escolha como fonte proteica em dietas para espécies de peixes carnívoros. No entanto, é esperado que o preço da farinha de peixe aumente drasticamente ao longo dos próximos anos, com a produção aquícola e pecuária a aumentar, o que vai certamente afetar a rentabilidade e sustentabilidade económica do sector da aquacultura. A indústria da aquacultura sustentável implica necessariamente a substituição de farinha de peixe por ingredientes alternativos, de preferência com um valor nutritivo equivalente a farinha de peixe. Farinha de Carne de Aves (PBM) é um subproduto de origem animal que tem um teor relativamente elevado de proteína e um perfil de aminoácidos equilibrado. Este foi proibido na alimentação em aquacultura na Comunidade Europeia desde a crise da doença das vacas loucas (BSE). Recentemente, com a reautorização de proteínas animais processadas não ruminantes na alimentação na Aquacultura Europeia, espera-se que estas pesquisas contribuam significativamente para a sua utilização e para o desenvolvimento da Aquacultura Europeia. A dourada (*Sparus aurata*) é uma espécie com elevada importância comercial na Europa, particularmente no Mar Mediterrâneo, mas para manter a sua produção é necessário considerar novas estratégias, como o desenvolvimento de dietas mais ambientais a um preço reduzido. Por isso, o estudo presente tem como objetivo avaliar o efeito gradual da substituição da farinha de peixe por um subproduto animal local, farinha de carne de ave (PBM), no crescimento, eficiência alimentar, digestibilidade e eficiência económica

em douradas juvenis. Foram formuladas seis dietas isoproteicas e isolipidicas, com 45% de proteína bruta e 18% de lípidos totais, fazendo variar a quantidade de incorporação de PBM: dieta controlo (PBM0), em que a farinha de peixe é a fonte de proteína, e PBM7.5; PBM15; PBM22.5; PBM30; PBM37.5 onde a farinha de peixe foi substituída pela inclusão de PBM a 7.5%, 15%, 22.5%, 30% e 37.5%, respetivamente. Cada uma das dietas foi fornecida, em triplicado, a grupos de juvenis de dourada, com um peso médio de 63g, durante 70 dias. No final do ensaio de alimentação, taxa de crescimento, ganho de peso, consumo de ração voluntário, utilização da ração e das proteínas, não foram afetados pelo tratamento dietético. Similarmente, a composição corporal não foi afetada por nenhuma das dietas experimentais. Os coeficientes de digestibilidade aparente da matéria seca, proteína, energia e fósforo também não foram afetadas pela substituição da Farinha de Peixe pela Farinha de Carne Ave nas dietas. Além disso, não houve efeito das dietas sobre a atividade digestiva das enzimas protease, tripsina, quimotripsina, lipase e amilase, tanto na região anterior como na região posterior do intestino. No entanto, o intestino posterior apresenta maior atividade enzimática do que o intestino anterior. O rácio de eficiência económica decresce como aumento da substituição da farinha de peixe por PBM. No geral, os resultados mostram que para douradas, até 37.5% de PBM pode ser incorporada nas dietas experimentais, reduzindo o nível dietético da farinha de peixe a 7.5%, sem afetar o desempenho do crescimento, a digestibilidade e a utilização da ração e da proteína. A análise económica revelou que as dietas com PBM são mais baratas e que têm o mesmo índice de lucro económico que as dietas à base de farinha de peixe. Novos estudos são necessários para determinar o efeito na modulação da inclusão dietética de PBM em outros aspetos importantes, tais como o estado de imunidade e de saúde do animal, bem como as características da qualidade do peixe/filete.

Palavras-chave: Aquacultura; dourada; fontes proteicas alternativas; farinha de carne de aves; crescimento; digestibilidade; enzimas digestivas; eficiência económica

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Abbreviations

AA: Amino acid

ADC: Apparent digestibility coefficient

BSE: Bovine spongiform encephalopathy

CL: Crude lipid

CP: Crude protein

EAA: essential amino acid

ECR: Economic efficiency ratio

EPI: Economic profit index

EU: European Union

FBW: Final Body Weight

FeM: Feather meal

FM: Fishmeal

IA: Anterior intestine

IP: Posterior intestine

MBM: Meat and bone meal

PBM: Poultry by-product meal

SEM: Polled standard error of the mean

WG: Weight Gain

Introduction

Aquaculture and its role

Given the continued growth of the human population, finding new ways to meet its food needs is one of the biggest challenges of our time. Even with problematic economic scenarios, fish continues to be one of the most-traded food commodities worldwide and its production is constantly increasing due to population growth and rising consumer demand (FAO, 2016). A healthy diet, rich in protein, is essential for the growing population and to avoid food shortages (Sargent *et al.*, 2001). Aquaculture plays an important role and, due to its rapid growth, its production rather the fisheries industry seems to be the source of future supplies of fish for human consumption (Watanabe, 2002; FAO, 2016).

Aquaculture is the production of aquatic organism, including fish, molluscs, crustaceans and aquatic plants, using techniques designed to increase the production, beyond the natural capacity of the environment. Aquaculture continues to be the fastest growing animal food-producing sector, outpacing the population growth (FAO, 2016). Since 1970, the share of aquaculture production in total fish and shellfish production has grown steadily and nowadays it provides more than half of all the fish consumed worldwide. Aquaculture production is the major cause of the world per capita fish consumption increase, from an average of 9.9 kg in 1960s to 20 kg in 2014 (FAO, 2016). Moreover, aquaculture has been playing a significant role in eliminating hunger, promoting health, reducing poverty and providing jobs in many underdeveloped and developed countries.

The Status of Aquaculture Worldwide

Global fish production has growth in the last five decades, with fish supplies increasing at an average annual rate of 3.2 percent in the period 1961-2013, outpacing world population growth at 1.6 percent (FAO, 2016). Fishery and aquaculture production totalled, in 2014, 167 million tons (**Fig.1**), being 146 million tons used for human consumption and the rest for production of fishmeal and fish oils (FAO, 2016). As aforementioned, from all other animal food-production sectors, aquaculture is the fastest growing activity, contributing currently to 50% of the supply of fisheries products worldwide. Despite the continued growth of the sector, its actual expansion slowed down

relatively to the growth observed in the previous decade (1995-2004) (FAO, 2016). In the last decade, world aquaculture production of fish increased form 31.1% in 2004 to 44.1% in 2014, corresponding to an annual growth of 5.8%, down from the 7.2% attained in the previous decade (1995-2004).

Global aquaculture production attained 73.8 million tonnes in 2014, and the Asian countries have maintained a dominant position in the aquaculture sector worldwide, representing 88.9% of the total production. China, the largest producer of aquaculture products, was responsible for producing 61.6%, in 2014, with 45.5 million tons' food fish produced (FAO, 2016). Some developed countries, as the United States of America, have reduced their aquaculture output in recent years, mainly owing to the competition from countries with lower labour and production costs.

Despite the long tradition of aquaculture practices in some countries, globally it still is a relatively new sector that grew rapidly in the last fifty years. The aquaculture sector is now considered of strategic importance in the European Union and Mediterranean countries, contributing to food security and providing consumers with a source of high nutritional quality protein at a relatively low price (Barazi-Yeroulanos, 2010).

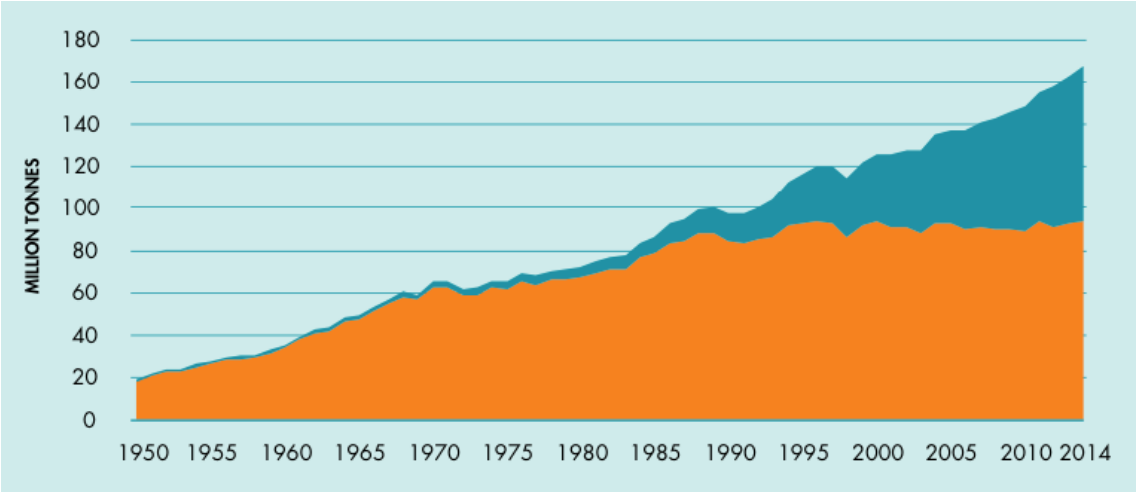

Fig. 1: World capture fisheries and aquaculture production. Source: FAO (2016)

Aquaculture in Portugal

The geographic location of the Portuguese coast, due to the influence of the Mediterranean Sea and Atlantic Ocean, allows for a wide range of habitats and is therefore considered one of the richest areas in biological terms, considering, in

particular, the quality of its waters and the diversity of species (DGRM, 2016). Because of that, Portuguese coast has a set of characteristic considered ideal for the development of aquaculture (DGRM, 2016).

Portugal has a long-standing tradition and history in the fisheries sector, however, in 2013 aquaculture represented only 6% of the total production. According to the European Maritime and Fisheries Fund (EMFF), Portuguese aquaculture could become an important activity for the fisheries sector in a near future. Aquaculture in Portugal is a relatively old activity and it began with the extensive production of marine and brackish species in coastal inland waters, estuaries and costal lagoons, namely in the salt pans (FAO, 2016). Regarding the evolution of the species, there are three different important periods, 1970's, 1980's and 1990's. Until the decade of 70, aquaculture production was dominated by *Mugilidae*, typically forages species with low commercial value, accounting for about 80% of aquaculture production. The 1980's were characterized by a large increase in fish farming in inland waters, particularly rainbow trout, accompanied by shellfish, especially cockles (*Ruditapes decussatus*), in brackish and marine waters. In the 1990's, there was a strong growth and modernization of the aquaculture of marine species, initially focused on seabass and seabream and, more recently, turbot (*Scophthalmus maximus*) and sole (*Solea vulgaris*) (INE, 2016). Aquaculture production accounts for only 3% of national fish production, while in other European countries come to represent about 20% of total fish production (DGRM, 2016).

In Portugal, since the early 90's, aquaculture production increased from 4.457 tons in 1990, to 10.791 tons in 2014, representing 50 million € in the last year (**Table 1**). During this period, there was an increasing diversification of production. While in 1990, the production was limited to two species, trout and clams, in 2014 there were significant productions of various species of fish and bivalves, especially the production of turbot with 3.6 tons, followed by clams with 2.3 tons (DGRM, 2016; INE, 2016).

Currently, the most produced species are turbot, gilthead seabream, European seabass, clams and oysters. Turbot is the most produced species with a total of 3588 tonnes per year, followed by clams (*Ruditapes decussatus*) with a total of 2252 tons per year and gilthead seabream (*Sparus aurata*) with a total of 1071 tonnes per year, as shown in **Table 2** (INE, 2016). The production of oysters (*Crassostrea gigas*, *Crassostrea angulata* and *Ostrea edulis*) with a total of 1085 tonnes produced, increased 36.6% in 2014 due to a new paradigm of investments that have been observed from north to south, in nurseries and in areas that were previously being used for fish production (INE, 2016).

Table 1: Evolution of Aquaculture Production by production conditions in Portugal (tons). Source: INE, 2016

Production conditions	1990	1995	2000	2005	2010	2014
Fresh waters	2.266	958	1.296	845	950	788
Brackish & Marine waters	2.191	4.081	6.240	5.850	7.063	10.003
Total	**4.457**	**5.039**	**7.536**	**6.695**	**8.013**	**10.791**

. The fish production has been growing over the years, and there has been an increase in turbot production which offset the sharp downturn in the European seabass and gilthead seabream productions. The reduction in the production of these two species was due to the deactivation of some establishments and also the passage of semi-intensive production systems to extensive production systems, as a way found by the producers to better accommodate the increase in production costs, enabling their developments. Another reason may due to seabream having an optimum temperature of 22-25 ºC, temperatures that are only found in Algarve.

Table 2: Evolution of Aquaculture Production by Species in Portugal (tons). Source: INE, 2016

Production Species	2000	2004	2008	2010	2012	2014
Rainbow trout	1.293	915	930	949	479	787
Turbot	379	275	351	2.424	4.406	3.588
Gilthead seabream	1.815	1.685	1.635	851	895	1.071
European seabass	653	1.234	1.069	396	531	400
Clams	2.416	2.014	2.299	2.539	2.394	2.252
Oysters	252	432	1037	548	736	1.085
Total	**6.808**	**6.555**	**7.321**	**7.707**	**9.441**	**9.183**

Aquaculture production has a huge potential to growth much due to natural conditions that Portugal offers and therefore, is important to understand how can the country take advantage of that potential and grow as an industry (FAO, 2016).

Gilthead seabream (*Sparus aurata* L.)

Gilthead seabream*,* (*Sparus aurata*, Linnaeus, 1758) is a perciform fish that belongs to the family Sparidae and to the genus Sparus (**Fig.2**). Its has a relatively deep and lateral compressed body with an oblong shape, a head with small eyes and an upper jaw reaching no further than the middle part of the eye. Their palate is smooth and has a teeth pattern that is adapted to carnivorous feeding, based on molluscs and other benthic organisms. It possesses a silver-grey colour all over the body with a big dark spot at the beginning of the lateral line that spread to the upper part of the opercular bone. Another major characteristic of this species is a golden coloured stripe founded between the eyes (Basurco *et al*., 2011).

Gilthead seabream is common in the Mediterranean Sea. It also appears in the Eastern Atlantic coasts from Great Britain to Senegal but in smaller amounts and its rarer in the Black Sea (Moretti *et al*., 1999). In Portugal, they are found only in continental waters. Regarding the habitat, this is a species that lives in coastal areas of rocky, sandy and seaweed to depths of 30 m were adults can occur up to 150 m deep. Over the initial stages of its life, this species is also found in marine and brackish water environments such as coastal lagoons and estuarine areas and rivers because they are eurihaline species (Parisi *et al*., 2014). Nevertheless, they are sensitive to low temperatures, 4 ºC being the lethal minimum and also at very low salinities.

Individuals of gilthead seabream species reach sexual maturity at 1-2 years (20-30 cm) for males and 2-3 years (33-40 cm) for females. Relatively to spawning, it takes place in the mouths of October to December.

Fig.2: Gilthead seabream specimen (*Sparus aurata* L.). Source: FAO

Gilthead seabream Aquaculture Production

Due to its biological characteristics and high commercial value, gilthead seabream occupies an important place in both fishing and marine aquaculture. Initially, gilthead seabream has been produced in coastal lagoons and saltwater pounds, such as the Italian “vallicoltura” or the Egyptian “hosha” that are a kind of natural traps, taking advantage of juveniles migration from the sea into coastal lagoons (FAO, 2016). The development of intensive rearing systems, during the 1980’s, allowed the beginning of the mass production of this species, especially in sea cages, and nowadays, has become one of the most produced species in European aquaculture.

Initially, its production involved the capture of the juveniles, but nowadays, most of the seabream production occurs under intensive conditions, involving the production of juveniles in technologically sophisticated hatcheries, requiring skilled staff. The control of all of the life cycle allowed an increase of the growth rate and the resistance to environmental conditions and culture. In captivity, this species can be kept in extensive, semi-intensive or intensive production conditions. Its production occurs in many European Countries, mainly in Mediterranean, being Greece responsible for 49% of production and the largest producer. It follows Turkey and Spain, with 15% and 14% respectively, and these tree countries are the main producers in the Mediterranean region (EUMOFA, 2016).

In Portugal, gilthead seabream was the first species to be commercial produced, and its production increased at a steady pace until the 2008 European market crisis. Since then, the gilthead seabream production appears to have stabilized.

Aquaculture Challenges – Reducing Fishmeal Content

More than 70% of the total fish and shellfish aquaculture production is dependent upon the supply of external feed inputs. To maintain the current aquaculture growth, aquafeed production will have to grow at similar rate, while fisheries by-products (fishmeal and fish oil) remains static and other sectors compete for same feed resources (Tacon *et al*., 2015).

Fishmeal has been the major protein ingredient in aquatic feeds. More than 73% of global fishmeal production was destined to the production of aquatic feeds in the last decade (OECD, 2015). Fishmeal represents the “ideal” feed ingredient for aquafeeds

due to its nutritional profile that is similar to the nutritional requirements of the most aquaculture species. It has a high protein content, with an excellent amino acid profile, a high essential fatty acids levels, particularly the polyunsaturated fatty acid of long chain, nucleotides, phospholipids, minerals, and trace elements (including calcium, phosphorus, magnesium, zinc, manganese, selenium, iodine, molybdenum, and chromium), and lipid soluble and water soluble vitamins (Tacon *et al*., 2009). Moreover, fishmeal is highly digestible and palatable to fish and lacks anti-nutritional factors normally found in plant feedstuffs (Gatlin *et al*. 2007; NCR 2011). According to FAO (2012), "fishmeal is the crude flour obtained after milling and drying fish or fish parts, and is produced from whole fish, remains or other fish by-products resulting from processing". The reduction of dietary incorporation of fishmeal in aquafeeds represents a major challenge for the aquaculture development. Fishmeal inclusion level in aquafeeds has been decreasing, but for some aquaculture species its incorporation level may still reach up to 50% (Metts *et al*., 2011; Oliva-Teles, 2015). Due to the increasing human population and fisheries pressure, the global production of fishmeal has been declining (OECD, 2015). There is also a competitive demand for marine protein sources by human and other livestock (Millamena, 2002). In fact, due to its rising prices, stagnation of supply, environmental issues and low efficiency of fish stock utilization, it is essential to replace fishmeal in aquafeeds, partial or totally, with a more sustainable, environmentally-friendly and less expensive protein source (Hardy, 1996, 2003; Pauly *et al*., 2002; Naylor *et al*., 2003; OECD, 2015). To be a viable alternative for fishmeal or fish oil, a candidate ingredient must possess certain characteristics, including nutritional suitability, ready availability, and ease of handling, shipping, storage, and use in feed production (Kaushik, 1990; Gatlin *et al*., 2007; Lim *et al*., 2008). However, despite the feed formulation, feed has to ensure the fish health and performance, consumer acceptance, minimal pollution and ecosystem stress, and human health benefits. Finally, competitive pricing is essential for the adoption of non-fish alternatives in feeds. However, given limited supply and increasing demand, the long-term outlook appears to be one of rising fishmeal and fish oil prices, a trend that could facilitate the substitution of non-fish alternatives, depending on relative price trends (Naylor *et al*., 2009; FAO, 2014).

As aforementioned, fishmeal is becoming limited due to increasing demand and decreasing marine fisheries resources. Tacon (1995) listed a large number of possible fishmeal replacers, including invertebrate animal by-products (e.g. silkworm pupae, earthworms, zooplankton), vertebrate animal by-products (e.g. blood meal, liver meal, meat and bone meal, poultry by-products), single-cell proteins (mainly from fungal and

bacterial sources), oilseeds (e.g. soybean, rapeseed, sunflower, cottonseed), legumes (e.g. beans, peas, lupine) and miscellaneous plant protein products (e.g. corn gluten meal and concentrates made from potatoes and leaves). In general, poorer digestibility, lower availability of some essential amino acids, palatability problems, and, in some cases, the presence of anti-nutritional factors, have limited the replacement of fishmeal by plant proteins or animal by-products. In fact, studies have been showing that they have significant limitations and because of that, these cannot be used at extremely high levels in the diet of most fish species (New, 2001; Bureau, 2016).

Among the potential alternative to fishmeal, plant protein feedstuffs have been the most extensively studied. Despite the efforts, high inclusion levels of plant feedstuffs has been limited due to deficiencies in essential amino acids, anti-nutrient factors and/or poor palatability (Gatlin *et al*., 2007; Oliva-Teles *et al*., 2015). Furthermore, high inclusion levels of plant protein are also associated with reduced feed intake, nutrient utilization and increasing environmental waste (Gomes *et al*. 1995; Karapanagiotidis, 2014). Also the presence of high level of fibre and anti-nutritional factors may also affect the intestinal functional and integrity, as well as general health and immune status of the fish (Krogdahl *et al*., 2010; Oliva-Teles, 2012). Moreover, the aquafeed industry competes for the use of plant feedstuffs with the sector of terrestrial animal production and biofuel production as well as with the use for direct human consumption.

Actually, with the expansion of the aquaculture sector worldwide, the demand for aquafeeds and its main protein source, fishmeal, is rapidly increasing. This continuous increasing demand together with the decreasing supplies of fishmeal have been forced the manufactures and aquacultures to investigate for alternative protein sources of good nutritional quality, which are ideally less expensive than fishmeal as the terrestrial animal by-products. Animal by-products may become a promising suitable raw alternative to fishmeal that is both economically viable and environmentally friendly for aquafeed production.

Animal by-products used in aquaculture

Processed animal proteins, also known as animal by-products have been categorised by the species from which they are made: pork, poultry (chicken, turkey, duck), feather and blood meal. Rendered animal by-products include livestock and poultry carcasses plus offal, spent cooking fats and oils, fat trimmings, bones, and other

meat and poultry processed materials. These by-products contain significant levels of protein and oils and provide a ready source of nutritious, digestible animal protein and fat.

Animal by-products are protein-rich feedstuffs derived from the rendering of animal tissues providing very useful and cost effective protein to animal feed manufacture (Karapanagiotidis, 2014). Indeed, comparatively to fishmeal they are significantly less expensive per kg of crude protein than fishmeal. Comparatively to plant proteins feedstuffs, animal by-product meals have a significantly higher protein level, a more complete amino acid profile and, some of these products contain high levels of available lysine and phosphorous, representing a good alternative to the imported plant protein feedstuffs, such as soybean meal. Moreover, due the new processing techniques, the quality of the final animal by-products has increased and indeed, the digestibility of these products has increased over the last 30 years to 80-90%. Besides, nowadays, it is possible to guarantee the consistency of the product quality and safety, which is of crucial importance for the aquafeed market.

The highly variable raw quality of these products and low digestibility limited its use in the 1970s and 1980s (Cho *et al*., 1979; Cho *et al*., 1982; NCR, 1993). After that, another issue that limited its use was the food crisis of the 1990s, due to the bovine spongiform encephalopathy (BSE) epidemic. Animal by-products was pointed out as the vehicle of certain infectious diseases, including BSE, being prohibited to be included in aquafeeds, under the European Union (EU) regulation (Regulation (EC) No 178/2002). However, recently the EU decided to take a first step by lifting the feed ban for non-ruminant processed animal by-products use in fish feed in 2013 (EFPRA, 2014).

Transformation of animal by-products into feed ingredients will contribute to the mitigation of environmental problems caused by animal by-products processing. The proper processing of these by-products avoids the release into the environment, being considered vectors for insects, vermin, bacteria and viruses, which may result in water contamination (leaching of nutrients and pathogenic microorganisms) and air pollution (noxious gases and nuisance odorants, Gerber *et al*., 2007). Therefore, it is expected that the inclusion of processed animal by-products in aquafeeds will Europe will contribute for the sustainable growth of European aquaculture, also contributing for the reduction of the carbon footprint of aquafeeds, since the carbon footprint of animal by-products is much lower than plant feedstuffs (EFPRA, 2014).

Among the animal by-products, hydrolysed feather meals, poultry meal, non-ruminate meal or meat and bone meal have been subject of several studies.

Hydrolysed feather meals (FeM)

Hydrolysed feather meal is a by-product resulting from treatment under pressure of clean, undecomposed feathers from slaughtered poultry, free of additives and/or accelerators. It has high protein content, with relatively high digestible protein content for fish, well balanced essential amino acid profile, with exception of lysine and tryptophan, lack of anti-nutritional factors and contains 18 types of free amino acids (Zhang *et al*., 2004). However, information is lacking regarding the nutritional value of hydrolysed feather meals in aquafeeds. Without supplementation with the limiting essential amino acids, FeM by itself cannot provide the required amount of lysine and tryptophan for most fish species. Indeed, several research studies have indicated that supplementing FeM based diets with limiting essential amino acid can only partially (up to 50 percent) alleviate reduction in fish weight gain when compared to the fishmeal control group (Pauly *et al*., 2002). More research is needed in amino acid supplementation in order to improve the efficiency of FeM based diets.

Overall, hydrolysed feather meal is relative more suitable for non-carnivorous fish species. It has been tested with juveniles of several species such as chinook salmon, *Oncorhynchus tshawytscha* (Fowler, 1990), *Japanese flounder*, *Paralichthys olivaceus* (Kikuchi *et al*., 1994), Indian major carp, *Labeo rohita* (Hasan *et al*., 1997), common carp, *Cyprinus carpio* (Jahan *et al*., 2001), tench, *Tinca* (Gonzales-Rodrigues *et al*., 2014), hybrid tilapia (Zhang *et al*., 2014) showing that adequate inclusion levels of FeM in diet are species-dependent.

Earlier studies found that replacing fishmeal with limited amounts of feather meals in trout or salmon diets did not negatively impact fish growth and feed utilization (Higgs *et al*., 1979; Fowler, 1990; Bishop *et al*., 1995; Bureau *et al*., 2000). Recent studies conducted with high quality FeM revelled that protein digestibility is relatively higher, ranging from 67 to 87% (Bureau *et al*., 1999; 2000; Cheng *et al*., 2004; Davies *et al*., 2009; Poppi *et al*., 2011), in opposition to the low levels reported earlier in the 1970's (Cho *et al*., 1979), suggesting a significant improvement in the digestibility of FeM through the improvement of FeM processing technologies (Bureau *et al*., 1999).

Meat and Bone meal (MBM) and Poultry by-product meal (PBM)

According to the American Feed Control Officials (AFCO, 2003), non-ruminants' animal by-products meal, including meat meal and meat and bone meal is the rendered product form of non-ruminant mammal tissues, exclusive of any added hair, hoof, horn, hide trimmings, manure and stomach contents except in such amounts as many occur unavoidably in good processing practices. PBM consists of the ground, rendered, clean parts of the carcass of slaughtered poultry, such as necks, feet, undeveloped eggs, and intestines, exclusive of feathers. Unlike poultry meal, poultry by-product meal may include the bone. If a particular species of bird is used, it may be declared by the more common name, such as "chicken" or "turkey" (AFCO, 2003).

These by-products are economically animal protein sources (Abdel-Warith *et al*., 2001; Hardy *et al*., 2002) and are derived from slaughtered farmed livestock (swine and/or poultry). They have a relatively higher protein content (50-85%), a good balance essential amino acids, are available in large quantities year-round and costs less than half the price of fishmeal (NCR, 2012; FAO, 2016). Nutritional value of these by products is inversely related to the ash content (Hardy *et al*., 2002), but so is the price. Of concern is the lake of consistency of nutritional quality of these by-products that is greatly influenced by the quality and specific combination of the raw materials and by the processing methods used to manufacture these products (Forster *et al*., 2006; Rossi *et al*., 2014). Previously, it was pointed out that better manufacturing practices may improve the quality of animal by-products (Bureau *et al*., 1999, 2000). Indeed, nowadays the new developed processing technology, such as flash drying, has improved the nutritional value of these animal protein sources.

This new generation of animal by-products with high consistency in freshness, quality and digestibility significantly improved its potential as fishmeal replacement. Even though research worldwide has evidenced that these by-products have great potential to be included in aquafeeds, limited work has been done with European fish species, due to restriction of its use as feedstuff in the EU, as aforementioned.

Nutritional composition of PBM is similar to fishmeal, but slightly lower in some essential amino acids, such as methionine and lysine, has high protein content (60-80%), is highly digestible and is commercially available in the market in high volumes and controlled quality. These feedstuffs have been successfully used to partially or totally replace fishmeal in the diets for some fish (Robinson *et al*., 1993; Allan *et al*., 2000).

Generally, the fishmeal replacement by MBM or PBM is less than 300g kg^{-1} in aquafeeds (Pongmaneerat *et al*., 1991; Robaina *et al*., 1997; Bureau *et al*., 2000; Kureshy *et al*., 2000). Some studies showed that MBM or PBM could partially replace dietary FM without affecting growth and feed efficiency of experimental fish (Davies *et al*., 1991; El-Sayed, 1998; Bureau *et al*., 2000; Webster *et al*., 2000). However, MBM or PBM have some limitations, particularly due to the amino acid profile, especially methionine and lysine content, and high ash content (up to 10 to 40%) that is reported to reduce feed digestibility (NCR, 1993; McGoogan *et al*., 1996; Wu *et al*., 1999; Allan *et al*., 2000). Indeed, studies conducted in Japan and Portugal indicated that meat meal (e.g. high protein, low ash MBM) is highly digestible for several freshwater and marine species when compared to the MBM (Gomes *et al*., 1995; Watanable *et al*., 1996; Silva *et al*., 1998). Low palatability is another cause for poor performance in some fish species fed diets containing MBM (Rodriguez *et al*., 1996; Robaina *et al*., 1997). According to Xue *et al*. (2001), some dietary factors, such as, feed quality, availability and feed utilization capacity, and temperature of the water, could make diets with high levels of MBM less palatable than FM-based diets. Also, the inclusion of feeding stimulants in the diet may improve the palatability of diets containing meat and bone meal (Xue *et al*., 2001).

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Objectives of this study

The main objective of the present study is to evaluate the potential of poultry by-product meal as a fishmeal replacer in diets for gilthead seabream, contributing to the circular economy by adding value to waste products of slaughtering processes, resulting in a more sustainable feed production chain and reduced feed prices.

For that purpose, a growth and a digestibility trial were performed to evaluate the impact of dietary fishmeal replacement by increasing levels of PBM on voluntary feed intake, growth performance, nutrient availability and digestibility in gilthead seabream juveniles.

Material and Methods

Experimental diets

The proximate composition of the main ingredients used to formulate the experimental diets is present in **Table 3**.

Table 3: Proximate analysis of the ingredients used (% dry matter).

Description	Dry Matter	Protein	Lipids	Ash
Poultry by-product meal[1]	95.1	68.1	17.7	13.1
Fishmeal 68%-70%	88.1	76.2	10.7	8.8
Fishmeal 60%	90.6	62.7	11.5	22.2
Wheat meal	85.5	12.1	2.5	1.4
Dehulled horse bean	87.8	29.5	4.5	3.5
Rapeseed meal non-GMO[2]	87.5	34.7	6.4	7.3
Hemoglobin AP310[3]	87.2	94.0	3.8	4.6
Corn gluten meal	90.2	67.3	6.6	1.1
Dehulled Pea meal	87.7	50.6	6.9	4.7

All the ingredients were provided, Soja de Portugal, Ovar, Portugal

[1]Savinor, Soja de Portugal, Ovar, Portugal

[2]Non-GMO: non-genetically modified

[3]Hemoglobin powder AP310P; APC Europe S.A.

Based on the ingredients composition, six experimental diets were formulated to be isoproteic (45% protein) and isolipidic (18% crude lipid), including poultry by-product meal (PBM) levels from 0 (control diet) to 37.5% in replacement of FM (**Table 4**). All dietary ingredients, including poultry by-product meal (PBM) as shown in **Fig.3** and **Fig.4**, were finely ground all well mixed and then, this mixture was dry pelleted in a laboratory pellet mill (CPM), through a 3 mm die. The pellets were dried in an oven for 24 h, at 35 ºC. Then, the diets were sieved and storage in the freezer until use.

For the digestibility trial, the same experimental diets were used, including 0.5% of chromium oxide (Cr_2O_3) as an inert digestibility marker. The ingredients and proximate composition of the experimental diets are presented in **Table 4** and **5**, respectively.

Table 4: Ingredients composition of the experimental diets (g/ kg dry matter)

Ingredients	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5
Poultry by-product meal	—	75	150	225	300	375
Fishmeal 68%-70%	275	225	175	125	75	75
Fishmeal 60%	175	150	125	100	75	0
Wheat meal	144	142	140	138	136	135
Dehulled horse bean meal	60	60	60	60	60	60
Feather meal	50	50	50	50	50	50
Rapeseed meal	50	50	50	50	50	50
Hemoglobin AP310	40	40	40	40	40	40
Corn Gluten	35	35	35	35	35	35
Dehulled Pea meal	25	25	25	25	25	25
Squid Meal	10	10	10	10	10	10
Fish and poultry oil[1]	120	120	120	120	120	120
Mineral premix	4	4	4	4	4	4
Vitamins premix	3	3	3	3	3	3
Binder	1	1	1	1	1	1
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Stay C	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin E	0.25	0.25	0.25	0.25	0.25	0.25
Lysine	-	1	2.2	3.5	4.8	5.2
Methionine	-	0.7	1.3	2	2.7	3.1
Constant components[2]	7.45	7.45	7.45	7.45	7.45	7.45

[1] Fish and poultry oil 50/50.

[2] Constant components: mixture of additives; due to the confidential agreement with the feed company the exact amount of each one is not present.

Fig. 3: Poultry meal (PBM) before grinding

Fig.4: Poultry meal (PBM) after grinding

Table 5: Composition analysis of the experimental diets (% dry matter).

	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5
Dry Matter (%)	92.7	92.6	92.6	94.0	93.7	93.8
Protein	47.9	49.1	50.3	50.4	48.1	47.4
Lipids	18.5	17.9	18.8	18.4	19.0	20.0
Ash	11.5	11.1	12.0	11.1	11.2	10.2
Phosphorus	1.59	1.49	1.44	1.34	1.19	1.12
Energy (kJ g^{-1} DM)	35.1	34.8	35.2	35.4	35.9	35.7

Growth Trial

Experimental fish

The growth trial was conducted at the Marine Zoological Station, Faculty of Science, University of Porto. Gilthead seabream were obtained from a commercial hatchery.

After transportation, gilthead seabream were treated with formaldehyde, to eliminate any external parasites, and held quarantine system during fifteen days. After

this period, fish were transferred to the experimental system of growth trial and acclimatized to those conditions during fifteen days. During this period, fish were fed a commercial diet twice a day until the start of the trial.

Growth trial

The growth trial was performed in a thermoregulated recirculation water system equipped with a battery of 18 fiberglass tanks (100 L water capacity each) and supplied with continuous flow of filtered seawater (4 to 5 L/min) (**Fig.5**). Optimal water quality was maintained throughout the growth trial, due the filtration system, composed by a sedimentation basin equipped with a mechanical filter (sponge), to collet large particles (faeces), two units of bio filtration and a sand filter. Each bio filtration unit contains high density plastic bio balls used as substrate for nitrifying bacteria that oxidize ammonia into nitrite and then into nitrate. A water pump was used to transfer the seawater from the filtration unit to the rearing tanks. An air pump (blower) supplied a continuous air flow in all tanks and biological filters. All the water of the growth trial system was completely renovated once a day. To pump the seawater from the sea, the Marine Zoology Station has two water pumps allowing the direct seawater pumping.

Fig. 5: Thermoregulated recirculation water system used for the growth trial.

At the beginning of the growth trial, the average body weight was determined to be 63 g. Then, 18 homogeneous groups of 10 gilthead seabream were established, under a light anaesthesia (ethylene glycol monophenil ether, 0.3 ml L^{-1}), and randomly distributed to the 18 tanks of the system, one group per tank (**Fig.6**). Each experimental diet was randomly assigned to three tanks. Fish were fed by the hand, twice a day (morning meal at 9 a.m. and the afternoon meal at 15 p.m.), 6 days a week, until visual apparent satiety during a 12-week period.

Fig.6: Gilthead seabream in the experimental tanks

During the trial, the water temperature was regulated to 22 ± 1 ºC (near the optimum water temperature for the species). Photoperiod was maintained at a schedule of 12/12 hours of light/dark. Salinity average 33 ± 2% and dissolved oxygen was above 90% of saturation. Feed consumed was recorded daily and mortality was also monitored before each meal.

Fish were group-weighed and counted every three weeks, and at the end of the trial, after one day of feed deprivation. At the beginning of the trial, 6 fish from the initial stock and at the end of the trial 3 fish per tank were randomly collected, and pooled for whole-body composition analysis. Wet weight, liver and viscera weights were recorded for the determination of hepatosomatic and visceral index.

After the final weighing, fish were fed for more 3 days and then the intestine was sampled for measuring digestive enzymes activity in full fed fish, to avoid effects of fasting, following previous recommendations (Krogdahl *et al*., 2005) (**Fig.7**). Three fish per tank were randomly sampled, euthanized with a sharp blow to the head, and immediately eviscerated in an ice-cooled tray. The digestive tract was excised, adherent adipose and connective tissues were carefully removed, and the intestine divided in two

portions: anterior and posterior intestine. The posterior intestine was distinguished from the anterior intestine by the increased diameter, dark mucosa and annular rings. The anterior portion of intestine was obtained by the division of the remaining intestine in two identical parts. The anterior intestine is the portion directly after the stomach and included the pyloric caeca. The intestine sections were immediately frozen in liquid nitrogen and then stored at -80 ºC until measurement of enzymes activity.

Fig 7: Collecting the gilthead seabream intestine for determination of enzymatic activity.

After the final weighing and all the sampling work, the remaining fish continued to be fed a commercial diet for later use in the digestibility trial.

Digestibility Trial

The digestibility trial was conducted at Marine Zoological Station, Faculty of Science, University of Porto.

The experimental system consists in another thermoregulated recirculating water-system equipped with 12 tanks (50 L water capacity each), designed according to the Guelph system (Cho *et al*., 1990). A faeces settling column was connected to the outlet of each tank (**Fig. 8**). A continuously water-flow was established, at a rate of about 4.5 L/min. During the trial, water temperature averaged around 22 ± 1ºC.

Fig.8: Thermoregulated recirculation water system used for the digestibility trial.

At the beginning of the trial, twelve groups of six gilthead seabream, with an average weight of 180 g were established (**Fig.9**). Each experimental diets was randomly assigned to duplicate tanks and fish were fed by hand to apparent satiation, twice a day (8.30 a.m. and 14.30 p.m.). The digestibility measurements were carried out in two periods, and at each one the six experimental diets were randomly assigned to two different groups. The first 5 days of each period were used for gilthead seabream adaptation to the experimental diets and after faeces were collected once a day for 15 to 20 days, until a representative sample of faeces was collected. Before the morning meal, faeces accumulated in each settling column tank were collected, centrifuged (3000 g) and the supernatant discarded and faeces were pooled for each tank and stored at -20 ºC until analysis. Thirty minutes after the afternoon meal, all tanks, water pipes and the sediment column were cleaned to remove any residue of faeces or diet.

Fig.9: Gilthead seabream in experimental tanks

Apparent digestibility coefficients (ADC) of organic matter, dry matter, protein, lipid and energy of the diets were determined by the following formula:

ADC = [1-((dietary Cr_2O_3 level × faeces nutrient or energy level) / (faeces Cr_2O_3 level × dietary nutrient or energy level))] ×100

Chemical analyses performed in ingredients, diets and faeces

Sample Preparation

Before the analysis, faeces and whole-fish were dried in an oven at 100 ºC to constant weight and then, the samples were ground to obtain a homogeneous sample. Chemical analysis of the ingredients, experimental diets, faeces and whole-fish were made in triplicate according to the following procedures:

Dry Matter

Approximately 500 mg of homogeneous sample was placed in pre-weighed crucibles. The moisture content was determined by the total weight loss of the sample after drying at 100 ºC until constant weight.

Ash Content

After the determination of the moisture content, sample were placed in an muffle furnace and after the incineration at 450 ºC for 16 h, the ash content is calculated by the weight of inorganic residue present in the crucibles.

Crude Protein

The protein content (N x 6.25) were determined by Kjedahl method following acid digestion, using a Kjeltec digester and distillation units (Tecator Systems, Höganäs, Sweden; model 1015 and 1026, respectively). Crude protein was calculated by multiplying the total nitrogen content by the factor 6.25 (16 gN/100).

Approximately 500 mg of sample (200 mg for faeces samples) was added to the tubes of digestion. Two tablet containing 1 g of sodium sulphate (Na_2SO_4) plus 0.05 g of selenium (Se) were added to each sample as catalyst. The tubes were placed in the digest unit and the water tap was turned on to create a suction effect of the sulphuric acid (H_2SO_4) vapours. The samples were digested for one hour at 400 ºC with concentrated sulphuric acid (15 or 5 ml H_2SO_4, for macro or micro system, respectively) which converts organic nitrogen to ammonium sulphate. After cooling for 30 min, the tubes containing the samples were transfer to the Kjeltec distillation unit. For each digestible tube with a sample was prepared one flask with 25 ml of boric acid and 10 drops of the methyl orange pH indicator and placed in the Kjeltec distillation unit. After 2, 3 minutes, the tube is removed from the distillation unit and the amount of ammonia was determined by the titration with hydrochloric acid (HCL) (0.5 or 0.2 N, for macro or micro system, respectively), in presence of the methyl orange pH indicator.

Lipids (Soxtec method)

The lipid content of ingredients, diets and carcasses was determined by the Soxtec method, involving a continuous extraction with petroleum ether in a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046).

Approximately 500 mg of sample was placed in a cartridge and positioned in the extraction unit with the aid of a support. Samples were boiled for one hour in petroleum ether, rinsed for two hours and then, the extracted lipids were completely collected in the extraction cups. The extraction cups were place in an oven at 100 ºC for 24 hours and thereafter were weighed. Lipid content was defined through the difference in weight of the cups before and after extraction.

Chromium oxide

Chromium oxide content in faeces and diets used in the digestibility trial was determined by acid digestion to Furukawa *et al*. (1966). 300 mg of sample were weighed and placed in 100 ml volumetric flasks. Then, 5 ml of nitric acid (HNO_3) was added and the heating mantle was turned on. Flasks content was digested until the acid volume was reduced to half (approximately 30 minutes). Then, another 5 ml of nitric acid (HNO_3) was added to be sure that all organic matter was completed digested. After a cooling period, 3 ml of perchloric acid ($HCIO_4$) were added to each cold flask. If flasks are still hot and/or an incomplete digestion of the organic matter has occurred, an explosive reaction may happen. The flaks are placed again in the heating mantle until the green solution becomes yellow. After cool down the flasks for a few minutes, its content was placed into 25 ml volumetric flaks. The spectrophotometer was adjusted at 350 nm with a blank and then, the reading of the samples was realized. Chromium oxide content of the sample was calculated using the following standard line:

$$y = 0{,}2089\ x + 0{,}0032$$

Where *y* represents optimal density at 350 nm and *x* represents the content of chromium oxide of the sample in mg/100 ml.

Phosphorus

Total phosphorus content in diets and faeces was determined based on a colorimetric method (Silva *et al*., 2002). Approximately 200 mg were weighted and placed in 100 ml volumetric flaks. Then, 5 ml of nitric acid (HNO_3) was added to each flask and the heating mantle was turned and content was digested. After a cooling period, 1 ml of perchloric acid ($HClO_4$) was added to each cold flask and heated until the sample turn into a white solution. After a cooling period, the samples were transferred into 25 ml volumetric balloons and completed its volume with distilled water. Then, an aliquot of this solution was mixed and reacted with 5 ml of ammonium molybdate and 2 ml of ascorbic acid. The amount of phosphorus was determined by measuring the intensity of blue colour, which is produced by the formation of colloidal oxides. The colour intensity developed by phosphomolydate depends on its phosphorus content, which was determined after reading of the samples and standard solution at 750 nm.

Digestive Enzymes activities

For the enzymatic activity measurement, each intestine portion was homogenized in ice, with an Ultra Turrax, and centrifuged at 12000 rpm, for 30 min at -4 ºC. Supernatant was collected and stored at -80 ºC, until analyses. All enzyme activities were determined using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Protease activity

Digestive proteases are hydrolases that break the peptide bonds of proteins. To know the protease activity, casein hydrolysis method was used and the test carried out on 0.1 KCl with buffer in pH 1.8. The reaction mixture containing casein (1% w/v; 0.4 ml), buffer (1.6 ml) and homogenate supernatant (20 ml) was incubated for 40 minutes at 37 ºC and stopped by adding 1 ml trichloroacetic acid (15% w/v) solution. Samples were centrifuged and the supernatant absorbance was read at 280 nm against blanks. A control blank for each sample was prepared adding the supernatant from the homogenates after incubation. Tyrosine solution was used to establish a calibration curve (181.19 g/mol).

α-Amylase activity

The α-Amylase is a key enzyme that acts on complex polysaccharides as glycogen and starch, catalysing the hydrolysis of α-glucoside bond, releasing glucose and maltose. In mammals, it is produced by the salivary glands and the exocrine pancreas. In fish, amylase is produced by pancreas and liver but it was detected in pancreas juice, stomach and in the intestines (NRC, 2011).

The method comprises in the hydrolysis of 2-chloro-4-nitrophenyl-α-D-maltotrioside by α-amylase; this reaction releases 2-chloro-4-nitrophenol (CNP) and forms 2-chloro-4nitrophenyl-α-D-maltoside (CNPG2), maltotriose (G3) and Glucose (G). The rate of 2chloro-4-nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of α-amylase present in the sample:

$$10\ \text{CNPG3} \xrightarrow{\text{Amilase}} 9\ \text{CNP} + 1\ \text{CNPG2} + \text{G3} + \text{G}$$

The reaction mix consisted of 200 µl of amylase reagent (2-chloro-4-nitrophenyl-α-Dmaltotrioside, CNPG3) and 10 µL of sample homogenate. This mixture was incubated at 37 ºC during 30 seconds and absorbance (Δ DO/min) was read at 1 minute intervals during 3 minutes at 405 nm and 37 °C.

Lipase activity

Lipase is an enzyme necessary for pancreatic digestion and absorption of lipids and their function is to catalyse the hydrolysis of glycerol esters of fatty acids.

In this method, the pancreatic lipase, along with the colipase, desoxycholate and calcium ions, hydrolyses the substrate 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'methylresorufin)-ester.

$$\text{1-2-O-dilauryl-rac-glycero-3-glutaric-(6' -methylresorufin)-ester} \xrightarrow{\text{Lipase}}$$

$$\text{1-2-O-dilauryl-rac-glycerol + Glutaric-6'-methylresorufin-ester (no stable)} \xrightarrow{OH^-}$$

Glutaric acid + Methylresorufin

The rate of methylresorufin formation was quantified photometrically and it is proportional to the concentration of catalytic lipase present in the sample homogenate. The reaction mix consists in 100 µl of reagent 1, 20 µl of reagent 2 and 5 µl of sample. This mixture was incubated for 30 seconds and the sample absorbance (Δ DO/min) was then read at 10 seconds intervals, during 11 minutes, at 580 nm and 37 °C.

Trypsin activity

Trypsin is a protease of pancreatic origin, that breaks the bonds of proteins by hydrolysis to form smaller peptides and amino acids. The activity was measured at 37 ºC with toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME) substrate. The extracts were incubated for 2 minutes in 2 ml of Tris/$CaCl_2$ 8.1.

Trypsin enzyme analysis was performed for both the anterior region to the posterior region of the intestine with 70 µl of reagent and 10 µl of diluted sample 1/5. The absorbance was read in the spectrophotometer at 410 nm.

Chymotrypsin activity

Chymotrypsin is another protease. The assay was carried out with benzoyl-L-tyrosine ethyl ester (BTEE) 0.001M to 37 ºC. Extracts (30ml) were incubated for 2 minutes in 2 ml of Tris/$CaCl_2$ pH 7.8. One enzyme unit was defined as the amount of enzyme required to hydrolyse 1 g of substrate (TAME or BTEE) for 1 minute per mg protein. In both regions of the bowel enzymatic analysis was performed with 70 µl of BTEE solution and 10 µl of diluted sample 1/50.

Specific enzymatic activity

Enzyme activity of total proteases, amylase, lipase, trypsin and chymotrypsin was expressed as specific activity (units per milligram of soluble protein; one unit (U) of activity was defined as µmol of product generated per minute). Soluble protein concentration was determined using Bradford's method (1976), with bovine serum albumin solution as standard. Amylase and lipase activities were determined using the formula:

$$mU\ mg\ protein^{-1} = \frac{(\Delta\ DO/\ \Delta\ t)\ \times\ Vt\ \times\ f}{Ex \times\ 10^{-3}\ \times 10^{-9}\ \times\ Ve\ \times\ d\ \times\ P}$$

Where (Δ DO/ Δ t) is the decrease or increase of optical density / minute, V_t is the total reaction volume, *f* is the correction factor for the dilution of the extract, E_x is the molar extinction coefficient, 10^{-3} is the conversion factor of litre to millilitre, 10^{-9} is the conversion factor from mol to nmol, *Ve* is the volume of added extract in ml, *d* is the length of the light beam through the microplate (0.79 for lipase activity and 0.675 for amylase activity) and *P* is the mg of protein per ml.

Economic Analyses

The economic analyses of the experimental diets were performed at the end of the growth trial. To evaluate that, we determinate:

Economic Conversion Ratio: ECR (€ kg of fish^{-1}) = FCR (kg diet kg of fish^{-1}) × diet price (€ kg)

Economic profit index: EPI (€ fish^{-1}) = weight gain (kg) × seabream selling price (€ kg^{-1}) – ((weight gain (kg) × diet price (€ kg of diet^{-1}))

Statistic analyses

All statistical analyses were done using the SPSS 21.0 software package for Windows. Data were presented as means and standard error of the mean (S.E.M.) and were checked for normal distribution and homogeneity of variances and normalized when appropriate.

Statistical evaluation of growth performance, feed utilization efficiency and whole-body composition of gilthead seabream fed the experimental diets was done by one-way variance analysis of variance (ANOVA). Digestive enzymes activity was statistically analysed by a two-way analysis of variance (Two-Way ANOVA). Statistical evaluation of the apparent digestibility coefficients was done by analysis of variance according to a randomized complete block design ANOVA, using as a block the two periods.

Significant differences between means were calculated through Tukey's multiple range test ($p< 0.05$).

Results

Growth trial: performance and feed utilization efficiency

All experimental diets were well accepted by the fish. No pathological signs or mortality were observed during the trial **Table 6**. Growth performance, expressed as final body weight, weight gain and daily growth index, was unaffected by dietary treatment. Similarly, voluntary feed intake, feed efficiency and protein efficiency ratio were also similar irrespectively of the dietary treatments (**Table 6**).

Table 6: Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets.

	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5	SEM
Initial Body Weight (g)	63.3	63.3	63.3	63.3	63.3	63.3	0.1
Final Body Weight (g)	174.3	179.5	170.4	171.8	181.4	165.4	2.7
Weight gain (g kg ABW^{-1}day^{-1})	13.4	13.7	13.1	13.1	13.8	12.7	0.2
Weight Gain (% IBW)[1]	175.6	183.7	169.3	171.5	186.8	161.4	4.19
Daily Growth Index[2]	2.29	2.36	2.23	2.24	2.39	2.15	0.04
Feed intake (g kg ABW $^{-1}$day^{-1})[3]	19.8	21.1	18.5	19.7	20.9	20.3	0.35
Feed Efficiency[4]	0.67	0.65	0.71	0.67	0.66	0.63	0.01
Protein Efficiency Ratio[5]	1.11	1.04	1.11	1.06	1.08	1.14	0.02
Survival Rate (%)	100	100	100	100	100	100	0.00

Values presented as means (n=3). SEM: pooled standard error of the mean

Absence of superscript letters within a row represents no significant differences between treatments (P> 0.05).

Average body weight (ABW): initial body weight (IBW) + final body weight (FBW)/2.

[1]Weight gain, % = [(Final weight – Initial weight) / Initial weight] x 100

[2] DGI = [(Final weight$^{1/3}$ – Initial weight$^{1/3}$) / time in days] x 100

[3]FI = Total dry feed intake / Average body weight / days

[4]FE = wet weight gain / dry feed intake

[5]PER = weight gain / crude protein intake

Whole Body Composition

At the end of the growth trial, whole-body composition was unaffected by the inclusion of PBM in the experimental diets (**Table 7**). There were no significant differences in both hepatosomatic and visceral indices of gilthead seabream fed the different experimental diets.

Table 7: Whole-body composition (% wet weight), hepatosomatic and visceral indices of seabream fed the experimental diets.

	Initial	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5	SEM
Dry Matter (%)	26.2	35.2	35.7	33.8	34.5	35.7	34.7	0.33
Protein	14.6	17.1	17.7	17.1	17.2	17.6	17.7	0.12
Lipids	6.5	14.2	15.0	13.0	13.8	15.0	12.8	0.34
Ash	6.1	3.8	3.7	4.0	4.0	3.9	4.3	0.07
HSI[1] (%)	_	1.98	1.92	1.90	1.83	2.01	1.82	0.03
VI[2] (%)	_	10.23	8.88	9.28	8.95	8.86	9.72	0.22

Values presented as means (n = 3). SEM: pooled standard error of the mean (SEM).
Absence of superscript letters within a row represents no significant differences between treatments (P> 0.05).
[1]Hepatosomatic index: (Liver weight / body weight) × 100.
[2]Visceral index = [Viscera weight (g) / body weight (g)] x 100

Digestibility Trial: ADC (%) of the experimental diets

The apparent digestibility coefficients (ADC) of the experimental diets are presented in **Table 8**. Apparent digestibility coefficients of dry matter, protein, phosphorus and energy were generally high and not affected by the dietary treatment.

Table 8: Apparent digestibility coefficients (ADC, %) of seabream fed the experimental diets.

	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5	SEM
Dry Matter	80.7	81.4	79.4	74.5	75.2	73.8	0.8
Protein	92.8	92.7	91.5	90.4	89.8	89.7	0.4
Phosphorus	79.7	83.8	82.8	81.4	80.3	79.4	0.9
Energy	90.8	90.6	89.2	87.8	87.4	87.4	0.5

Values presented as means (n = 4) and pooled standard error of the mean (SEM).
Absence of superscript letters within a row represents no significant differences between treatments (P> 0.05).

Digestive enzymes

The specific activities of proteases, trypsin, chymotrypsin, lipase and amylase in the anterior, posterior and total intestine of gilthead seabream juveniles fed the experimental diets are present in **Table 9**. The proteolytic activity was measured at pH 8 and total intestine activity was obtained by the sum of the anterior and posterior intestine activities.

Independently of dietary treatment, digestive enzymes activity was higher in posterior intestine than in the anterior intestine, excepted for the proteases activity that was similar for both portions. The dietary treatment did not affect the activity of the measured digestive enzymes.

Table 9: Specific activities of protease, trypsin, chymotrypsin, lipase and amylase (mU mg protein^{-1}) in different intestine sections (anterior (AI), posterior (PI) and total) of gilthead seabream fed the experimental diets.

	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5	SEM
Protease							
AI	60.7	70.2	74.8	73.3	71.0	66.6	5.2
PI	60.5	65.3	64.0	81.9	80.4	70.5	9.6
Total[2]	121.2	136.6	138.8	154.9	151.4	137.1	4.9
Trypsin							
AI	19.0	21.1	23.8	23.3	18.2	19.4	4.5
PI	79.5	106.6	105.9	75.8	92.7	90.1	16.3
Total[2]	98.5	127.8	129.7	99.1	111.0	109.4	20.5
Chymotrypsin							
AI	1349.1	1330.2	1574.0	1394.2	1216.5	1319.7	190.8
PI	1974.3	1941.6	2062.4	2017.7	1687.1	2005.0	307.9
Total[2]	3323.4	3271.9	3636.4	3411.9	2903.6	3324.6	498.7
Lipase							
AI	0.49	0.50	0.51	0.44	0.35	0.47	0.06
PI	2.30	2.70	2.35	2.47	2.34	2.85	0.88
Total[2]	2.79	3.20	2.86	2.91	2.69	3.32	0.10
Amylase							
AI	28.3	25.6	32.1	44.9	37.7	24.4	7.8
PI	60.9	54.9	76.2	63.7	46.9	49.4	11.5
Total[2]	89.2	80.5	108.4	108.6	84.6	73.8	6.0

Two-Way ANOVA[1]			
	Section	Diet	Interaction
Protease	ns	ns	ns
Trypsin	***	ns	ns
Chymotrypsin	***	ns	ns
Lipase	***	ns	ns
Amylase	***	ns	ns

Values presented as mean (n=3). SEM: pooled standard error of the mean (SEM).
[1]Two-Way ANOVA: ns P>0.05; * P>0.05; ** P<0.01; *** P<0.001
[2]Total intestine tract: sum of the activity in anterior and posterior intestine sections.

Nutrient and amino acid budget

Nitrogen balance of fish fed with the experimental diets are presented in **Table 10**. Dietary fishmeal replacement by PBM did not significantly affect nitrogen intake and retention, expressed as (g kg^{-1} ABW day^{-1}) or in percentage of the nitrogen intake. Even thought, faecal nitrogen losses increased with the dietary fishmeal replacement by PBM, the lowest value was attained with the control diet and the highest values with diets including 22.5% or more of PBM.

Table 10: Nitrogen balance of gilthead seabream fed the experimental diets.

	PBM 0	**PBM 7.5**	**PBM 15**	**PBM 22.5**	**PBM 30**	**PBM 37.5**	**SEM**
Intake (g kg ABW $^{-1}day^{-1}$)	1.52	1.66	1.49	1.59	1.61	1.54	0.03
Faecal Loss (g kg ABW $^{-1}day^{-1}$)	0.11^{a}	0.16^{bc}	0.15^{b}	0.19^{cd}	0.20^{d}	0.21^{d}	0.01
Metabolic Loss (g kg ABW $^{-1}day^{-1}$)	1.02	1.07	0.96	1.00	0.98	0.93	0.02
Retention (g kg ABW $^{-1}day^{-1}$)	0.39	0.42	0.39	0.39	0.42	0.40	0.01
Retention (% Intake)	26.0	25.5	26.3	24.9	26.5	26.0	0.42

Values presented as means (n = 3) and pooled standard error of the mean (SEM).
Absence of superscript letters within a row represents no significant differences between treatments (P> 0.05).

Economic Analysis

The economic analysis at the end of the growth trial are presented in **Table 11**. Both economic efficiency ratio (ECR) and the economic profit index (EPI) increases about 17% with the dietary replacement of FM by 30% of poultry meal, even though significant differences were only observed in the ECR.

Table 11: Economic analysis of the experimental diets.

	PBM 0	PBM 7.5	PBM 15	PBM 22.5	PBM 30	PBM 37.5	SEM
Diet Price (€ kg^{-1})	1.22€	1.17€	1.11€	1.06€	1.00€	0.98€	-
ECR (€ kg^{-1})	1.81^{b}	1.80^{ab}	1.57^{a}	1.58^{ab}	1.52^{a}	1.56^{a}	0.02
EPI (€ $fish^{-1}$)	0.35	0.37	0.37	0.37	0.41	0.35	0.00

Values presented as mean (n=3) and pooled standard error of the mean (SEM).

Absence of superscript letters within a row represents no significant differences between treatments (P> 0.05).

Diet price determined based on the cost of each feed ingredient and its inclusion rate. ECR, Economic efficiency ratio; EPI, Economic profit index.

Discussion

Aquafeeds are still highly depend of high inclusion levels of fishmeal, particularly for carnivorous species. However, due to the scarcity and rising cost of fishmeal it is of utmost importance to find viable alternative ingredients to support the sustainable development of aquaculture. Many studies have been conducted to evaluate the potential use of non-ruminant processed terrestrial animal proteins as fishmeal replacers in different aquaculture fish species with variable results. However, as the utilization of this type of feedstuffs in European aquafeeds was prohibited until very recent years due to BSE crisis, the research conducted with species of interest for European aquaculture is very limited and date back to the 90s.

The nutrient content of PBM can be quite variable and depends on the substrate that is being processed (Watson, 2006). However, recent advances in processing technology have greatly improved it nutritional quality and an effort has been made to guarantee the consistency of its quality. In general, it is a highly palatable and high quality feedstuff with high protein content, balanced essential amino acid profile, fatty acids profile, vitamins and minerals content. For fish species, PBM has been shown to be of high nutritional quality and has been considered as a valuable protein source for many species, especially for carnivorous fish species (Nengas *et al*., 1999). The potential of PBM to replace dietary fishmeal has been studied in tench (Gonzalez-Rodriguez *et al*., 2016); Japanese sea bass (*Lateolabrax japonicus*) (Wang *et al*., 2015); Florida pompano, *Trachinotus carolinus* (Riche, 2015); golden pompano, *Trachinotus ovatus* (Ma *et al*., 2014); cobia, *Rachycentron canadum* (Watson *et al*., 2014); chinook salmon, *Oncorhynchus tshawytscha* (Fowler 1981, 1982, 1991); rainbow trout, *Oncorhynchus mykiss* (Alexis *et al*., 1985; Steffens, 1994; Pares-Sierra *et al*., 2014); coho salmon, *Oncorhynchus kisutch* (Higgs *et al*., 1979); European eel, *Anguilla* (Gallagher *et al*., 1988); gilthead seabream (Alexis, 1997; Nengas *et al*., 1999); red sea bream, *Chrysophrys major* (Takagi *et al*., 2000); sunshine bass, *Morone chrysops* x *M. saxatilis* (Webster *et al*., 1999) and red drum, *Sciaenops ocellatus* (Kureshy *et al*., 2000). Even thought, the replacement of fishmeal by PBM in diets for gilthead seabream has been evaluated in a limited number of studies to date.

In the present study, the results obtained shown that PBM can be an alternative protein source to fishmeal in gilthead seabream diets, in accordance with the results obtained previously (Alexis, 1997; Nengas *et al*., 1999; Robaina *et al*., 1997). The

present study showed that fishmeal can be replaced up to 37.5% by PBM without negatively affect growth performances. Despite the slightly reduced growth of fish fed the 37.5% PBM based diet, this is the first study demonstrating that PBM can be efficiently used as a main protein source in gilthead seabream diets, incorporating only 7.5% of fishmeal. Regardless PBM was used to replace both high and moderate protein level fishmeal (70% and 60% protein level fishmeal, respectively), growth of fish in this study, measured as daily growth index (DGI of 2.3-2.1) was within the higher range obtained in other studies with the species, all of them using high quality fishmeal (ranging from 1.0 to 2.5; Couto *et al*., 2016; Dias *et al*., 2009; Peres and Oliva-Teles, 2011), highlighting the possibility to reduce fishmeal incorporation rates in diets of gilthead seabream.

Previously, it was observed that gilthead seabream performed well with diets including up to 50% of PBM, in diets with 35% of fishmeal, while higher replacement levels caused a severed growth retardation (Nengas *et al*., 1999). For other fish species, the viability of poultry by-products in fish diets was found to depend on fish species and size as well as on the nutritional quality of PBM, determined by the quality of raw materials and processing techniques used. For rainbow trout, it was observed that dietary PBM inclusion was beneficial up to 40% of the diet, whereas at 59%, a slightly decrease could be observed (Pares-Sierra *et al*., 2014). Fowler (1991), reported that addition of 20% PBM could replace 50% of the fishmeal in a diet for chinook salmon without any negative effects.

Gallagher (1994) reported that hybrid striped bass fed a diet containing 12% fishmeal and 36% low-ash poultry by-product meal had a weight gain similar to fish fed a diet containing 47% fishmeal (control diet). Steffens (1994) noted that, a diet containing 53% poultry by-product meal, as the only animal protein source, did not cause any significant differences in growth and feed efficiency when compared to an isonitrogenous control fishmeal based diet. Sfeffens (1994) and Yang *et al*. (2004) also reported similar results, showing that poultry by-product meal may, efficiently substitute up to 50% fishmeal protein in diets. Moreover, Booth *et al*. (2012) on Australian snapper (*Pagrus auratas*); Yang *et al.* (2004) on *Macrobrachium nipponense*; Abdel-Warith *et al.* (2001) on African catfish; Gallagher *et al.* (1988) on European Eels; Muzinic *et al.* (2006) on sunshine bass; Saoud *et al.* (2008) on redclaw crayfish (*Cherax quadricarinatus*); Turker *et al.* (2005) on black sea turbot (*Scophthalmus maeoticus*); Saadiah *et al.* (2011) on cobia and Guo *et al.* (2007) on cuneate drum (*Nibea miichthioides*) reported that a dietary fishmeal replacement with PBM up to 50% did not affect growth.

Other studies reported that the replacement level may increase up to 70% of fishmeal without amino acid supplementation, as it was observed for red drum (Kureshy *et al*., 2000). Takagi *et al*. (2000) reported that fishmeal can even be replaced by PBM at a level of 75% or even 100% without a significant depression in fish performance by using a high-quality PBM in red sea bream. Yu (2004) and Yang (2006) also proved that fishmeal can be replaced at a level of 80% by PBM for tilapia, trout, and gibel carp, respectively.

By contrast, a reduction of growth was observed with mirror carp, *Cyprinus carpio*, even at the minimum replacement level tested (12% PBM; Emre *et al.*, 2003). Similarly, decrease of growth performance of Florida pompano were observed when fishmeal was replaced with poultry by-product meal, irrespectively the inclusion level, but dietary supplementation with taurine increased the performance of PBM based diets (Rossi *et al.*, 2012).

Different aspects may contribute for the differences in the maximum dietary replacement level of fishmeal by PBM among the studied species. Of primordial importance is the nutritional quality of PBM that is greatly affected by the quality and combination of raw material, processing techniques used to produce PBM (Forster *et al*., 2006; Rossi *et al*., 2014), resulting in an inconsistent and unpredictable final product, being more variable than between fishmeal. The proportion of bone and so the ash content is also a limiting issue, being, inclusively used to assess the nutritional quality of PBM. Besides the quality of this feedstuff, dietary aspects may also affect the performance of PBM based diet as: unbalanced EEA profile, insufficient dietary essential amino acid and energy content, digestibility and palatability problems, particle size distributing or a combination of these factors is one of the possible reasons for obtaining different results on these parameters related to the fish's growth performance (Forster *et al*., 2003).

When working with an alternative protein source to fishmeal such as PBM, palatability is an extremely important factor. Low diet palatability leads to a decrease in voluntary feed consumption and consequently decrease fish growth performance. In the present study, irrespectively the dietary PBM inclusion level, voluntary feed intake was not affected. Similar results were previously reported for gilthead seabream (Robaina *et al*., 1997). In general, for the majority of fish species it was observed a decrease in voluntary feed intake with the high dietary PBM inclusion level. This is the case of Australian snapper (Booth *et al.*, 2012), gilthead seabream (Nengas *et al.*, 1999), African catfish (Abdel-Warith *et al.*, 2001), gibel carp (Yang *et al.*, 2004), juvenile red drum

(Kureshy *et al.*, 2000), mirror carp (Emre *et al.*, 2003), Florida pompano (Rossi *et al.*, 2012), sunshine bass (Muzinic *et al.*, 2006) and redclaw crayfish (Saoud *et al.*, 2008). Contrarily, other authors reported no effect on feed intake (Gou *et al*., 2007), while other reported inclusively an increase in feed intake as it was observed for gibel carp (Yang *et al*., 2004). One of the possible causes for the reduction of voluntary feed intake in PBM based diets is related to its content in saturated fatty acids. The increased concentration of saturated fatty acids in the fish diet may contribute to the reduction of palatability of these diets for fish (Rodriguez-Sena *et al*., 1996; Robaina *et al*., 1997). However, that was not the case of the present study, as it was used a mixture of both fish and poultry oil (50/50) as main lipid sources, and so the incorporation up to 37.5% of PBM had little contribution to the final dietary saturated fatty acid level. The effect of dietary inclusion of poultry oil to replace fish oil has not been investigated in as much detail as fish oil replacement by vegetable oils (Castro, 2016).

The present study show that fishmeal can be replaced by including up to 37.5% of PBM without negatively affect feed utilization, measured as feed efficiency, protein efficiency ratio and nitrogen retention. As fish composition is related to diet composition, protein and essential amino acid retention have been considered the most sensitive indicators of an inadequate supply of amino acids (Rodehutscord *et al*., 1995), suggesting a good protein and EEA profile of all the experimental diets regardless of the PBM inclusion level. Similarly results were previously reported on gilthead seabream (Robaina *et al*., 1997). Some authors observed in some fish species no effect on feed efficiency with the dietary PBM inclusion level. This is the case of sunshine bass (Webster *et al*., 2000), gibel carp (Yang *et al*., 2006), African catfish (Abdel-Warith *et al*., 2001), striped bass (Rawles *et al*., 2006) and Malabar grouper (Li *et al*., 2009). Others, reported in some fish species a significant decrease in feed efficiency with the dietary PBM inclusion indicating that feed utilisation was reduced. This is the case of Australian snapper (Booth *et al*., 2012), gilthead seabream (Nengas *et al*., 1999), redclaw crayfish (Saoud *et al*., 2008), Florida pompano (Rossi *et al*., 2012), African catfish (Goda *et al*., 2007), cuneate drum (Guo *et al*., 2007), sunshine bass (Muzinic *et al*., 2006), mirror carp (Emre *et al*., 2003), black sea turbot (Turker *et al*., 2005) and cobia (Saadiah *et al*., 2011).

In line with the effect of PBM meal in feed efficiency, the digestibility of dry matter, protein, phosphorus and energy was also not affected by the increased replacement level of fishmeal by PBM. However, faecal nitrogen losses were increased whatever the replacement level. The reduction of protein digestibility from 92.8% to 89.7% (control and 35.7PBM diets, respectively) suggests that protein digestibility of the ingredient was

around 83%. This is in accordance with previous reports for gilthead seabream, which evaluated the digestibility of poultry meal to be around 80% (Nengas *et al*., 1995; Lupatsch *et al*., 1997). For other fish species, relatively lower values have been reported such as for turbot (Wei *et al*., 2015); rainbow trout (Alexis *et al*., 1985), chinook salmon (Fowler 1991), cobia (Zhou *et al*., 2004) and red drum (Gaylord *et al*., 1996). Digestibility of PBM varies considerably depending of the quality of the raw material and processing conditions, including particle size, temperature of heating, drum drying or spray drying. Also, the quantity of bone may affect significantly the digestibility and the reduction of the ash fraction is an essential approach to develop high digestible/low-pollution feeds (Riche *et al*., 1999; Sugiura *et al*., 2000; Jahan *et al*., 2003). In the present study, ash content of PBM (13%) was higher than that of high protein fishmeal (9%), but lower than that of moderate protein fishmeal (22%).

Enzymatic investigations are necessary to clarify the effect of fishmeal replacement by PBM, as any changes in digestive enzyme activity influence digestion and absorption of food (Lemieux *et al*., 1999). Several factors may affect digestive enzyme production in fish, such as feeding habits, food preferences, diet formulations and anti-nutritional factors (ANFs) (Pavasovic *et al*., 2007). In fish, nutrient absorption is known to take place in anterior intestine and, to a lower extent, in the posterior intestine (Gai *et al*., 2012). To the best of our knowledge, there are no studies regarding the effect of dietary fishmeal replacement by PBM on the activity of digestive enzymes. In the present study, the activity of all digestive enzymes tested show no significant differences among treatments. This indicates that the five experimental diets where PBM levels progressively increased, have a similar effect on enzymatic activity as the control diet, which completely lacks PBM. These results suggest that the reduction of the apparent digestibility coefficient of PBM based diets was probably due to differences in chemical composition and physical properties of the digesta rather than a reduction on the production of the digestive enzymes.

In relation to the intestine portion, with exception of proteases, the remaining four enzymes have shown higher enzymatic activity in the anterior intestine than in posterior intestine. Digestive enzymes that are synthesized in exocrine pancreas are secreted into the anterior intestine, being consider with higher digestive and absorptive action than in the more distal portion of intestine (Buddington *et al*., 1987; Bakke *et al*., 2010). Therefore, the higher activity of digestive enzymes in the posterior intestine may be a consequence of a possible drag of secreted mucous, as was reported previously (Magalhães *et al*., 2015; Gai *et al*., 2012; Pérez-Jiménez *et al*., 2009).

At the end of growth trial, whole-body composition was unaffected by the increase of PBM inclusion level in the experimental diets. Previously, it was observed that for this species, the dietary inclusion of PBM lead to a significant reduction of whole body-lipid content (Nengas *et al*., 1999) and an increase of ash content (Robaina *et al*., 1997). Turker *et al.* (2005) also reported significantly lower lipid content of black sea turbot fed diets with 25% PBM. Inversely, other authors reported an increase of body lipid content with the dietary inclusion of PBM (Gouveia, 1992); Goda *et al.*, 2007; Yang *et al.*, 2006; Robaina *et al.*, 1997 and Guo *et al.*, 2007).

Most of the works reviewed have evaluated FM replacements from biological or nutritional viewpoints. Little attention has been paid to the economic analysis of these protein sources. The cost of diet formulation decreased with the dietary inclusion of PBM, resulting in a lower economic efficiency ratio. Diet PBM 37.5 (0.98€) was less expensive than PBM 0 diet (1.22€). Therefore, on an economic basis, 37.5% inclusion of PBM was feasible and less expensive, with the same economic profit index. The cost-benefit analysis of the present study indicated that PBM diets were better protein sources and less expensive than fishmeal.

Conclusion

In conclusion, present study confirms the suitability of PBM to replace fishmeal in diets to gilthead seabream, highlighting the importance of using good PBM quality. Under present conditions, up to 37.5% of PBM can be incorporated in practical diets, reducing the dietary fishmeal level to 7.5%, without affecting growth performance, digestibility, feed and protein utilization of gilthead seabream juvenile. Economic analysis revealed that PBM diets were less expensive and have a higher economic efficiency ratio and similar economic profit index than fishmeal basis diets.

Overall, present results indicated that, PBM is a good nutritive value ingredient and may became a valuable alternative protein source for gilthead seabream and for other marine carnivorous species. Therefore, the potential of using PBM in aquafeeds seems very promising.

However, further investigations are required aiming to maximise the incorporation of PBM in the diets, including a deeper characterization of its nutritional profile, as the study of the AA digestibility and fatty acid composition, as well as the fine-tuning of the diet formulation by adjusting the digestible EAA profile of the diets. Still, further investigations are required to determine the modulation effect of the dietary inclusion of PBM on other important aspects, such as the immunity and health status of the animal. Moreover, the analysis of the impact of fishmeal replacement by PBM on the body and fillet quality traits is need, particularly with large gilthead seabream.

This study contributes proactively to the development of a more sustainable aquafeed production. It also contributes to increase the fundamental nutritional know-how regarding the effects of dietary incorporation of processed animal by-products, allowing its reintroduction in the feed chain and so contributing to the reduction of the environmental footprint of livestock production.

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