

SC

2.^o
CICLO

FCUP
UPV
2015

U.
PORTO

Potential use of meat and bone meal in diets for
gilthead seabream (*Sparus aurata*) juveniles

Sara Patrícia Pinto Moutinho

FC

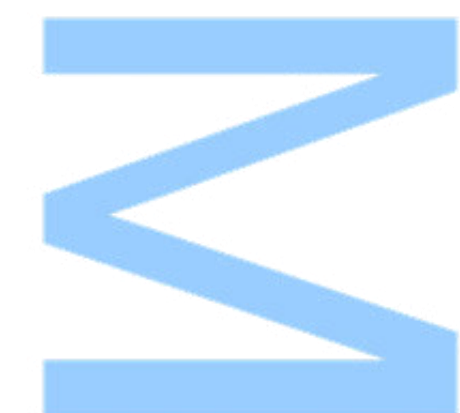
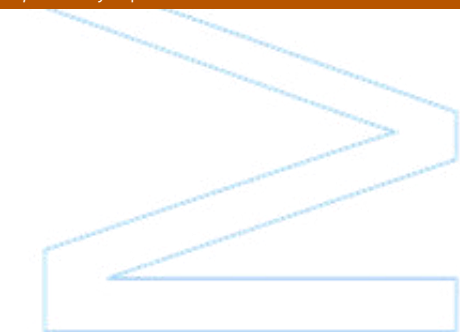
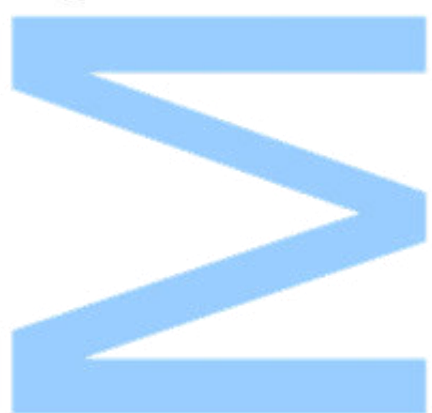


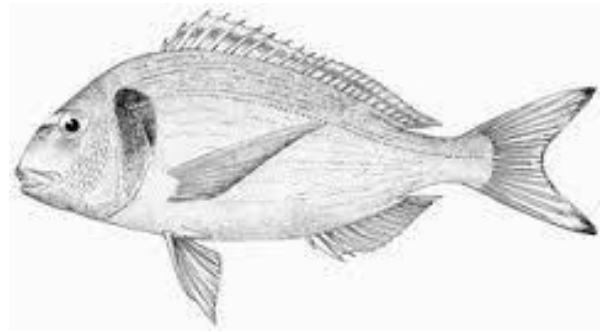
Potential use of meat and bone meal in diets for gilthead seabream (*Sparus aurata*) juveniles

Sara Patrícia Pinto Moutinho

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto, Universitat
Politécnica de València
Recursos Biológicos Aquáticos

2015





Potential use of meat and bone meal in diets for gilthead seabream (*Sparus aurata*) juveniles

Sara Patrícia Pinto Moutinho

Mestrado em Recursos Biológicos Aquáticos
Departamento de Biologia
2015

Orientador

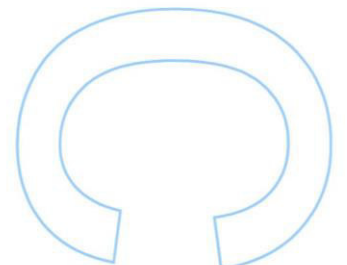
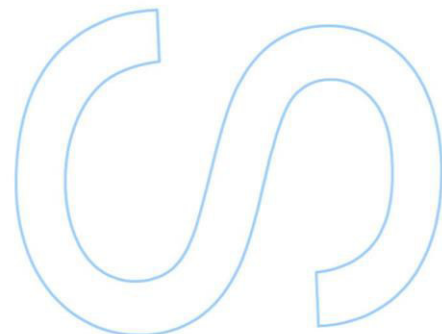
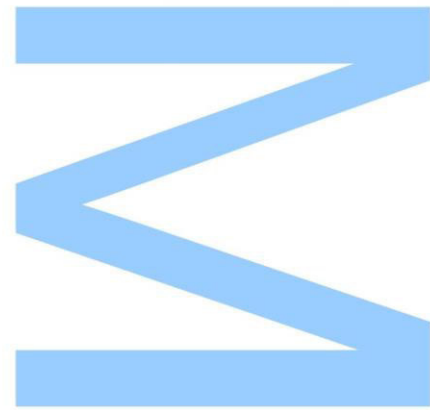
Doutora Helena Peres, Investigadora Auxiliar, CIIMAR

Coorientador

Professor Miguel Jover Cerdá, Professor Catedrático, UPV

Coorientador

Doutora Silvia Martínez-Llorens, Investigadora, UPV

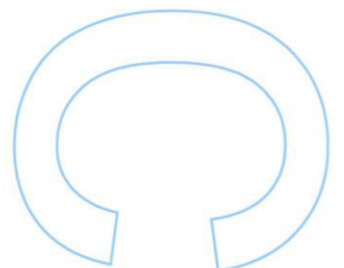
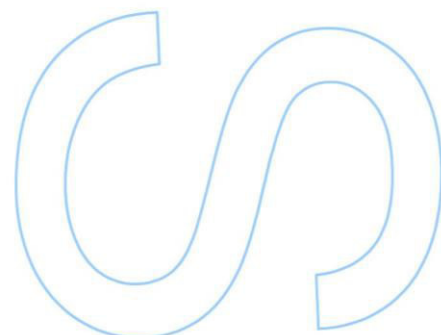
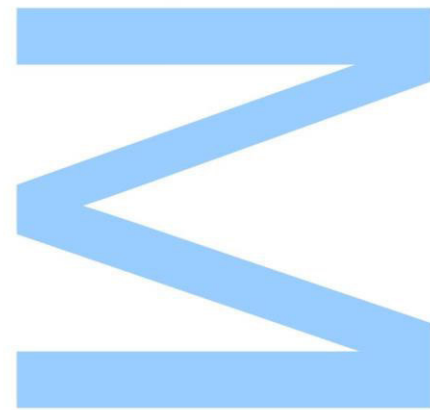




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____ / ____ / ____



Acknowledgements

First of all, I would like to express my sincere gratitude to Prof. Dr. Aires Oliveira-Teles, not only for accepting me as a Master student and giving me the opportunity to begin my journey in this field but also for all his support, advice and scientific knowledge shared during this year.

Secondly, I would like to thank Prof. Miguel Jover Cerdá, of the Polytechnic University of Valencia, for accepting and allowing me to develop my thesis in collaboration with the GAB research group.

A very special thank you to Prof. Silvia Martínez-Llorens whose care, guidance and knowledge ensured the success of this work. I must also thank Prof. Ana Tomás Vidal, Raquel, Ana, Guillem and fellow colleagues at UPV for helping me whenever needed.

To “La Résistance”, a very kind thank you for the friendship that made my stay in Valencia a memorable experience.

A very special thank you to Cláudia Serra, for the dedication and patience and whose teachings were essential to make this work possible. To all my colleagues at NUTRIMU, thank you.

I would especially like to express my gratitude towards Dra. Helena Peres, as I am sure that without her tremendous support, encouragement, knowledge, dedication, and kindness, none of this would have been possible. I would especially like to thank her for the recognition in my work, motivating me to do better and pursue my goals.

Finally, I would like to show my sincere appreciation to my dear friends, family and my parents, Luciano e Maria, for all the love and support and for always being there for me whenever I needed the most and also to Gaspar, for being there in the final stages of my work. To everyone who, in some way, contributed to the success of this thesis, thank you.

Abstract

Fishmeal (FM) has been the preferred protein source for aquafeeds, in particular for carnivorous species. However, current FM inclusion levels threaten the expansion of the intensive production of those species. In this context, research has been focusing on evaluating more cost-effective and sustainable alternative ingredients to FM. Meat and bone meal (MBM) is a desirable product for carnivorous fish diets as it generally possesses a high protein content, relatively balanced amino acid profile, high digestibility and palatability and lacks anti-nutritional factors. Also, it is produced worldwide with a steady availability. Recent unban of the use of these ingredients inside the European Union emphasizes the necessity to evaluate it as new potential ingredient for FM replacement. Gilthead seabream (*Sparus aurata*) is an important economic species in Mediterranean aquaculture but overproduction, associated with increasing price of feeds, led to a decrease in profitability of the intensive production of this species. Therefore, present study aimed to evaluate FM replacement with MBM on growth, digestibility, feed efficiency utilization and gut microbiota of gilthead seabream juveniles. Three experimental diets were formulated (45% CP; 20% CL): a control diet (FM100), with FM as the main protein source, and MBM50 and MBM75 where FM was replaced at 50% and 75%, respectively. Triplicate groups of juvenile gilthead seabream (25 ± 0.72 g) were fed for 83 days with the experimental diets. A 50% substitution did not significantly affect growth (DGI of 2.48 and 2.51 for FM100 and MBM50 diets, respectively), feed utilization efficiency (FCR of 1.51 and 1.53; PER of 1.51 and 1.50, for FM100 and MBM50 diets, respectively). However, a 75% substitution led to a significant decrease on growth rate (DGI of 2.25) and feed utilization (FCR of 1.72; PER of 1.29), although feed intake ($\text{g kg ABW}^{-1} \text{ day}^{-1}$) was significantly higher (26.1 compared to 24.2 for diet MBM50). Whole-body composition was mostly unaffected by the experimental diets with the exception of lipid and energy content, which were significantly lower in fish fed the diet MBM75. Protein and essential amino acid retention were unaffected by the experimental diets while lipid and energy retention were significantly reduced with the increase of FM substitution. Crude protein digestibility was high (>89%) and unaffected by the experimental diets while energy digestibility was significantly higher for diet MBM50 (95.2 %), compared to the control (82 %). ADCs of essential amino acids were high (>92%) for all experimental diets and statistically similar or higher for diet MBM50, compared to the control diet, but lower for MBM75 when compared to MBM50.

MBM significantly modulated gastrointestinal microbiota with a decrease in operational taxonomic units (OTUs) and species richness but an increase in replicate similarity with increasing MBM inclusion rate. MBM appeared to promote the development of *Vibrio*, *Bacillus* and *Mycobacterium* genera while colonization by *Staphylococcus* and *Corynebacterium* genera appeared to decrease. Overall, results indicate that half of FM could be replaced by MBM, in diets for gilthead seabream juveniles, without compromising growth performance and feed utilization with good results in nutrient and EAA digestibility and retention. Further studies are required to study fatty acid profile, digestibility and retention, and the effect of dietary MBM inclusion on general intestine health fish, fish wellbeing and immune status as well as on flesh quality traits of gilthead seabream.

Keywords: Gilthead seabream; alternative protein sources; meat and bone meal; amino acids; digestibility; microbiota

Resumo

A farinha de peixe (FP) é a principal fonte proteica em dietas para aquacultura, em particular para espécies carnívoras. No entanto, os atuais níveis de incorporação da FP ameaçam a expansão da produção aquícola destas espécies. Sendo assim, a investigação tem centrado esforços no estudo de novos ingredientes, alternativos à FP, economicamente mais viáveis e sustentáveis. A farinha de carne e osso (MBM) é um ingrediente com elevado potencial para incorporação em dietas para peixes carnívoros, dado o seu elevado teor em proteína, perfil de aminoácidos relativamente equilibrado, elevada digestibilidade, boa palatabilidade e ausência de fatores anti-nutricionais. Para além disso, a MBM é produzida mundialmente e com disponibilidade contínua. A recente reautorização do uso destes ingredientes na alimentação de peixes na União Europeia salienta a necessidade da avaliação do seu potencial como alternativa à FP, em espécies produzidas na Europa. A dourada (*Sparus aurata*) é uma espécie de grande importância económica na aquacultura Mediterrânica mas o excesso de produção, associada ao aumento do preço das dietas, levou a uma diminuição na rentabilidade da produção intensiva desta espécie. Neste contexto, o presente estudo teve como objetivo avaliar o efeito da substituição da FP por MBM no crescimento, digestibilidade, eficiência de utilização do alimento e microbiota gastrointestinal de juvenis de dourada. Foram formuladas 3 dietas experimentais, com 45% de proteína bruta e 20% de lípidos totais, fazendo variar a taxa de incorporação da MBM: dieta controlo (FM100), com FP como

a principal fonte proteica, e MBM50 e MBM75 onde FP foi substituída em 50 e 75%, respetivamente. Cada uma das dietas foi fornecida, em triplicado, a grupos de juvenis de dourada (peso médio inicial 25 ± 0.72 g), durante 83 dias. A substituição de 50% da FM por MBM não afetou significativamente o crescimento (DGI de 2.48 e 2.51 para as dietas FM100 e MBM50, respetivamente) ou a eficiência de utilização do alimento (FCR de 1.51 e 1.53; PER de 1.51 e 1.50 para as dietas FM100 e MBM5, respetivamente). No entanto, uma substituição de 75% da FP acarretou uma diminuição significativa da taxa de crescimento (DGI de 2.25) e da utilização do alimento (FCR de 1.72; PER de 1.29), apesar da ingestão voluntária de alimento ($\text{g kg ABW}^{-1} \text{ day}^{-1}$) ter sido significativamente maior (26.1 comparado a 24.2 para a dieta MBM50). A composição corporal não foi, de uma forma geral, afetada pelas dietas experimentais, com a exceção do teor em lípidos e energia, que foram significativamente mais baixos nos peixes alimentados com a dieta MBM75. A eficiência de retenção proteica e aminoacídica não foi afetada pelas dietas experimentais enquanto a lipídica e energética foram significativamente reduzidas com o aumento da substituição da FP. O coeficiente de digestibilidade aparente da matéria seca, proteína, lípidos energia e aminoácidos foi avaliada através de um ensaio de digestibilidade. A incorporação de MBM não alterou significativamente a digestibilidade da proteína, que foi elevada ($> 89\%$), mas aumentou a digestibilidade da energia, sendo esta significativamente maior para a dieta MBM50 (95.2%), comparativamente ao controlo (82%). Os coeficientes de digestibilidade aparente dos aminoácidos essenciais foram elevados ($> 92\%$), para todas as dietas experimentais e estatisticamente semelhantes ou superiores para a dieta MBM50, comparativamente ao controlo, mas mais baixas para a dieta MBM75 quando comparadas à MBM50.

A incorporação de MBM nas dietas modulou significativamente o microbiota gastrointestinal, verificando-se um decréscimo em unidades taxonómicas operacionais (OTUs) e riqueza de espécies. A inclusão de MBM parece promover o desenvolvimento das bactérias dos géneros *Vibrio*, *Bacillus* e *Mycobacterium*, enquanto a colonização pelos géneros *Staphylococcus* e *Corynebacterium* diminui. De um modo geral, estes resultados indicam que metade da FP pode ser substituída por MBM, em dietas para juvenis de dourada, sem comprometer a desempenho de crescimento, eficiência de utilização do alimento, digestibilidade e retenção dos nutrientes e energia. Contudo, futuros estudos são necessários para avaliar o efeito da inclusão de MBM em dietas na saúde intestinal, bem-estar e estado imune de douradas, bem como avaliar a sua repercussão na qualidade da carne da dourada.

Palavras-chave: Dourada; fontes proteicas alternativas; farinha de carne e osso; aminoácidos; digestibilidade; microbiota

Contents

Abstract	1
Resumo	2
Tables list	6
Figure list	6
Abbreviations	7
Introduction	8
The State of Aquaculture	8
The State of European and Mediterranean Aquaculture	9
Gilthead seabream (<i>Sparus aurata</i> L.)	10
Biology	10
Aquaculture production	11
Feed formulation in aquaculture	12
Ingredients for aquafeeds: current situation	14
Plant protein sources in gilthead seabream	16
Animal by-products protein sources	17
The use of animal by-products in aquaculture	19
Objectives of this study	20
Materials and methods	24
Diet composition	24
Growth trial	25
Fish sampling	26
Sampling for GIT microbiota analyses	26
Digestibility trial	26
Chemical analyses	27
Amino acid determination	27
PCR-DGGE (Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis)	28

DNA extraction from GIT samples	28
Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE)	28
Data and statistical analyses	29
Ethics statements	30
Results	30
Growth trial: performance and feed utilization efficiency	30
Digestibility trial: ADC (%) of diets and amino acids	31
Whole body composition and biometric parameters	32
Nutrient and amino acid budget.....	32
Modulation of gilthead seabream gut microbiota	36
Discussion	41
Conclusion.....	48
References	50

Tables list

Table 1: Studies performed to evaluate fishmeal replacement with different animal by-products ingredients in gilthead seabream.....	19
Table 2: Studies performed with several farmed species using animal by-product ingredients for fishmeal replacement.	21
Table 3: Composition and proximate analysis of the experimental diets.....	24
Table 4: Amino acid composition (g 16 g ⁻¹ N) of the experimental diets and EAA requirement of gilthead seabream (g 16 g ⁻¹ N; Peres and Oliva-Teles 2009).....	25
Table 5: Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets.....	30
Table 6: Apparent digestibility coefficients (ADC, %) of the experimental diets.....	31
Table 7: Digestible amino acids content (g 100 g ⁻¹ DM) of the experimental diets	32
Table 8: Whole-body composition and biometric parameters of gilthead seabream fed the experimental diets.....	33
Table 9: Nitrogen, lipid and energy budget of gilthead seabream fed the experimental diets.....	34
Table 10: Amino acid budget of gilthead seabream fed the experimental diets	35
Table 11: Ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in stomach (STO) and intestinal sections (AI, anterior intestine; MI, middle intestine; PI, posterior intestine) recovered from gilthead seabream fed the experimental diets.....	39
Table 12: Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the GIT communities of gilthead seabream fed the experimental diets.	40

Figure list

Fig. 1: World capture fisheries and aquaculture production. Source: FAO (2014).....	8
Fig. 2: Total aquaculture production in the European Union. Source: EUMOFA (2014)	10
Fig. 3: Gilthead seabream specimen (<i>Sparus aurata</i> , L.). Source: FAO	11

Fig. 4: Retention (%) of ingested essential amino acid in gilthead seabream fed the experimental diets..... 34

Fig. 5: PCR-DGGE fingerprints of the microbiota found in stomach and intestinal sections recovered from gilthead seabream fed the experimental diets. Black numbers on top of the figure represent the different samples analyzed (from a pool of two fish each) while red numbers inside the figure correspond to bands removed for sequencing, which results are presented in Table 12..... 37

Fig. 6: Dendrograms and PCR-DGGE fingerprints of the microbiota found in stomach and intestinal sections recovered from gilthead seabream fed the experimental diets. 38

Abbreviations

AA: Amino acid	FeM: Feather meal
ADC: Apparent digestibility coefficient	FM: Fishmeal
AI: Anterior intestine	FW: Freshwater
BM: Blood meal	GIT: Gastrointestinal tract
BSE: Bovine spongiform encephalopathy	MBM: Meat and bone meal
CF: Crude fiber	MI: Middle intestine
CGM: Corn gluten meal	MM: Meat meal
CL: Crude lipid	MT: Million tones
CP: Crude protein	NEAA: Non-essential amino acid
DGGE: Denaturing Gradient Gel	NFE: Nitrogen free-extract
Electrophoresis	OTUs: Operational taxonomic units
DM: Dry matter	PAP: processed animal proteins
EAA: Essential amino acid	PBM: Poultry-byproduct meal
EU: European Union	PCR: Polymerase Chain Reaction
FA: Fatty acid	PI: Posterior intestine

Introduction

The State of Aquaculture

The ever growing human population and its inherent growing need for food has been putting pressure on natural fish stocks and is causing doubts about a sustainable future of seafood for human consumption. Fish global per capita consumption has increased from 9.9 kg in 1960 to 19.5 kg in 2012 (FAO 2014) and fisheries captures alone will not be able to meet the expected demand of seafood in the future. In fact, 28.8% of fish stocks were estimated to be overexploited by the fishery industry in 2011 (FAO 2014). Although it was reported that total capture fisheries reached a second all-time high of 93.7 million tons (MT) in 2011 (93.8 MT in 1996), overall, these numbers only represent a relatively stable situation that has been seen in the last decade, where total capture fisheries values have revolved around 90 MT (**Fig. 1**) (FAO 2012). In particular, marine captures have decreased from 82.6 MT in 2011 to 79.7 MT in 2012 (FAO 2014).

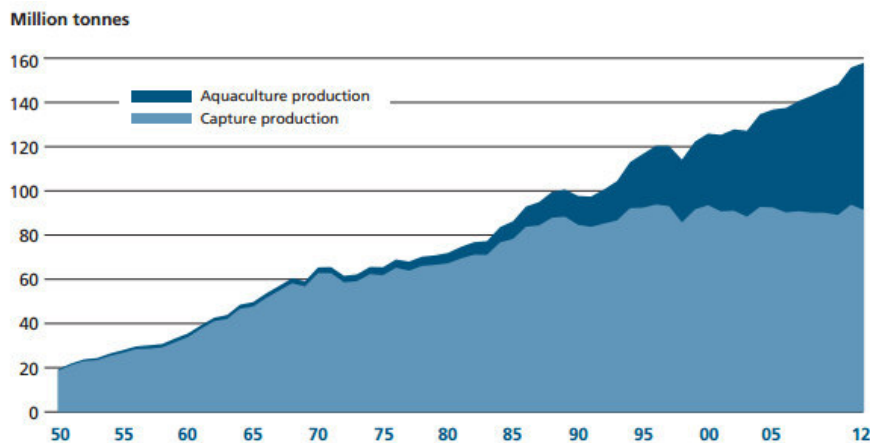


Fig. 1: World capture fisheries and aquaculture production. Source: FAO (2014)

Aquaculture plays now, more than ever, an important role in providing global food security by meeting the increasing demand while alleviating the negative effects of fishing. According to Haylor and Bland (2001), aquaculture is “the farming of aquatic organisms in inland and coastal areas, involving intervention in the rearing process to enhance production and the individual or corporate ownership of the stock being cultivated”. This production includes diverse practices and a wide range of farmed species, systems and techniques, where more than 567 species are currently being farmed, 354 of which are finfish (FAO 2014). The aquaculture industry also plays a vital role in reducing poverty as it creates employment in many underdeveloped and

developed countries and allows preservation of the natural ecosystems and improves environmental sustainability.

Historically, this type of farming has been practiced in many forms and degrees since it was firstly documented in China, with the freshwater carp, in 2000 B.C. As scientific knowledge and technologies develop, aquaculture became more efficient with the increase of intensive production systems. In fact, producers have gone from obtaining wild seeds of juveniles from their natural environment to a complete, closed production, contributing this way to sustain natural fish stocks.

Currently, aquaculture is considered to be the fastest growing food production sector (Yang et al. 2006), reaching an all-time high in 2012 with the total production of 90.4 MT, more than doubling since 2000 (32.4 MT) (FAO 2014). Despite the positive growing trend, it appears that aquaculture production is stagnating as it expanded more slowly in the period 2000-2012 (6.2%) than in the periods 1980–1990 (10.8%) and 1990–2000 (9.5%) (FAO 2014). Nevertheless, world food fish aquaculture production grew at an average rate of 6.2% in the period 2000-2012, but lower than in the period 1990-2000 at 9.5%, and it was estimated that in 2013, total production reached 70.5 MT of food fish, corresponding to an increase of 5.8% (FAO 2014). Compared to capture fisheries, farmed food fish worldwide contributed a record 42.2% of a total of 158 MT of fish produced in 2012 by both sectors (FAO 2014). However, these values are the result of an uneven production as, in 2012, Asia alone accounted for 88% of total aquaculture production, with 43.5 MT of food fish produced in 2013. The European Union (EU) only represented a small percentage of global production, corresponding to 4.3% (2.88 MT) in 2012 (FAO 2014) and it is still largely dependent on imports of fish and fishery products, showing production constrains (Karapanagiotidis 2014).

The State of European and Mediterranean Aquaculture

In the last 15 years, marine aquaculture became more intensive as a need to compensate for the stagnating capture fisheries, and was possible due to new technologies, expansion of suitable sites, improvement in feed technology, better knowledge of farmed species biology, increased water quality within closed farming systems and increase demand for seafood products (Read and Fernandes 2003). In 2011, the EU was the fifth largest fisheries and aquaculture producer worldwide, with a volume of 1.24 MT of aquaculture products, more than 20% of total EU fisheries production (EUMOFA 2014). Increase in finfish production due to diversification of farmed species allowed an increase in annual growth rate of 13% in 2000, compared to

only 4% in 1980 (IUCN 2007). However, despite the initial growth, in 2011, the volume of farmed products in the EU was the lowest registered since 2003 (**Fig.2**) (EUMOFA 2014). In 2011, Spain was the Member State with the highest volume of farmed products (274 225 tones), which represented an increase of 8% relatively to 2010, followed by France, United Kingdom, Italy and Greece (EUMOFA 2014).

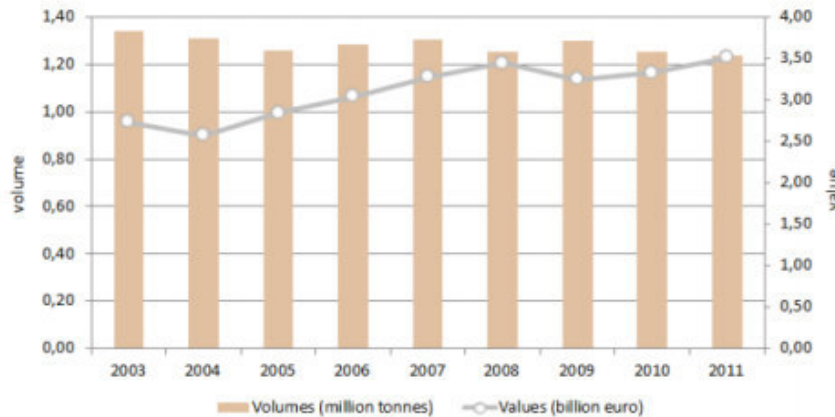


Fig. 2: Total aquaculture production in the European Union. Source: EUMOFA (2014)

Most recent data point that, in 2013, Portugal's total aquaculture production was of 9955 tones, providing a revenue of around 54 million euros, representing a 9% decrease in volume, mainly due to a reduction in turbot's production, but a 3.1% increase in revenue compared to 2012 (INE 2015). Finfish production in marine and brackish waters accounted for 41.9% of total production where 85% of those refer to gilthead seabream and turbot production (INE 2015). Also, in the EU, gilthead seabream was the sixth most produced aquaculture species, after salmon, trout, oyster, mussel and carp, representing 5.8% of the total European aquaculture production in 2011 (EUMOFA 2014).

Gilthead seabream (*Sparus aurata* L.)

Biology

Sparus aurata (Linnaeus, 1758) (**Fig. 3**) is a perciform fish that belongs to the family Sparidae. It possesses a relatively deep and compressed oval body, with thick lips and small eyes, and a generally curved head profile. The overall body color is silver-grey with a big dark blotch at the beginning of the lateral line that extends to the upper part of the opercular bone. The edge of the fork and caudal fins are black. This species also possesses a characteristic golden colored bar between the eyes, always narrower in the central part (Basurco et al. 2011).

This species is commonly found in the Mediterranean Sea but less frequently in the Eastern Atlantic coasts from Great Britain to Senegal and rarer in the Black Sea (Moretti et al. 1999). It has a demersal behavior and inhabits sea grass beds and sandy bottoms; young gilthead seabreams can be found at low depths (up to 30 m) while adults can occur in deeper waters (up to 150 m), living in solitary or in small schools (Basurco et al. 2011). Due to its euryhaline nature during the early stages of its life cycle, this species can also be found in brackish waters, such as coastal lagoons and estuaries. However, it is sensitive to low temperatures, 4 °C being the lethal minimum.

Gilthead seabream is a mainly carnivorous species, feeding of crustaceans and mollusks, as well as polychaetes, some teleost fish and echinoderms, but can be accessorially herbivorous (Wassef and Eisawy 1985). They are considered opportunist feeders, where they adapt their diets according to local availability and accessibility, and temporal variation (Pita et al. 2002). Regarding reproductive biology, this species is considered to be a protandric hermaphrodite, meaning that juveniles reach sexual maturity as males (during the first 2 years of life) and then become sexually mature females at sizes over 30 cm of length. Spawning occurs from December to April, during which the pelagic eggs hatch at open sea and, in early spring, juveniles migrate towards coastal areas where there is abundant food, protection and milder temperatures. In late autumn, they return to open sea to breed.



Fig. 3: Gilthead seabream specimen (*Sparus aurata*, L.). Source: FAO

Aquaculture production

Gilthead seabream is a species of great economic value for the Mediterranean aquaculture industry (Nengas et al. 1999; Libralato and Solidoro 2008). It has shown great adaptability to all kinds of farming systems and displays a homogenous growth under culture conditions (Montero et al. 2009). Historically, this species was extensively cultured in Mediterranean coastal lagoons and saltwater ponds such as the Italian “valli”

(Basurco et al. 2011) or the Egyptian “hosha”, which are natural traps that take advantage of the juveniles’ trophic migration from the sea to coastal lagoons. For a very long time, marine rearing of this species depended on the collection of wild juveniles and it was only up until the 1980’s that intensive fish rearing systems were developed, mostly due to successful artificially breeding techniques derived by a shortage of fry and juveniles, establishing the beginning of mass production of gilthead seabream (Moretti et al. 1999). Since then, this species has become one of the main products of European aquaculture.

Currently, grow out of gilthead seabream is performed on floating cages at open sea while most of the reproduction and growth phase is in intensive land systems (Merinero et al. 2005). In 2011, the EU produced around 74 000 tons of gilthead seabream, providing a revenue of 370 million euros. However, this represented a decrease of 19% in volume and 6% in value when compared to 2010 (EUMOFA 2014). Greece was the largest contributor, responsible for 67% of all volume produced. In 2013, Portugal reported a production of 1 201 tons of gilthead seabream (INE 2015).

Improvements of rearing techniques in the last several years, such as feeding systems automation, harvesting procedures and health management, have resulted in an overproduction of gilthead seabream that is having a toll on prices in the main European markets (Flos et al. 2002). From 1996 to 2005, production in Mediterranean countries rose from 30 000 tones to 90 000 tones (Martínez-Llorens et al. 2008) and this, associated with the decrease in sale price, has forced farmers to control the production costs in order to improve profitability (Merinero et al. 2005).

Feed formulation in aquaculture

About 40% of total aquaculture production is dependent on the supply of exogenous feeding (Deutsch et al. 2007). This is known as intensive aquaculture production and allows producers more control over the quality of the final product and more control over the culture conditions. From the period 1995-2008, the industrial aquafeed production increased more than threefold, as a consequence of the increase in intensive aquaculture, growing from 7.6 MT to 29.2 MT, at an average rate of 11% per year, and it is expected to reach 71 MT by 2020 (FAO 2011). Currently, the challenge in fish nutrition research is the formulation of sustainable diets, less dependent on marine ingredients that support maintenance, growth, reproduction, health and well-being of the animal, at a reduced cost, while providing food with a good nutritional value for humans.

Compared to terrestrial animals, aquaculture feeds possess a wide range of nutritional composition where ideal values in nutrients vary among the different species and life stages, and according to other factors such as production and environmental constraints, markets or manufacture's preferences and economic climate (Bureau 2006). Marine fish, due to developing in an aquatic environment where carbohydrates sources are scarce, have a digestive and metabolic system better adapted to use protein and lipids as energy source (Lovell 1998), which is required for life-sustaining processes, such as maintenance, movement, and tissue synthesis. Currently, commercial feeds for marine carnivorous fish contain between 40-50% protein and 12-26% lipids (Cerdá 2012). Fish also seem to use protein more efficiently than terrestrial animals, due to a more effective nitrogen excretion through the gills that requires much less energy than excretion as urea or uric acid (Lovell 1998). Fish, like other animals, do not have a true protein requirement but have a requirement for a well-balanced mixture of essential (EAA) and non-essential amino acids (NEAA). Amino acids are the structural components of proteins and are used, among other functions, to synthesize new protein and new muscle and a balanced amino acid profile is essential for fish growth and wellbeing (NRC 2011).

Dietary incorporation of lipids and carbohydrates is important to promote dietary protein sparing, so that protein is solemnly used for muscle growth instead of energetic purposes, as protein is considered to be most expensive component in a feed (Forster and Dominy 2006). It is important to note that the metabolic capacity to use either of these non-protein energetic components differs among fish species and feeding habits and environments, being necessary knowledge on the species' specific ability of its utilization.

Also, feeding is considered to be one of the main factors that can significantly modulate the gut microbial community in farmed fish (Estruch et al. 2015). The gastrointestinal tract (GIT) is a complex system not only involved in digestion and nutrient absorption but also in the animal's immune response and disease resistance (Cerezuela et al. 2012), which are largely influenced by the GIT microbiota (Silva et al. 2011). Bacteria are the most commonly found microorganisms in the GIT of fish (Rawls et al. 2004; Nayak 2010) with colonization beginning in the larval stages and consequent establishment, composition, and diversity being modulated through the rest of the fish's life cycle by many endogenous and exogenous factors including habitat, environmental/culture conditions, age and diet (Nayak 2010; Tapia-Paniagua et al. 2010). Therefore, it is important to study how dietary manipulations can modulate the diversity and composition of the GIT microbiota and its relationship with the host. This

information might be used as a strategy to improve nutrition but also as a way to prevent diseases (Navarrete et al. 2009) since a disruption in this delicate balance may lead to alterations in immune regulatory response, increasing disease susceptibility or reducing functionality (Perez et al. 2010; Dimitroglou et al. 2011; Tapia-Paniagua et al. 2011).

Up to this point, little is known about the diversity and functional role of gilthead seabream GIT microbial communities (Kormas et al. 2014). Most of the studies performed so far on several fish species are based on culture dependent methods which are laborious and time consuming (Tapia-Paniagua et al. 2010), and do not allow a true evaluation of the microbiota diversity and composition, as many bacteria species are unculturable (Navarrete et al. 2009; Feng et al. 2010). The devolvement of molecular culture-independent approaches, such as PCR-DGGE (Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis), has allowed a more complete and rapid assessment of the composition, diversity and functional relationships of the microbiota in fish GIT (Clements et al. 2014). PCR-DGGE is based on the amplification of equally sized PCR products of a hypervariable region of a highly conserved bacterial gene (V3-region of the 16S rRNA gene), which are then separated by electrophoresis on a denaturing gradient gel, based on their differential denaturation profile, i.e. their differential polymorphisms (Ercolini 2004). These DNA fragments can then be further analyzed, through sequencing of the excised gel bands and subsequent identification (Hovda et al. 2007).

Ingredients for aquafeeds: current situation

In order to formulate adequate feeds for farmed species, the industry has relied heavily on ingredients of marine origin such as fishmeal (FM) and fish oils. According to FAO (2012), fishmeal is “the crude flour obtained after milling and drying fish or fish parts, and it is produced from whole fish, remains or other fish by-products resulting from processing” and it is the preferred ingredient as it can support rapid fish growth and feed conversion, constituting the major dietary protein source in commercial aquafeeds for marine species (Gao et al. 2013). It has a high protein content, high nutrient digestibility and palatability, balanced amino acid profile, lack of anti-nutritional factors and it is generally widely available (Forster and Dominy 2006; Gatlin et al. 2007). It is also a highly tradable product, and its production can be an important source of revenue for some countries (FAO 2014).

Although in 2012, 35% of the worlds’ FM was produced from fish waste, a significant proportion derives from capture fisheries of small pelagic fish, such as

anchoveta, sardines, herring and mackerel (FAO 2014; Sun et al. 2014). As the main producing countries, responsible for 2/3 of the trade, Peru and Chile, are affected by natural phenomena, such as El Niño, the catches of these species are bound to fluctuate, affecting supply and prices of FM (Hardy 2000). In fact, over the last decade, the prices of this commodity have significantly risen associated with the increasing demand and scarcity, both for terrestrial and aquatic production, going from a little over 500 US\$/ton in 2003 to almost 2000 US\$/ton in 2013 (FAO 2014). Due to this, there has been general concern about future supply of FM and its sustainable use in feeds as the aquaculture sector remains the largest consumer, currently using 60% of total production (Karapanagiotidis 2014). At current inclusion rates, annual FM production is not enough to support the predicted growth of intensive rearing systems. Also there is a perceived inefficiency in catching fish, processing it into FM and then fed it back to fish (Yu 2004). Taking everything into account, it is, therefore, necessary to improve feeding strategies as an industry that is dependent on FM is vulnerable to collapse through the loss of profit margins (Read and Fernandes 2003).

In Mediterranean intensive aquaculture, feeding and diet formulation can account for as much as 45% of the overall production costs (Williams et al. 2003). FM supplies the largest portion of dietary protein for carnivorous fish in aquaculture (Kokou et al. 2012) and for some farmed species, inclusion levels are still around 50% (Glencross et al. 2007). In fact, intensive production of European sea bass and gilthead seabream still requires continuous supply of high quality marine products (Karapanagiotidis 2014) and, particularly for gilthead seabream production, feeding costs can go as high as 49% of total production costs (Merinero et al. 2005).

One way to increase profitability and reduce the environmental impact of FM is through the optimization of the nutrient levels in diets (Williams et al. 2003), for example, by achieving an optimal protein/energy ratio (García-Gallego et al. 1998), or by strategically replace FM with other less expensive protein sources (Martínez-Llorens et al. 2008). Research is ongoing to find the best sustainable replacements for FM, without compromising growth, quality and welfare of farmed fish while still ensure the best economic returns.

Throughout the years, there has been a great number of potential ingredients evaluated to be included in feeds for farmed species. According to Gatlin et al. (2007), a suitable replacer for FM must be widely available at a competitive price, easy to handle, ship, store and incorporate in fish diets. In terms of nutritive value, it must be low in fiber and carbohydrates, high in protein, with a balanced amino acid profile, as well as be highly digestible and palatable. Many of these ingredients are more complex than FM

and require thorough evaluation in order to determine their nutritional value, appropriate incorporation levels and nutritional limitations, and its practicality to include in commercial feed formulation (Glencross et al. 2007). Due to the high protein requirements of carnivorous marine species, such as gilthead seabream, the number of potential alternatives is restricted to ingredients with high protein content and digestibility (Yiğit et al. 2012).

Plant protein sources in gilthead seabream

Due to its wide availability, competitive price and relatively constant nutritional composition (Pereira and Oliva-Teles 2002), plant protein sources have been the subject of study for a great variety of farmed species. Because gilthead seabream is an economically important species in Mediterranean aquaculture (Kokou et al. 2012), there has been substantial effort to evaluate plant protein ingredients for FM replacement. Some of the studied plant ingredients include soybean meal (Robaina et al. 1995; Kissil et al. 2000; Kissil and Lupatsch 2004; Martínez-Llorens et al. 2007; Kokou et al. 2012), corn gluten meal (Robaina et al. 1997; Kissil and Lupatsch 2004; Yiğit et al. 2012), hazelnut meal (Emre et al. 2008), lupin seed meal (Robaina et al. 1995; Pereira and Oliva-Teles 2004), wheat gluten (Kissil and Lupatsch 2004), pea protein concentrate (Sánchez-Lozano et al. 2010), pea seed meal (Pereira and Oliva-Teles 2002), carob seed germ meal (Martínez-Llorens et al. 2012), rapeseed protein concentrate (Kissil et al. 2000), as well as mixtures of several plant ingredients (Venou et al. 2003; Kissil and Lupatsch 2004).

Despite its potential, plant protein sources have only seldom been able to fully replace FM (Pereira and Oliva-Teles 2002), or even being used at high levels in feeds as fish performance has been inversely related to the inclusion levels of test ingredients (Pereira and Oliva-Teles 2003). This trend also follows for most carnivorous fish species (Gómez-Requeni et al. 2004; Karapanagiotidis 2014).

Overall, when using plant protein sources individually, FM can be replaced up to 60% for Sparidae fish, while with mixtures, substitution levels can go up to 75% but with major negative effects on fish's health (Oliva-Teles et al. 2011). The high content of carbohydrates present in plants protein sources limits its use as most fish species, in particular carnivorous, cannot use them effectively as energy source (Li et al. 2010). Enes et al. (2011) suggested that diets for gilthead seabream juveniles should not include more than 20% digestible carbohydrates, as higher dietary inclusions may depress growth and feed utilization. Furthermore, the presence on anti-nutritional factors

in many plant sources has negative physiological effects, by damaging the gastrointestinal tract and reducing nutrient digestibility, growth performance, and increasing disease susceptibility (Baeza-Ariño et al. 2014). Also, some plant protein sources appear to be limited in some EAA, such as lysine, methionine and tryptophan (NRC 1993; Martínez-Llorens et al. 2012) that can restrain growth and protein accretion (Kissil et al. 2000). However, this limitation can be overcome by using a mixture of complementary protein sources (Pereira and Oliva-Teles 2004) or by dietary supplementation with crystallized amino acids, making the feed more complex and, sometimes, more expensive.

The aquaculture industry also competes in the international market for the use of these plant ingredients along with the animal husbandry sector, biofuel production and direct use for human consumption (Karapanagiotidis 2014). Additionally, sustainability issues arise where there is growing pressure to develop environmentally friendly aquafeeds as some of the most used plant sources are produced in tropical countries, causing destruction of the rainforest for soy production in South America or the native forest in south-east Asia for palm (Karapanagiotidis 2014).

Animal by-products protein sources

Animal by-products, also called processed animal proteins (PAP) or rendered animals ingredients, have received great attention worldwide as they appear to be more practical and cost-effective alternative to FM for aquaculture feeds (Booth et al. 2012).

These products are usually the result of processed slaughterhouses leftovers and in the EU, around 17 MT of these by-products are produced annually, corresponding to 3 million metric tons of protein (Woodgate and Veen 2004). Depending on the raw materials used to manufacture these ingredients, they can have various designations such as meat meal (MM), meat and bone meal (MBM), poultry by-product meal (PBM), feather meal (FeM), blood meal (BM) and even milk-byproducts and gelatin (Hardy and Barrows 2002). MM or MBM are widely used animal by-products, derived from slaughtered farmed livestock (cattle, swine, sheep, and/or poultry) (FAO 2011), and typically possess high protein content (50-85%) with a relatively good amino acid profile, (Wilson 2002), moderate fat level (7-15%), high ash content (10 to 40%), high digestibility, low carbohydrate content, and lack anti-nutritional factors (Hu et al. 2013). Meat meals are also a good source of calcium and trace minerals (Rossi and Davis 2014) and can be used as a source of phosphorous (P), even at low inclusion levels (Suloma

et al. 2013). It is a product produced worldwide with a steady availability, allowing a more flexible feed formulation and advantageous in countries where FM is not locally available.

Nutritional quality of animal by-products are greatly influenced by the quality and specific combination of the raw materials as well as by the processing methods used to manufacture these products (Forster and Dominy 2006; Rossi and Davis 2014) which can result in an inconsistent and unpredictable final product, being more variable than between fish meals. Meat meals are typically produced by a dry-rendering process where the raw material is cooked by dry heat at 135-140°C in a steam jacked cooker until all the moisture is evaporated, followed by fat removal by draining off and screw press and grinding. Variation in the proportion of bone and soft tissues used contribute to large variations in meal quality and their classification as MM (<55% protein and <4.4 P) or MBM (<55% protein and <4.4% P) (Bureau et al. 2000).

Compared to FM, protein digestibility of meat meals is generally lower, up to 20% for meals with high ash and fat content, and may be low in some EAA such as lysine, methionine, phenylalanine, isoleucine and/or histidine (Millamena and Golez 2001; Hu et al. 2008a; Wang et al. 2008), mainly caused by heat-damaged protein due to excessive heat during processing (Allan et al. 2000). In fact, an unbalance of EAA content may lead to poor results when high substitution levels are applied (Tidwell et al. 2005; Forster and Dominy 2006).

Indigestible inorganic matter content, depending on the quantity of bone in the raw material, may also be a limiting factor when using MM and MBM as high content may impair digestibility, reducing nutrient and energy availability. Robaina et al. (1997) determined that ash levels in MBM exceeding 12.5% could lead to a decrease in protein digestibility in gilthead seabream.

Animal by-products, particularly meat meals, usually possess high levels of saturated fatty acids (FA) and 18:2 n-6 polyunsaturated FA but are low in n-3 highly unsaturated FA (eicosapentaenoic, docosahexaenoic, and arachidonic acids) that are required by marine fish (Millamena 2002; Hu et al. 2013). Unbalances in saturated and unsaturated FA content can lead to body lipid accumulation and morphological alterations (Robaina et al. 1997) as well as contribute to reduced palatability of these diets for fish. Robaina et al. (1997) found that gilthead seabream fed increasing levels of MBM showed hepatocyte necrosis in the liver and a progressive decrease in lipid digestibility.

The use of animal by-products in aquaculture

The use of animal by-products in aquafeeds is highly variable depending on the region. In the 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 (BSE) in ruminants in Western Europe in the 1980-1990's. BSE is a disease caused by prion protein and is also linked to a human disease, Creutzfeldt-Jakob disease, believed to be transmitted from infected cows to humans (Friedland et al. 2009), including its PAP by-products derived from infected ruminants. In 2013, however, this prohibition was lifted allowing the use of only PAPs derived from non-ruminant animals (Category 3, including poultry, feather meal, porcine and porcine blood meal, PAP) for feeding of aquaculture animals (EU Commission Regulation, EC No. 56/2013). This opened doors to a whole new range of ingredients that can be used in aquafeeds inside the EU.

Up to this point limited work has been carried out towards the use of animal by-products in gilthead seabream feeds. **Table 1** summarizes all the literature found.

Table 1: Studies performed to evaluate fishmeal replacement with different animal by-products ingredients in gilthead seabream.

Animal by-product ¹	IBW (g) ²	Dietary protein content (%DM)	Recommended substitution level (%DM)	Reference
MBM ^a	5	51.8	40	Davies et al. (1991)
MBM ^b	5	49.2	36	Alexis (1997)
PMM	1.1	44.9	100	
MBM ^c	40	43.9	20	Robaina et al. (1997)
PBM	1.55	44.0	75	Nengas et al. (1999)
BM ^d	33/179	46.6	15	Martínez-Llorens et al. (2008)

¹ MBM: Meat and bone meal; MBM^a (46-59% CP; 9-12% CL; 25-33% Ash); MBM^b (60% CP; 9% CL; 25% ash); MBM^c (64% CP; 10.3% CL; 25.4% ash). PMM: Poultry meat meal; PBM: Poultry by-product meal; BM^d: Blood meal (98.8% CP; 0.2% CL; 1.0% ash).

² IBW (g): Initial body weight

Also, for other aquaculture fish species worldwide, research has demonstrated that these ingredients have potential to be included in aquafeeds. **Table 2** summarizes the studies that have been performed using animal by-products ingredients as fishmeal replacement.

Objectives of this study

The aim of this study is to evaluate the effects on growth performance, whole-body composition, digestibility, nutrient and amino acid retention, and gut microbiota modulation in gilthead seabream fed diets formulated to replace 50 and 75% of fishmeal (FM) by meat and bone meal (MBM).

Table 2: Studies performed with several farmed species using animal by-product ingredients for fishmeal replacement.

Species	Habitat/ Trophic level ¹	IBW (g) ²	Animal by-product (% DM) ³	Diet protein content (%DM)	Recommended FM substitution levels (% DM)	Reference
African catfish (<i>Clarias gariepinus</i>)	FW 3.8 ± 0.4	93.1	PBM	25.3	100	Goda et al. (2007)
		93.9	MBM	25.8	75	
Australian short-finned eel (<i>Anguilla australis australis</i>)	FW 4.3 ± 0.5	2.23	MM	43.6	23	Engin and Carter (2005)
Australian silver perch (<i>Bidyanus bidyanus</i>)	FW 3.0 ± 0.34	12.2	MM	34 (DP ⁴ basis)	52	Stone et al. (2000)
Australian snapper (<i>Pagrus auratus</i>)	Marine 3.6 ± 0.2	14.0	MM	53.6	35	Booth et al. (2012)
			PBM	80.8	36	
			18% PBM + 15% BM + 5% MM	52.7	84	
Black sea bream (<i>Acanthopagrus schlegelii</i>)	Marine 3.2 ± 0.45	7.90	Enzyme treated PBM	41.8	16	Gao et al. (2013)
Bluegill (<i>Lepomis macrochirus</i>)	FW 3.2 ± 0.2	22.0	38% MBM	44.0	100	Masagounder et al. (2014)
Climbing perch (<i>Anabas testudineus</i>)	FW 3.0 ± 0.4	0.53	18% MBM	40.2	67	Kader et al. (2011a)
Cuneate drum (<i>Nibea miichthioides</i>)	Marine 4.0 ± 0.7	28.0	17% PBM + 9% MBM + 3% BM	42.8	80	Guo et al. (2007)
			17% PBM + 6% MBM + 3% BM + 3% FeM	43.0	80	
Florida pompano (<i>Trachinotus carolinus</i>)	Marine 3.5 ± 0.6	2.99	10% MBM	37.1	67	Rossi and Davis (2014)
		5.87	14% MBM + 0.6% Tau	39	100	
		15.3	8% PBM + 2% BM + 4% MBM	37.9	67	
Gibel carp (<i>Carassius auratus gibelio</i>)	FW 2.5 ± 0.0	13.5	12% PBM + 6% MBM + Lys and Met	37.9	67	Hu et al. (2008b)
		4.88	PBM	37.9	67	Yang et al. (2006)

Grouper (<i>Epinephelus coioides</i>)	Marine 4.0 ± 0.0	6.10	32% MM + 8% BM	45.4	80	Millamena (2002)
Hybrid striped bass (<i>Morone chrysops</i> x <i>M. saxatilis</i>)	FW / -	15.0	29% MBM	38.4	100	Webster et al. (1999)
			28% PBM	40.5	100	
		55.0	MBM	41.0	83	Bharadwaj et al. (2002)
		35.0	Feed grade PBM + Met	47.3	100	Rawles et al. (2011)
Japanese flounder (<i>Paralichthys olivaceus</i>)	Marine 3.7 ± 0.62	4.30	MBM	48.4	20	Kikuchi et al. (1997)
		5.55	MBM	45.8	>40	Wei et al. (2006)
			Pet grade PBM	45.4	80	
Japanese seabass (<i>Lateolabrax japonicus</i>)	Marine 3.4 ± 0.43	76.3	40% PBM + 35% MBM + 20% BM + 5% FeM	43.4	19	Hu et al. (2013)
Large yellow croaker (<i>Pseudosciaena crocea</i>)	Marine 3.7 ± 0.56	1.88	MBM	43.1	45	Ai et al. (2006)
Largemouth bass (<i>Micropterus salmoides</i>)	FW 3.8 ± 0.4	3.10	MBM	43.05	<50	Tidwell et al. (2005)
			PBM	47.04	100	
		23.3	MBM / PBM	43.2 / 43.1	30	Li et al. (2010)
Malabar grouper (<i>Epinephelus malabaricus</i>)	Marine 4.2 ± 0.61	50.2	PBM / MBM / FeM	52.8 / 53.4 / 53.7	25	Li et al. (2009)
Nile tilapia (<i>Oreochromis niloticus</i>)	FW 2.0 ± 0.0	11.7	BM + MBM + FeM	41.4	100	Rodríguez-Serna et al. (1996)
		12.5	MBM	30.0	100	El-Sayed (1998)
			PBM	30.0	100	
Olive flounder (<i>Paralichthys olivaceus</i>)	Marine 3.7 ± 0.62	14.5	MBM	52.6	20	Lee et al. (2012)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	FW 4.1 ± 0.3	6.50	MBM	47.4-48.4	32	Bureau et al. (2000)
		11.0	FeM	55.0-56.7	30	
		0.96	MBM + Leather meal + Squid liver powder + FeM + MBM + PBM	45.1	20 (or 28% FM protein)	Lee et al. (2001)

Rainbow trout (<i>Oncorhynchus mykiss</i>)		1.93	7% BM + 7% MBM + 7% PBM + 7% FeM	46.8	75	Yanik et al. (2003)
		16.8	PBM	50.4	29	
		16.8	BM	48.4	13	El-Haroun et al. (2009)
		35.0	FeM + MBM / FeM + PBM / MBM + PBM	50.3 – 52.2	50	
Red drum (<i>Sciaenops ocellatus</i>)	Marine 3.7 ± 0.57	2.30	Flash-dried PBM / Enzyme-digested PBM	44.0	>67	Kureshy et al. (2000)
Sea bass (<i>Dicentrarchus labrax</i>)	Marine /	110	10% MBM + 4% BM	46.1	69	Altan et al. (2010)
Silver perch (<i>Bidyanus bidyanus</i>)	FW 3.0 ± 0.34	12.1	6% MM + 9% Provine ⁵ + BM	34.0	52	Hunter et al. (2000)
Spotted rose snapper (<i>Lutjanus guttatus</i>)	Marine 4.0 ± 0.2	11.0	High quality PBM	52.0	90	Hernández et al. (2014)
Sutchi catfish (<i>Pangasius hypophthalmus</i>)	FW 3.1 ± 0.46	4.80	MBM	28.7	67	Kader et al. (2011b)

¹ Source: <http://www.fishbase.org>. FW: freshwater

² IBW (g): Initial body weight

³ BM: Blood meal; MBM: Meat and bone meal; MM: Meat meal; PBM: Poultry by-product meal; FeM: Feather meal;

⁴ DP: digestible protein

⁵ Commercial high protein meat meal

Materials and methods

Diet composition

Three experimental diets were formulated to be isoproteic (45% crude protein) and isolipidic (20% crude lipid). A control diet (FM100), containing FM as the main protein source, was formulated. Two other diets were formulated to replace FM by increasing levels by the experimental ingredient, meat and bone meal, at 50% (MBM50) and 75% (MBM75) in the diets (dry matter basis). Diets were prepared using a cooking extrusion processing with a semi-industrial twin-screw extruder (CLEXTRAL BC-45; Firmity, St. Etienne, France). The processing conditions were as follows: 100 rpm speed screw, 110 °C temperature, and 40-50 atm pressure to form 2 to 3 mm diameter pellets. Ingredients and chemical composition of experimental diets are presented in **Table 3**. Amino acid composition of the experimental diets is presented in **Table 4**.

Table 3: Composition and proximate analysis of the experimental diets.

	Diets		
	FM100	MBM50	MBM75
Ingredients (g kg⁻¹ DM)			
Fish meal¹	574	287	143
Wheat meal²	263	176	132
Meat and bone meal³	-	409	615
Soy oil	94	33	3
Fish oil	49	74	87
Vitamin and minerals mix⁴	20	20	20
Proximate analysis (% DM)			
Dry matter (DM, %)	90.0	91.9	91.5
Crude Protein (CP)	44.0	43.8	45.3
Crude Lipid (CL)	21.4	19.0	20.6
Crude Fiber (CF)	2.20	1.84	1.66
Ash	10.3	18.8	20.1
Energy (kJ g⁻¹)	20.3	18.8	19.8
NFE (%)⁵	22.1	16.6	12.3

¹ Fish meal (93.2% DM, 70.7% CP, 8.9% CL, 15.1% Ash, 19.7 kJ⁻¹ Energy);

² Wheat meal (92.4% DM, 17.1% CP, 2.4% CL, 78.3% CHO, 2.4% Ash);

³ Meat and bone meal (97.0% DM, 53.1% CP, 15.3% CL, 4.7% CHO, 26.9% Ash, 17.69 kJ⁻¹ Energy); VALGRA S.A., Beniparrell, Valencia, Spain

⁴ Vitamin and mineral mix (g kg⁻¹): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; (PO₄)₂Ca₃, 5. Premix composition: retinol acetate, 1000000 IU kg⁻¹; calcipherol, 500 IU kg⁻¹; DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.

⁵ Nitrogen-free extract, NFE (%) = 100-%CP-%CL-%CF-%Ash

Table 4: Amino acid composition (g 16 g⁻¹ N) of the experimental diets and EAA requirement of gilthead seabream (g 16 g⁻¹N; Peres and Oliva-Teles 2009).

	FM100	MBM50	MBM75	Requirement ¹
Essential amino acids				
Arg	6.75	7.17	7.21	5.55
His	2.73	1.78	1.90	1.89
Ile	4.26	3.01	2.98	2.55
Leu	7.61	5.70	5.96	4.75
Lys	6.94	5.53	5.35	5.13
Met	2.83	2.30	2.21	2.60
Phe	4.20	3.14	3.17	-
(Phe + Tyr)	(7.10)	(5.24)	(5.29)	(5.76)
Thr	4.18	2.99	3.44	2.98
Val	5.37	4.13	4.49	3.21
Non-essential amino acids				
Ala	5.67	5.45	6.78	
Asp	8.45	6.68	7.57	
Cys	0.68	0.64	0.75	
Glu	14.1	12.1	13.9	
Gly	5.91	8.38	11.7	
Pro	4.03	4.85	6.20	
Ser	3.77	3.14	3.77	
Tyr	2.90	2.11	2.12	

¹ Determined by Peres and Oliva-Teles (2009).

Arg: arginine; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Phe: phenylalanine; Thr: threonine; Val: valine; Ala: alanine; Asp: aspartate; Cys: cysteine; Glu: glutamate; Gly: glycine; Pro: proline; Ser: serine; Tyr: tyrosine.

Growth trial

Gilthead seabream juveniles were provided by a local fish farm (Piscimar, S.L., Castellón, Spain) and transported alive to the Fish Nutrition Laboratory of the Polytechnic University of Valencia. Prior to the growth trial, all fish were 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). After the adaptation period, 405 gilthead seabream juveniles, with an initial body weight of 25 ± 0.72 g, were randomly distributed into 9 cylindrical fiberglass tanks (1.750 L) in a recirculation seawater system (65 m³ capacity) with a rotary mechanical filter and a gravity biofilter (approximately 6 m³).

Diets were randomly assigned to triplicate groups and fish were hand fed to apparent visual satiation, two times a day, six days a week, for a total of 83 days. Feed

consumption was recorded daily and water parameters (temperature, salinity, dissolved oxygen and pH) were measured weekly. All tanks were equipped with aeration. The water was heated by a heat pump installed in the system. Photoperiod was natural and all tanks had similar light conditions. 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 and pH ranged from 6.5 to 7.5.

Fish sampling

Individual weighing occurred under anesthesia (30 mg L⁻¹ of clove oil (Guinama®, Valencia, Spain) containing 87% of eugenol) every 4 weeks. At the end of the trial (83 days), 5 fish per tank were sacrificed by a lethal bath of clove oil, pooled and stored at -32 °C and biometric parameters recorded. For initial whole-body composition analyses, 5 fish were randomly sampled, pooled and stored at -32 °C, prior the beginning of the growth trial.

Sampling for GIT microbiota analyses

Fish were fed 10 to 12 hours before sampling (to allow food to be in the intestine) and then 2 hours before (to allow food to be in the stomach). All the following procedures were performed under aseptic conditions. Six fish per dietary treatment were anesthetized using clove oil dissolved in water (1 mg 100 mL⁻¹ of water), in order to minimize suffering, and then sacrificed by decapitation. The abdominal cavity was opened and four different sections were considered: stomach (STO), anterior (AI), middle (MI) and posterior intestine (PI). The GIT content was obtained by scrapping the gastric/intestinal mucosa with a spatula, whereby samples include the luminal and the mucosa-associated microbiota. Thus, a total of four gastrointestinal content samples were obtained per fish, placed in Eppendorff tubes and immediately frozen in liquid nitrogen. Later, they were stored at -80°C until DNA extraction.

Digestibility trial

At the end of the growth trial, the remaining fish were transferred to the digestibility system and adapted for one month. The digestibility system consisted in a thermo-regulated recirculating seawater system equipped with a battery of 9 fiberglass tanks of 55 L capacity, designed according to the Guelph System (Cho et al. 1982). At the beginning of the digestibility trial, 9 homogeneous groups of 5 fish were randomly

distributed to each tank. To estimate apparent digestibility coefficient, celite was added as an inert digestibility marker, at 10 g kg⁻¹, to the same diets used previously in the growth trial. Each diet was assigned to triplicate group and fish were hand fed in excess. After an adaptation period of 5 days to the experimental system, fecal collection was performed by stripping until a significant amount of sample was collected and fecal samples were placed to dry at 60 °C for 48h prior to analyses. Apparent digestibility coefficients (ADC) of dry matter, energy, protein and amino acids were determined with the following formula:

ACD (%) = 100 x [1 – (marker in diet/marker in feces) x (Y in feces/Y in diet)], where Y is the nutrient or energy content.

Chemical analyses

Chemical analyses of the dietary ingredients were performed prior to diet formulation. Diets, ingredients, feces, as well as 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 Höganäs, Marineeden), crude lipid extracted with methyl-ether (ANKOM^{XT10} Extractor), crude fiber by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator), and acid insoluble ash (ADC marker) following the method described by (Atkinson et al. 1984). 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.

Amino acid determination

Total amino acid composition of ingredients, diets, feces 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 °C for 24 h. Aminobutyric acid was added as an internal standard before hydrolyze. 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).

PCR-DGGE (Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis)

DNA extraction from GIT samples

DNA extraction from GIT samples, from a pool of 2 fish per tank to reduce variation, was performed according to Pitcher et al. (1989) with some modifications. Briefly, approximately 300 mg of each sample were resuspended in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) vigorously mixed and pelleted by centrifugation at 13000 *g* for 5 min. After 2 washes with 1 mL TE, cell pellet was resuspended in 200 μ L of TE containing 50 mg/mL of lysozyme and incubated for 30 min at 37 °C. A second 30 min incubation at 37°C was performed with the addition of 10mg/mL RNase, followed by a 30 min incubation at 55°C with 20 mg/mL Proteinase K and 10% SDS. After 10 min on ice in the presence of 500 μ L of GES (Pitcher et al. 1989) and 250 μ L of ammonium acetate (7.5 M), a phenol-chloroform extraction was performed by adding 500 μ L phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was re-extracted with 500 μ L of chloroform-isoamyl alcohol (24:1) and the DNA of the subsequent aqueous phase was precipitated with 0.6 volumes of isopropanol. After 10 min centrifugation at 13000 *g*, the DNA pellet was washed with ice-cold 70 % ethanol and dried at room temperature. DNA was resuspended in 50 μ L ultrapure water.

Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE)

Bacterial 16S rRNA gene fragments were amplified by a touchdown PCR on a T100™ Thermal Cycler (Bio-Rad), using primers 16S-358F (which has a GC clamp at the 5' end) and 16S-517R (Muyzer et al. 1993), yielding a 233bp DNA fragment. PCR mixtures (50 μ L) contained 24.75 μ L of water (Sigma), 10 μ L of GoTaq Buffer 5X (PROMEGA), 5 μ L of each dNTPs (2 mM, PROMEGA), 2.5 μ L of each primer (10 μ M Forward and Reverse), 0.25 μ L of GoTaq polymerase (PROMEGA), and 5 μ L of DNA template were subjected to a touchdown PCR. A 94°C incubation for 5 min was followed by 10 cycles of 64°C, 1 min, 65°C, 1 min and 72°C, 3 min. The annealing temperature was decreased at every cycle 1°C, until reaching 55°C. Thus, final 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Final extension was at 72°C, 10 min. PCR

products were resolved by electrophoresis on 1 % (w/v) agarose gels containing Gel Red (Biotium) to check for product size. 300 ng of each PCR product were loaded on an 8% polyacrylamide gel composed by a denaturing gradient of 40 to 80% 7M urea/40%formamide. Electrophoresis occurred on a DCode™ universal mutation detection system (Bio-Rad), during 16h at 60°C, 65V in 1×TAE buffer. Gels were stained for 1 hour with SYBR-Gold Nucleic Acid Gel Stain, and imaged on a Gel Doc EZ System (Bio-Rad) with the Image Lab software v4.0.1 (Bio-Rad). Selected bands were excised from the gel and eluted in 20 µl ultrapure water prior to DNA re-amplification using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (<http://www.ncbi.nlm.nih.gov>). Only sequences higher than 100 bp reads and 80–100% query coverage were considered a valid identification.

Data and statistical analyses

Before analysis, all data obtained was checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene’s test). If necessary, variables were normalized by log transformation or arcsin square root transformation, for data expressed as decimal fractions or percentage, respectively. DGGE banding patterns were transformed into presence/absence matrices and band intensities measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Lda. Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef’s measure of richness, and species diversity was assessed by the Shannon–Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA).

Statistical analysis of data was done by one-way analysis of variance (ANOVA). Newman–Keuls test was used to assess significant differences among diets at 0.05 significant levels (Stat graphics, Statistical Graphics System, Version plus 5.1, Herndon, VA, USA). DGGE parameters were subjected to a two-way ANOVA, with section and diet as fixed factors.

Ethics statements

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).

Results

Growth trial: performance and feed utilization efficiency

At the end of the growth trial, survival rate was high (>95%) and unaffected by the experimental diets. Growth performance and feed utilization of fish fed the experimental diets are presented in **Table 5**. Diet MBM50 showed similar results to the control diet with no significant differences in terms of final body weight, weight gain, daily growth index, feed conversion ratio and protein efficiency ratio. However, increasing the inclusion rate of MBM to 75%, growth performance and feed utilization were significantly reduced. Feed intake was significantly higher for diet MBM75 when compared to the control diet but not statistically different from MBM50.

Table 5: Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets¹.

	FM100	MBM50	MBM75	SEM
Initial Body Weight (g)	24.3	23.8	24.3	0.3
Final Body Weight (g)	121.4 ^a	121.7 ^a	108.2 ^b	2.4
Weight Gain (%) ²	399.4 ^a	412.4 ^a	346.1 ^b	12.5
Daily growth index ³	2.48 ^a	2.51 ^a	2.25 ^b	0.05
Feed intake (g kg ABW ⁻¹ day ⁻¹) ⁴	24.2 ^b	24.6 ^{ab}	26.1 ^a	0.4
Feed conversion ratio ⁵	1.51 ^b	1.53 ^b	1.72 ^a	0.04
Protein efficiency ratio ⁶	1.51 ^a	1.50 ^a	1.29 ^b	0.04
Survival Rate (%)	100	94.8	99.3	1.5

¹ Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

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

² Weight gain, % = [(Final weight – Initial weight) / Initial weight] x 100

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

⁴ FI = Total dry feed intake / Average body weight / days

⁵ FCR = Dry feed intake (g) / Weight gain (g)

⁶ PER = weight gain / crude protein intake

Digestibility trial: ADC (%) of diets and amino acids

The apparent digestibility coefficients (ADC) of diets and amino acids were evaluated (**Table 6**). ADC of dry matter, crude protein and energy were the highest for diet MBM50, averaging 89, 97 and 95%, respectively. ADC of crude protein was not statistically affected by the experimental diets while ADC of dry matter was significantly lower for both MBM75 (68%) and the control (75%) diets. ADC of energy was lower for fish fed diet MBM75 (87%) but not significantly different from the control diet (82%). The ADCs of EAA were all high (>93%) but significantly lower for fish fed diet MBM75 when compared to diet MBM50, which were significantly higher. Digestible amino acid content of the experimental diets is presented in **Table 7**.

Table 6: Apparent digestibility coefficients (ADC, %) of the experimental diets¹.

	FM100	MBM50	MBM75	SEM	
Dry matter	74.6 ^b	86.9 ^a	68.4 ^b	3.3	
Crude Protein	95.7	96.7	88.6	1.9	
Energy	82.2 ^b	95.2 ^a	87.0 ^{ab}	2.5	
Amino acids					
Essential amino acids	Arg	95.7 ^{ab}	96.7 ^a	94.1 ^b	0.5
	His	95.2 ^b	97.8 ^a	95.2 ^b	0.6
	Ile	95.9 ^{ab}	97.2 ^a	94.0 ^b	0.6
	Leu	95.9 ^{ab}	97.0 ^a	93.9 ^b	0.6
	Lys	97.3 ^{ab}	97.9 ^a	95.4 ^b	0.5
	Met	96.7 ^a	96.6 ^a	92.5 ^b	0.9
	Phe	94.4 ^a	97.0 ^a	93.3 ^b	0.7
	Thr	95.5 ^{ab}	96.6 ^a	93.6 ^b	0.6
	Val	95.6 ^{ab}	96.8 ^a	93.8 ^b	0.6
Non-essential amino acids	Ala	95.5	96.0	93.3	0.7
	Asp	92.0 ^b	96.4 ^a	93.0 ^b	0.8
	Cys	91.1 ^{ab}	92.6 ^a	87.6 ^b	0.6
	Glu	96.5 ^a	96.7 ^a	93.9 ^b	0.7
	Gly	92.2	94.4	91.9	0.6
	Pro	95.9 ^a	95.0 ^{ab}	91.8 ^b	0.8
	Ser	95.0 ^{ab}	95.9 ^a	92.9 ^b	0.6
	Tyr	97.1 ^a	97.2 ^a	94.1 ^b	0.7

¹ Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean

ADC (%) = $100 - 100 \times [(\text{marker in diet}/\text{marker in feces}) \times (\text{AA in feces}/\text{AA in diet})]$

Table 7: Digestible amino acids content (g 100 g⁻¹ DM) of the experimental diets¹.

Diet	Essential amino acids									Non-essential amino acids							
	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Asp	Cys	Glu	Gly	Pro	Ser	Tyr
FM100	2.84	1.15	1.80	3.12	2.97	1.20	1.78	1.75	2.26	2.38	3.42	0.27	6.01	2.40	1.70	1.58	1.24
MBM50	4.80	0.80	1.35	2.56	2.50	1.02	1.41	1.33	1.85	2.42	2.97	0.27	5.39	3.66	2.13	1.39	0.95
MBM75	3.41	0.84	1.30	2.60	2.37	0.95	1.37	1.50	1.96	2.94	3.27	0.30	6.07	4.99	2.65	1.63	0.93

¹ AA in the diet (g 100g⁻¹) x ADC (%) of AA / 100

Whole body composition and biometric parameters

At the end of the growth trial, whole-body composition was unaffected by the dietary inclusion of MBM, with the exception of crude lipid and energy content, which were significantly lower for fish fed the diet MBM75 (**Table 8**). There were no significant differences in whole-body amino acid composition (g 100 g⁻¹) and in the measured biometric parameters of gilthead seabream fed the different experimental diets.

Nutrient and amino acid budget

Nitrogen, lipid and energy balance of fish fed the experimental diets are presented in **Table 9**. Results show that the inclusion of MBM did not significantly affect nitrogen retention (% intake), while daily nitrogen intake was significantly higher for fish diet MBM75. Daily lipid and energy intake were significantly higher for diet MBM75 and lower for diet MBM50 but neither were significantly different from the control diet. Lipid retention (% intake) was significantly higher for fish fed diet MBM50 but lower for diet MBM75 than that of the control diet. Compared to the control diet, energy retention was significantly lower for fish fed the diet with the highest inclusion of MBM while with MBM50 it was not significantly different.

Amino acid budget is presented in **Table 10** and in **Fig. 4**, it is represented the efficiency of EAA retention (% intake) of fish fed the different experimental diets. Results show no significant changes in EAA retention, daily or per percentage of intake, for the different experimental diets.

Table 8: Whole-body composition and biometric parameters of gilthead seabream fed the experimental diets¹.

	FM100	MBM50	MBM75	SEM
Whole-body composition (% wet weight)				
Dry matter (%)	35.0	34.6	33.1	0.4
Crude protein	16.1	15.9	15.9	0.2
Crude lipid	16.1 ^a	16.4 ^a	14.5 ^b	0.3
Ash	2.10	1.91	2.01	0.1
Energy (kJ g ⁻¹)	9.94 ^a	9.68 ^a	8.67 ^b	0.23
Essential amino acid (g 100g⁻¹ wet weight)				
Arg	1.24	1.14	1.05	0.08
His	0.34	0.32	0.28	0.11
Ile	0.60	0.58	0.56	0.16
Leu	1.08	1.04	0.99	0.22
Lys	1.13	1.05	0.97	0.18
Met	0.49	0.51	0.53	0.07
Phe	0.55	0.52	0.51	0.13
Thr	0.61	0.54	0.56	0.13
Val	0.74	0.72	0.70	0.13
Non-essential amino acids (g 100g⁻¹ wet weight)				
Ala	0.92	0.84	0.87	0.21
Asp	1.32	1.35	1.27	0.19
Cys	0.10	0.11	0.12	0.02
Glu	2.11	2.04	1.97	0.27
Gly	1.11	0.91	1.00	0.81
Pro	0.64	0.52	0.61	0.32
Ser	0.57	0.55	0.53	0.09
Tyr	0.44	0.38	0.39	0.10
Biometric indices				
Condition factor (g cm ⁻³) ²	1.74	1.81	1.75	0.03
Visceral index (%) ³	8.57	8.53	9.31	0.22
Hepatosomatic index (%) ⁴	2.75	2.87	2.40	0.32
Visceral fat index (%) ⁵	1.23	1.17	1.22	0.13

¹ Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

² CF = [Wet weight (g) / Length³ (cm)] x 100

³ VSI = [Visceral weight (g) / wet weight (g)] x 100

⁴ HSI = [Liver weight (g) / wet weight (g)] x 100

⁵ VFI = [Visceral fat (g) / wet weight (g)] x 100

Table 9: Nitrogen, lipid and energy budget of gilthead seabream fed the experimental diets¹.

	FM100	MBM50	MBM75	SEM
Nitrogen				
Intake (g kg ABW ⁻¹ day ⁻¹)	1.71 ^b	1.72 ^b	1.89 ^a	0.03
Retention (g kg ABW ⁻¹ day ⁻¹)	0.43	0.42	0.40	0.01
Retention (% intake)	25.0	24.5	21.3	0.8
Lipid				
Intake (g kg ABW ⁻¹ day ⁻¹)	5.17 ^a	4.68 ^b	5.37 ^a	0.11
Retention (g kg ABW ⁻¹ day ⁻¹)	2.88 ^a	2.94 ^a	2.47 ^b	0.08
Retention (% intake)	55.8 ^b	62.9 ^a	46.0 ^c	2.6
Energy				
Intake (kJ kg ABW ⁻¹ day ⁻¹)	5.47 ^a	5.03 ^b	5.64 ^a	0.10
Retention (kJ kg ABW ⁻¹ day ⁻¹)	1.72 ^a	1.67 ^a	1.40 ^b	0.06
Retention (% intake)	31.5 ^a	33.2 ^a	24.9 ^b	1.4

¹ Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

Nutrient intake (g kg ABW⁻¹ day⁻¹) = [Nutrient intake (g DM) / 1000] / (ABW (g) x number of days)

Nutrient retention (g kg ABW⁻¹ day⁻¹) = [(FBW x final whole-body nutrient content) - (IBW x initial whole-body nutrient content) / 1000] / [ABW x number of days]

Nutrient retention (% intake) = Nutrient retention / Nutrient intake x 100

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

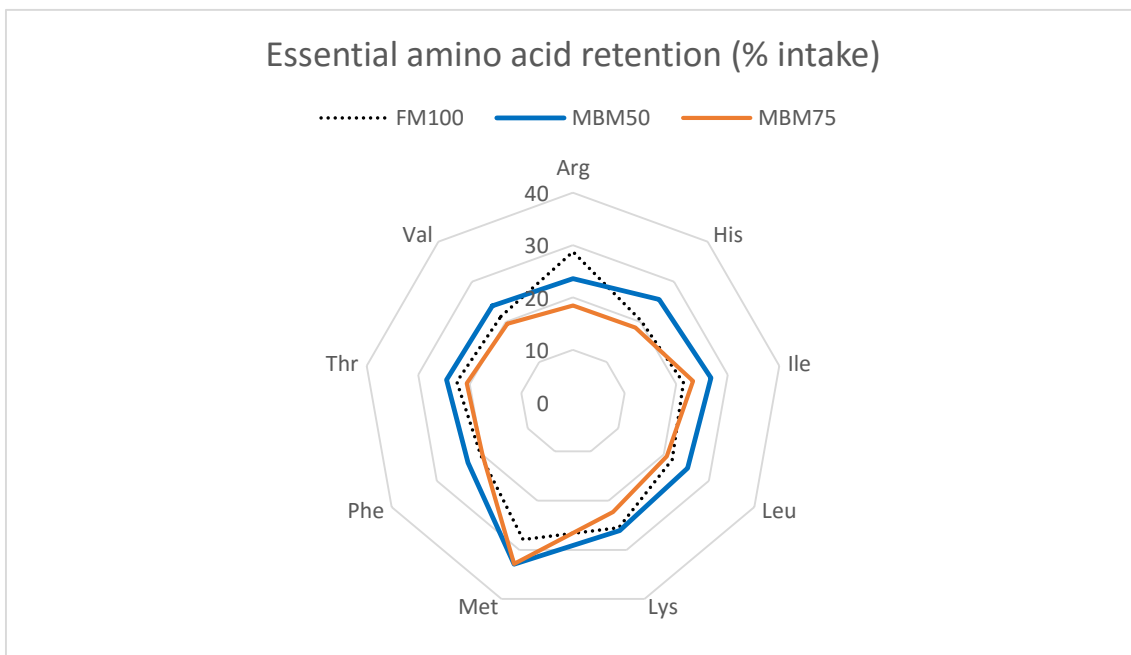


Fig. 4: Retention (%) of ingested essential amino acid in gilthead seabream fed the experimental diets.

Table 10: Amino acid budget of gilthead seabream fed the experimental diets¹.

		FM100	MBM50	MBM75	SEM	
Essential amino acids	Arg	Int (mg kg ⁻¹ day ⁻¹)	719.5 ^b	801.2 ^a	857.3 ^a	22.6
		Ret (mg kg ⁻¹ day ⁻¹)	205.2	187.9	158.1	13.2
		Ret (%Int)	28.7	23.6	18.5	2.3
	His	Int (mg kg ⁻¹ day ⁻¹)	291.3 ^a	209.7 ^c	231.9 ^b	12.6
		Ret (mg kg ⁻¹ day ⁻¹)	58.2	53.6	43.1	5.7
		Ret (%Int)	20.2	25.6	18.6	2.3
	Ile	Int (mg kg ⁻¹ day ⁻¹)	453.9 ^a	355.8 ^b	364.1 ^b	16.5
		Ret (mg kg ⁻¹ day ⁻¹)	96.6	94.4	84.6	5.4
		Ret (%Int)	21.5	26.7	23.3	1.51
	Leu	Int (mg kg ⁻¹ day ⁻¹)	810.9 ^a	673.1 ^b	728.1 ^b	22.2
		Ret (mg kg ⁻¹ day ⁻¹)	176.3	169.7	150.4	10.5
		Ret (%Int)	21.9	25.3	20.7	1.5
	Lys	Int (mg kg ⁻¹ day ⁻¹)	739.6 ^a	653.2 ^b	652.4 ^b	17.0
		Ret (mg kg ⁻¹ day ⁻¹)	186.6	170.4	145.2	15.0
		Ret (%Int)	25.5	26.1	22.3	2.1
	Met	Int (mg kg ⁻¹ day ⁻¹)	301.4 ^a	270.7 ^b	269.7 ^b	6.4
		Ret (mg kg ⁻¹ day ⁻¹)	84.0	88.8	88.5	2.1
		Ret (%Int)	27.9	33.0	32.9	1.2
Phe	Int (mg kg ⁻¹ day ⁻¹)	447.4 ^a	370.2 ^b	386.7 ^b	12.8	
	Ret (mg kg ⁻¹ day ⁻¹)	90.2	85.5	77.1	5.3	
	Ret (%Int)	20.3	23.1	20.0	1.4	
Thr	Int (mg kg ⁻¹ day ⁻¹)	444.3 ^a	352.6 ^b	420.0 ^a	14.7	
	Ret (mg kg ⁻¹ day ⁻¹)	99.4	86.0	86.3	6.3	
	Ret (%Int)	22.5	24.9	20.6	1.7	
Val	Int (mg kg ⁻¹ day ⁻¹)	572.7 ^a	487.8 ^b	547.5 ^a	14.4	
	Ret (mg kg ⁻¹ day ⁻¹)	121.6	116.9	106.7	6.6	
	Ret (%Int)	21.4	23.7	19.5	1.4	
Non-essential amino acids	Ala	Int (mg kg ⁻¹ day ⁻¹)	603.9 ^b	643.7 ^b	827.6 ^a	35.6
		Ret (mg kg ⁻¹ day ⁻¹)	153.9	139.3	135.9	7.0
		Ret (%Int)	25.7 ^a	21.7 ^{ab}	16.4 ^b	1.8
	Asp	Int (mg kg ⁻¹ day ⁻¹)	900.4 ^a	788.7 ^b	924.1 ^a	23.7
		Ret (mg kg ⁻¹ day ⁻¹)	216.2	221.3	192.2	12.4
		Ret (%Int)	24.2	28.1	20.9	1.8
	Cys	Int (mg kg ⁻¹ day ⁻¹)	72.6 ^b	75.0 ^b	91.1 ^a	3.1
		Ret (mg kg ⁻¹ day ⁻¹)	16.7	17.5	18.6	0.6
		Ret (%Int)	23.1	23.4	20.4	1.0
	Gly	Int (mg kg ⁻¹ day ⁻¹)	629.6 ^c	989.2 ^b	1427.9 ^a	116.3
		Ret (mg kg ⁻¹ day ⁻¹)	192.0	152.2	162.5	14.9
		Ret (%Int)	30.8 ^a	15.4 ^{ab}	11.4 ^b	3.8
	Glu	Int (mg kg ⁻¹ day ⁻¹)	1507.2 ^b	1423.3 ^b	1697.8 ^a	45.3
		Ret (mg kg ⁻¹ day ⁻¹)	342.9	332.9	299.2	19.5
		Ret (%Int)	22.9	23.4	17.7	1.7
	Pro	Int (mg kg ⁻¹ day ⁻¹)	429.6 ^c	572.0 ^b	757.0 ^a	48.0
		Ret (mg kg ⁻¹ day ⁻¹)	110.2	86.5	98.3	5.3
		Ret (%Int)	25.8 ^a	15.2 ^b	13.0 ^b	2.3
Ser	Int (mg kg ⁻¹ day ⁻¹)	402.1 ^b	371.0 ^b	460.2 ^a	14.1	
	Ret (mg kg ⁻¹ day ⁻¹)	92.4	88.4	79.7	6.9	
	Ret (%Int)	23.2	23.9	17.3	2.1	
Tyr	Int (mg kg ⁻¹ day ⁻¹)	309.3 ^a	248.7 ^b	258.9 ^b	10.0	
	Ret (mg kg ⁻¹ day ⁻¹)	69.2	59.0	56.6	3.9	
	Ret (% Int)	22.5	23.5	21.9	1.4	

¹ Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean; Int: intake; Ret: retention.

Modulation of gilthead seabream gut microbiota

The microbial community profiling of the stomach (STO) and intestinal samples (AI, MI, PI) recovered from gilthead seabream fed the experimental diets was studied by polymorphism analyses of the variable V3 region of the 16S rRNA gene using DGGE. Similar banding patterns between the 3 replicates for each diet were not always evident, with one replicate constantly failing to cluster with the other 2 in the Bray–Curtis dendrogram (**Fig. 5**). The figure further shows that the bacterial communities obtained from the AI of fish fed diet MBM75 seem to be more closely related (percentages of similarity around 70% between 2 out of 3 samples) than those recovered from fish fed the control and MBM50 diets, which seem to diverge more (percentages of similarity below 50% between 2 out of 3 samples for each diet). Nevertheless, variations on the average number of OTUs (Operational Taxonomic Units), microbial richness, microbial diversity and similarity indices between samples were detected with statistical significance between experimental diets and between gastrointestinal sections (**Table 11**). With exception on the average number of OTUs, there were significant differences ($p < 0.05$) on the indices of microbial richness, microbial diversity and similarity between the different gastrointestinal samples analyzed. The AI samples presented the highest microbial richness, the PI samples the highest microbial diversity, while the STO samples revealed the lowest microbial diversity and richness. PI and STO samples were the ones with higher similarity between replicates, that is, were the most homogeneous samples (**Table 11**). Replacement of FM by MBM lead to a significant decrease ($p < 0.01$) on the average number of OTUs and on the microbial richness, and to a significant increase on the SIMPER similarity ($p < 0.001$) (**Table 11**). Sequence analysis from the DGGE bands (**Fig. 5, Table 12**) showed that the detectable dominant bacteria present in the stomach and intestines of gilthead seabream fed the experimental diets were most closely related to uncultured bacteria (bands 13, 15, and 16) or bacteria belonging to the *Corynebacterium* (bands 6, 8, 12, and 18), *Staphylococcus* (bands 1, 10, and 11), *Vibrio* (bands 9 and 14), *Weissella* (bands 4 and 5) or *Bacillus* (bands 2 and 3) genus. *Mycobacterium* (band 7) and uncultured *Plantibacter* (band 17) were also detected.

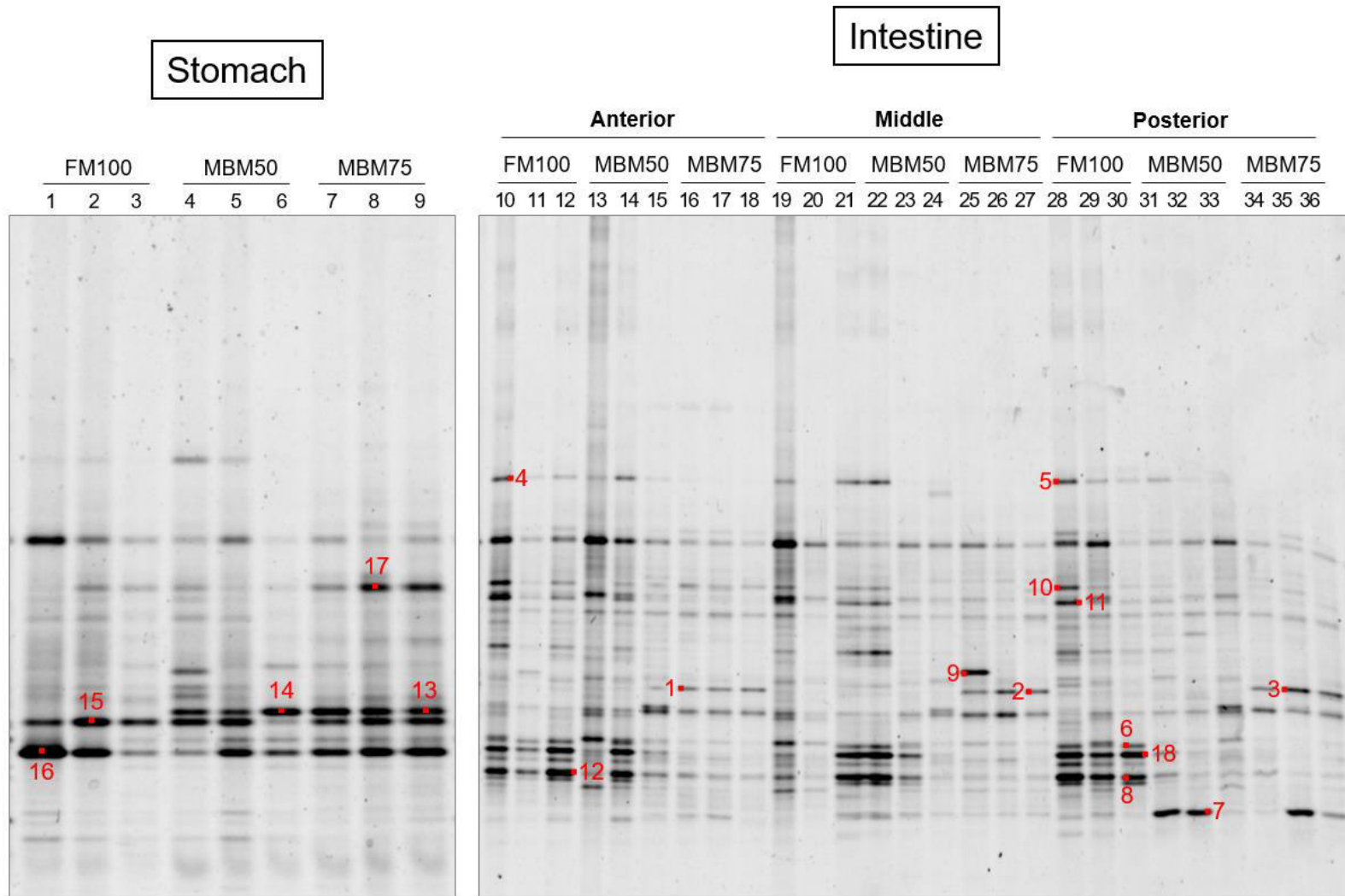


Fig. 5: PCR-DGGE fingerprints of the microbiota found in stomach and intestinal sections recovered from gilthead seabream fed the experimental diets. Black numbers on top of the figure represent the different samples analyzed (from a pool of two fish each) while red numbers inside the figure correspond to bands removed for sequencing, which results are presented in Table 12.

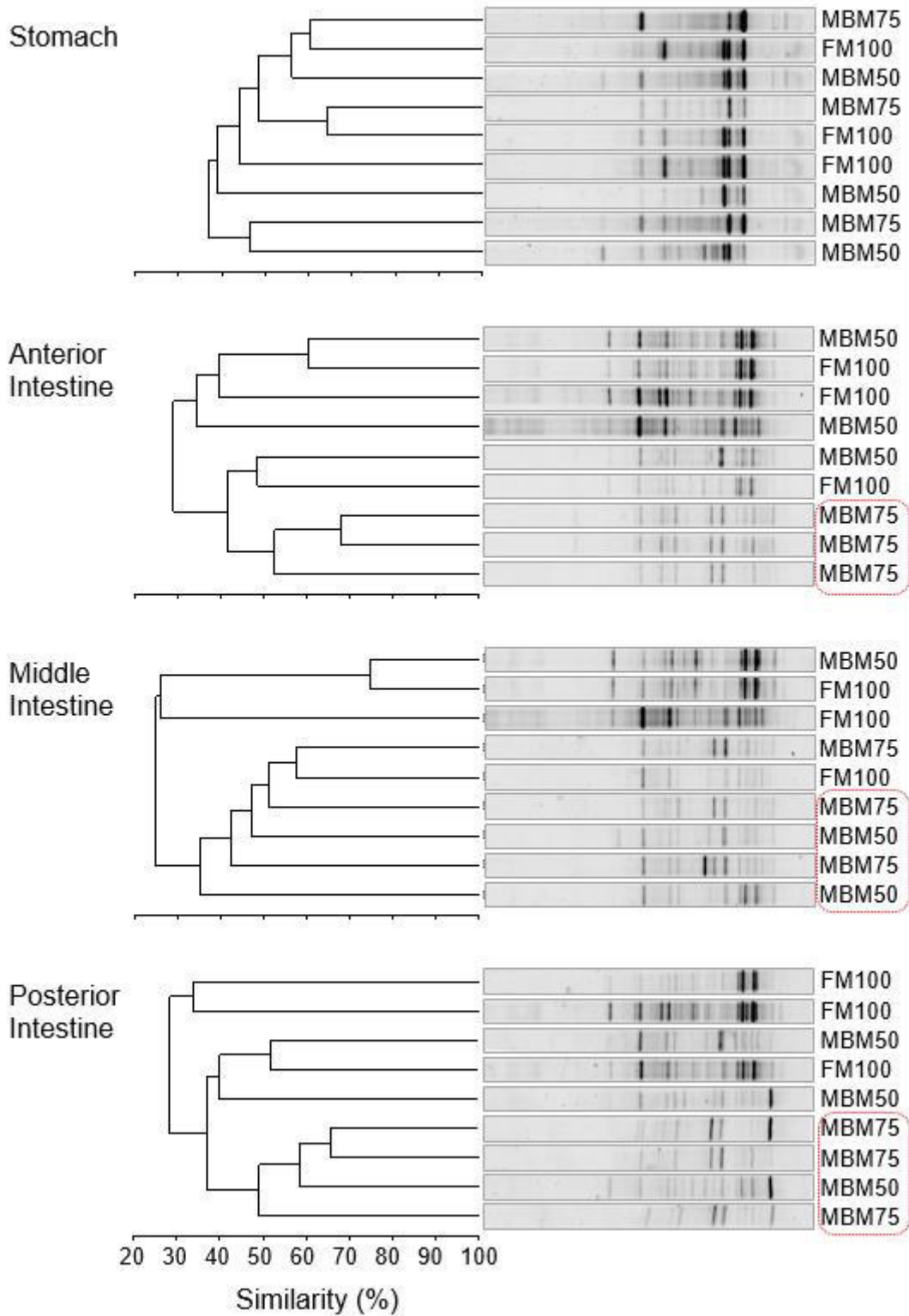


Fig. 6: Dendrograms and PCR-DGGE fingerprints of the microbiota found in stomach and intestinal sections recovered from gilthead seabream fed the experimental diets.

Table 11: Ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in stomach (STO) and intestinal sections (AI, anterior intestine; MI, middle intestine; PI, posterior intestine) recovered from gilthead seabream fed the experimental diets.

Diets	STO			AI			MI			PI		
	FM100	MBM50	MBM75	FM100	MBM50	MBM75	FM100	MBM50	MBM75	FM100	MBM50	MBM75
OTUs¹	17 ± 3.6	18.7 ± 4.5	17.3 ± 2.1	23 ± 4.4	24.7 ± 2.5	19 ± 2	23.3 ± 5.7	19.0 ± 4.0	18.0 ± 1.7	26.0 ± 3.6	19.3 ± 0.6	14.7 ± 3.2
Richness²	1 ± 0.2	1.1 ± 0.3	1 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	1.4 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.5 ± 0.2	1.2 ± 0.03	0.9 ± 0.2
Diversity³	2.3 ± 0.3	2.6 ± 0.2	2.3 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	2.5 ± 0.4	2.7 ± 0.2	2.5 ± 0.1	2.6 ± 0.2	2.8 ± 0.1	2.8 ± 0.1	2.4 ± 0.3
SIMPER Similarity (%)⁴	45.4 ± 5.1	42.5 ± 12.3	52.8 ± 7.3	36.0 ± 11.7	38.0 ± 4.9	57.6 ± 9.3	25.7 ± 3.5	30.7 ± 9	45.9 ± 17	38.4 ± 4.2	41.1 ± 1.5	58.8 ± 6.7

Two-Way ANOVA	Variation source ⁵			Section				Diet			
	Variation source	Section	Diet	Interaction	STO	AI	MI	PI	FM100	MBM50	MBM75
OTUs¹		ns	**	ns	-	-	-	-	a	ab	b
Richness²		*	**	ns	b	a	ab	ab	a	ab	b
Diversity³		*	ns	ns	b	ab	ab	a	-	-	-
SIMPER Similarity (%)⁴		*	***	ns	a	ab	b	a	b	b	a

Values presented as means ± standard deviation (±SD) (n = 3 per treatment pooled from 6 fish)

¹ OTUs: Average number of operational taxonomic units.

² Margalef species richness: $d=(S-1)/\log(N)$

³ Shannons diversity index: $H'=-\sum(pi\ln(pi))$

⁴ SIMPER, similarity percentage within group replicates.

⁵ ns, non-significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Table 12: Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the GIT communities of gilthead seabream fed the experimental diets.

Band	Nearest neighbor	Similarity to nearest neighbor (%)	Accession number of nearest neighbor
1	<i>Staphylococcus</i> sp. THK-6.1 16S ribosomal RNA gene, partial sequence	97	KM100592.1
2	<i>Bacillus subtilis</i> strain C14 16S ribosomal RNA gene, partial sequence	100	KP050498.1
3	<i>Bacillus subtilis</i> strain C14 16S ribosomal RNA gene, partial sequence	100	KP050498.1
4	<i>Weissella paramesenteroides</i> strain FT369 16S ribosomal RNA gene, partial sequence	96	KM207814.1
5	<i>Weissella paramesenteroides</i> strain FT369 16S ribosomal RNA gene, partial sequence	100	KM207814.1
6	<i>Corynebacterium</i> sp. S1-30 16S ribosomal RNA gene, partial sequence	99	KP114217.1
7	<i>Mycobacterium</i> sp. Iso-37 16S ribosomal RNA gene, partial sequence	98	KC768749.1
8	<i>Corynebacterium</i> sp. MU10 16S ribosomal RNA gene, partial sequence	98	KF631233.1
9	<i>Vibrio</i> sp. SF096-4 16S ribosomal RNA gene, partial sequence	99	JX549389.1
10	<i>Staphylococcus</i> sp. HB1 partial 16S rRNA gene, strain HB1	99	AM268420.1
11	<i>Staphylococcus arlettae</i> strain BAN98 16S ribosomal RNA gene, partial sequence	96	JX960429.1
12	<i>Corynebacterium</i> sp. MU10 16S ribosomal RNA gene, partial sequence	98	KF631233.1
13	Uncultured bacterium clone S14-hap 0613 16S ribosomal RNA gene, partial sequence	78	FJ373480.1
14	<i>Vibrio</i> sp. AB336d partial 16S rRNA gene, isolate AB336d	97	FR821229.1
15	Uncultured bacterium isolate DGGE gel band 16 16S ribosomal RNA gene, partial sequence	97	HQ876077.1
16	Uncultured bacterium clone B112_218 small subunit ribosomal RNA gene, partial sequence	100	KM500154.1
17	Uncultured <i>Plantibacter</i> sp. isolate DGGE gel band 2R12-2 16S ribosomal RNA gene, partial sequence	93	KF051512.1
18	<i>Corynebacterium variabile</i> partial 16S rRNA gene, strain PG-Z	99	HG798646.1

Discussion

In order for European aquaculture industry to expand, it is necessary to find viable alternatives to FM as its use is becoming less sustainable at current inclusion rates. A number of investigations have been carried out to evaluate the potential use of non-ruminant processed terrestrial animal proteins, MBM, in diets for aquaculture species worldwide. However, due to the prohibition in the EU of the use of terrestrial animal ingredients for aquafeeds in 2001 (Karapanagiotidis 2014), most of the literature found with the use of these ingredients are of studies performed outside the EU, with species not produced in this area. Besides that, there has been a wide range of results regarding the ideal substitution level due to the different habitats, feeding habits, and different processing techniques and raw materials used, most of the times, secret of the company, which can result in an unpredictable final product (Hendrick et al. 2005; Xavier et al. 2014). However, improvements in processing technologies, and the utilization of raw materials of higher nutritive value (e.g., blood meal or low bone meat meal) can lead to the production of a more nutritious MBM (Bureau et al. 2000; Rossi and Davis 2014).

Overall results of this study show that MBM is efficient in promoting good growth and feed performance in gilthead seabream juveniles. Fish fed a diet with half of the FM replaced by MBM showed a slightly higher weight gain, approximately 1.2% higher than that of fish fed the non-MBM control diet. On the other hand, increasing the replacement level to 75% decreased growth performance in about 9.3%, relatively to the non-MBM control diet. Issues related to the EAA profile, availability of protein, energy and amino acid of MBM may have, at least, contributed to the lower performance of MBM75 diet, relatively to the others, as it will be discussed.

MBM is a desirable dietary component for carnivorous and omnivorous fish species, containing high levels of protein and fat (Allan and Rowland 2005; Rossi and Davis 2014). Present results are in agreement with previous studies where 20-80% of dietary FM could be replaced by MBM without negatively affecting growth performance. For grouper, 80% of FM could be replaced by high quality animal protein, a blend of MM and BM, with similar growth rate as the control group (Millamena 2002). High FM replacement (of around 80%) with MBM was also achieved with hybrid striped bass (Bharadwaj et al. 2002).

For large yellow croaker and Australian silver perch, 45 and 50% of FM could be successfully replaced by meat meals (MBM/MM), respectively (Stone et al. 2000; Ai et al. 2006). Likewise, for other fish species such as sutchi catfish and African catfish, the

replacement level may be increased up to 67-75%, respectively, with no negative effects on growth (Goda et al. 2007; Kader et al. 2011b).

On the contrary, lower replacement levels of FM by MBM or MM were observed for Australian snapper (up to 35%; Booth et al. 2012), largemouth bass (up to 30%; Li et al. 2010), rainbow trout (up to 30%; Bureau et al. 2000), Malabar grouper (up to 25%; Li et al. 2009), Australian short-finned eel (up to 23%; Engin and Carter 2005), olive flounder (up to 20%; Lee et al. 2012), and Japanese flounder (up to 20%; Kikuchi et al. 1997). Very low results were also obtained by Kureshy et al. (2000) in red drum as more than 16.6% FM substitution with low ash MM decreased performance. For Florida pompano, in soybean meal based diets, MBM was effective to reduce FM from 15 to 5% (Rossi and Davis 2014). In part, at least, this discrepancy of results may be due to variations on MBM composition largely influenced by the raw material composition and quality as well as to the processing conditions during rendering and the feeding habit of the species (Rossi and Davis 2014; Xavier et al. 2014).

Palatability is an important issue when working with alternative protein sources to FM, as low palatability decreases feed consumption, and can result in poorer growth performance. In the present study, MBM did not compromise feed palatability, as fish fed diet MBM75 had significantly higher feed intake than that of fish fed the other diets. However, it did not translate to a higher feed efficiency ratio, as fish fed diet MBM75 had the lowest weight gain, or daily growth index. The higher feed intake of MBM75 diet may, in part at least, be related to its lower digestible protein content, compared to the other diets. Even though it is generally accepted that fish eat primarily to satisfy their energy requirements (Cho and Kaushik 1990; Kaushik and Medale 1994), in the present study an attempt to adjust feed intake to a certain level of digestible protein intake, irrespectively the diet, was observed (digestible nitrogen intake 1.64-1.67 g N kg⁻¹day⁻¹), as previously reported for other fish species (Peres and Oliva-Teles, 1999). Generally, results of most feeding trials indicate that replacement of FM with MBM has minimal effect on feed consumption (Allan and Rowland 2005; Ai et al. 2006; Booth et al. 2012; Hu et al. 2013; Rossi and Davis 2014).

Apparent digestibility values of MBM are generally lower than those of FM for different fish species (Silva and Oliva-Teles 1998; Wei et al. 2006; Booth et al. 2013; Xavier et al. 2014), contributing to a lower performance of MBM based diets. Nevertheless, ADC of energy was high for both diets containing MBM (95% and 87% for diets MBM50 and MBM75, respectively) but lower for the MBM75 diet than for the other diets, which may be correlated to its higher ash content. Indeed, high levels of indigestible inorganic matter have been reported to be a limiting factor when using animal

by-products, as it may increase intestinal transit (less time for nutrients' digestion), leading to a higher feed intake but poor feed efficiency and growth performance (Goda et al. 2007; Xavier et al. 2014), as it was observed in this trial. In the present study, ash content of MBM based diets (19.5% DM) was almost double the quantity present in the non-MBM control diet (10% DM), but ash content alone could not be accounted for as the only factor contributing for the poor growth performance of diet MBM75, as ash content of diet MBM50, similar to the one of the MBM75 diet, did not limited its performance.

Fish are able to effectively use lipids as an energy source (Sullivan and Reigh 1995). However, although it was not determined for this study, it is acknowledged that processed animal by-product meals possess high levels of saturated fats (Millamena 2002), which are less digestible than unsaturated fats (NRC 2011) as lipid digestibility is negatively correlated with the degree of saturation (Takeuchi et al. 1979). The presence of high levels of saturated fats in diet MBM75, comparatively to the other diets, could have led to a lower lipid digestibility and a lower energy digestibility, contributing to the observed growth reduction of fish fed diet MBM75. In fact, others authors have reported moderate lipid digestibility values when using these type of ingredients: 77.2% with MM (Mabrouk and Nour 2011), 58% and 73% with MBM (Bureau et al. 1999).

Dietary ash content may have also condition protein digestibility. Robaina et al. (1997) determined that there is a negative correlation between ash content and protein digestibility of a diet. In this study, even though protein and EAA digestibility of MBM based diets were high, protein and EAA digestibility of diet MBM75 were lower than those of MBM50. A wide range of protein ADC in diets formulated with animal by-products have been reported, indicating a dependence on the nutritional quality of the ingredient as well as on the species. Protein ADC of MBM based diets averaged 65.2% for gilthead seabream (Mabrouk and Nour 2011) but for mullet fish it averaged 87.2% (Booth et al. 2013). Booth et al. (2005) obtained a protein ADC of 75.3% when replacing 50% of FM in diets with MM in Australian snapper. For Nile tilapia, nutritional quality of the MBM significantly influenced protein digestibility that ranged from 50 to 87%, depending on the protein and ash content of the ingredient (Xavier et al. 2014). Protein ADC of the MM ingredient was determined to be around 75.1% in diets for sea bass (Silva and Oliva-Teles 1998).

Protein quality, evaluated in terms of amino acids' availability and profile, may have also affected MBM's nutritional quality (Peres and Oliva-Teles 2006; 2007). Indeed, more than protein content itself, fish require a well-balanced amino acid profile in feeds to achieve an optimal growth (Berge et al. 1999; Peres and Oliva-Teles 2009).

Unbalanced levels of EAA in diets have been reported as one of the causes for growth depression in several farmed fish when fed animal by-products (García-Gallego et al. 1998; Millamena 2002; Sun et al. 2014; Xavier et al. 2014), as protein deposition is closely related to weight gain. García-Gallego et al. (1998) reported that, for European eel, MM diets led to lower feed intake and utilization due to some EAA deficiency. However, in the present study, almost all EAA of the experimental diets exceeded the requirement levels for gilthead seabream, as determined by Peres and Oliva-Teles (2009), with the exception of methionine and phenylalanine + tyrosine, in accordance with previous studies using animal by-products (Nengas et al. 1999; Wang et al. 2008).

Besides the rendering composition, the technological processes may also condition protein digestibility, as heat and other processes can damage protein (Booth et al. 2005; Rossi and Davis 2014; Xavier et al. 2014). Tidwell et al. (2005) reported that when FM was replaced by 50% with MBM, growth reduction of largemouth bass was attributed not to the dietary amino acid composition but to their availability. Lysine is considered to be one of the first limiting amino acids in alternative ingredients to FM in aquafeeds (Kaushik and Seiliez 2010; NRC 2011) and, in processed animal ingredients, lysine is considered to be the amino acid most sensitive to heat damage during the rendering process (Nengas et al. 1999). Indeed, lysine availability may greatly differ among different batches of MBMs, ranging from 73 to 91% (Parsons et al. 1997). In the present study, even though lysine availability of the MBM ingredient was not determined, the obtained lysine digestibility coefficient and lysine retention efficiency for diet MBM50 suggested that amino acid availability of the MBM ingredient was little affected by the rendering process.

In the present study, EAA retention was not different for the three experimental diets. Lysine intake was significantly lower for the MBM diets but, for diet MBM50, the retention efficiency of lysine was even slightly higher (2.3%) than the control diet, suggesting a similar lysine efficiency utilization at a 50% replacement level. However, for diet MBM75, lysine retention was 12.5% lower than that of control diet. Despite the significant higher arginine intake for fish fed MBM diets, arginine retention in fish fed diet MBM75 decreased about 35.5%, though not statistically significant, compared to the non-MBM control diet. This lower arginine retention efficiency may be due to the high arginine content of the MBM based diets (>7 g 16 N⁻¹). A reduction of arginine utilization efficiency with an increase in intake is indeed to be expected due to a reduction of the absorption rate or to an increased metabolic utilization for other purposes than muscle growth, or both (Peres and Oliva-Teles 2008).

At the end of this trial, whole body composition was unaffected by the dietary MBM inclusion, with the exception of crude lipid and energy which were significantly lower for fish fed diet MBM75. Nutrient deposition in the body is related to the efficiency of its retention and, in this trial, whole-body crude protein and nitrogen retention efficiency were not significantly affected by the experimental diets although daily nitrogen intake was significant higher for diet MBM75. However, whole-body lipid content and retention, as well as energy retention, decreased in fish fed the diet with the highest level of MBM, suggesting a lower lipid and energy utilization efficiency with increasing MBM, as diets had similar crude lipid content (approximately 20% DM) and intake was significantly higher. Also for gilthead seabream, Robaina et al. (1997) reported a decrease, though not statistically significant, in both lipid digestibility and whole-body lipid content in gilthead seabream with increasing dietary MBM. Similar results were also obtained by Ai et al. (2006) where diets with more than 45% MBM caused a decrease in whole-body lipid content in large yellow croaker. On the contrary, juvenile snapper had a slight increase, although significant, of whole-body lipid content as dietary MBM increased (Booth et al. 2012), while other studies shown no significant differences in whole-body composition of fish fed diets with different levels of animal by-products (Bureau et al. 2000; Bharadwaj et al. 2002; Goda et al. 2007; Jamil et al. 2007), suggesting that the lipid utilization efficiency is influenced by either the species, quality of the ingredient or both.

Even though GIT microbiota modulation action due to the dietary incorporation of plant ingredients (Heikkinen et al. 2006; Refstie et al. 2006; Ringø et al. 2006b; Dimitroglou et al. 2010; Silva et al. 2011) and pre and probiotics (Dimitroglou et al. 2010; Cerezuela et al. 2012; Cerezuela et al. 2013; Kormas et al. 2014) has been previously reported, from all the literature found, this is the first study evaluating the effect of dietary animal by-products inclusion. In the present study, the inclusion of MBM in the diets for gilthead seabream modulated its GIT microbiota with significant changes in composition, and richness, while diversity was not significantly affected. A 50% FM replacement with MBM did not cause significant changes on the microbiota parameters analyzed, when compared to the non-MBM control diet, whereas an increase of the substitution level to 75% lead to a significant decrease in GIT microbial richness and OTUs, and to a significant increase in the similarity between replicates (i.e. homogeneity between individuals under the same treatment). Results from this study indicate that a 75% FM replacement with MBM may increase fish susceptibility to diseases as reduction of GIT microbial richness and diversity is often associated with higher susceptibility to diseases in both humans and animals (de Vos and de Vos 2012; Thomas et al. 2014). Also,

reduced diversity can compromise intestinal functionality as a diverse microbiota allows better adaptation to changing environmental conditions, such as those in aquaculture production (Cerezuela et al. 2012).

Besides the dietary effect on general GIT microbiota, significant differences in microbiota composition of different GIT sections (stomach, anterior, middle or posterior intestine) were observed, with an increase in the microbial diversity and richness towards the end of GIT. The lower microbial diversity and richness observed in the stomach might be explained by the harsh acidic stomach environment, which does not allow the establishment of bacteria unable to growth at low pH (Navarrete et al. 2009). Indeed, the pH variation of the different GIT compartments of juvenile fish can act as a selective mechanism, allowing colonization of some species and not others (Grisez et al. 1997). Besides the pH effect, the availability of digested nutrients, which is higher in the intestine than in the stomach, might also help to explain the higher microbial richness encountered in the last sections of the GIT, independent of the diet (Navarrete et al. 2009).

PCR-DGGE, followed by DGGE bands sequencing, is a powerful tool to determine the predominant bacteria present in GIT samples (Tapia-Paniagua et al. 2010). In present study, the predominant bacteria found from the sequenced bands belonged to the phyla Firmicutes (38.9%), followed by Actinobacteria (27.8%), uncultured bacteria (22.2%) and Proteobacteria (11.1 %). This is in accordance with previous studies where Proteobacteria, Firmicutes, and Actinobacteria prevailed in the gut of wild, organically or conventionally reared gilthead seabream, determined by pyrosequencing (Kormas et al. 2014), as well as in other farmed species such as grass carp (Han et al. 2010), yellow grouper (Zhou et al. 2009; Feng et al. 2010) and olive flounder (Kim and Kim 2013). Bacteroidetes, a predominant group found in gilthead seabream by Kormas et al. (2014), was not detected in this study.

Among the phyla described, the detectable predominant bacteria present in the stomach and intestine of gilthead seabream fed the experimental diets were most closely related to bacteria belonging to the *Staphylococcus*, *Vibrio*, *Corynebacterium*, *Weissella*, or *Bacillus* genera. A similar study reported *Diaphorobacter* as the dominant genus in wild and commercially reared gilthead seabream but this was not the case in the present study (Kormas et al. 2014).

Vibrio spp. and *Bacillus* spp. are particularly common genera found in the GIT of fish (Perez et al. 2010; He et al. 2013). In this study, *Vibrio* is the only genus that appears both in the intestine and stomach. While in the intestine it seems to appear in just in one replicate (middle intestine of fish fed diet MBM75), in the stomach, *Vibrio* appears to be

absent in the control diet and its bands become more pronounced with increasing MBM, suggesting that the inclusion of this ingredient promoted its appearance. Other authors have reported *Vibrio* as the dominant genus in juveniles and adult marine fish gut (Grisez et al. 1997; Tapia-Paniagua et al. 2010), but this was not observed in present study. Additionally, *Vibrio* is a common genus in aquatic environments, and its predominance in the stomach could also be attributed to the ingestion of the surrounding water since it is recognized that bacteria from water can survive and multiply in the digestive tract (Navarrete et al. 2009). Despite some species of this genus being pathogenic for fish (Heikkinen et al. 2006; Feng et al. 2010), others, such as *V. alginolyticus*, are beneficial for seabream larvae, competing with opportunist pathogenic bacteria (Grisez et al. 1997).

Similarly, MBM inclusion appears to potentiate the appearance of *Bacillus subtilis* as these bands become more pronounced (or only appear) in fish fed diet MBM75 in all intestinal sections. Other authors have also reported an increase in the presence of *Bacillus* spp. in the intestinal microbiota of rainbow trout fed diets with SBM (Heikkinen et al. 2006) and in Atlantic salmon fed diets with chitin (Askarian et al. 2012). Although Cerezuela et al. (2013) reported negative changes in the intestinal morphology of gilthead seabream when supplementing with a particular strain of *B. subtilis*, other strains are currently being used as probiotics in humans and animals (Cutting 2011), with different studies reporting the probiotic properties in fish, by enhancing fish immune response, growth performance and disease resistance (Nayak 2010; Sun et al. 2010; He et al. 2011, 2013; Liu et al. 2012).

The inclusion of MBM also appears to promote the development of *Mycobacterium* spp. as it is clearly more pronounced, though replicates were not homogenous, in the posterior intestine of fish fed diet MBM75. Bacteria from this genus are known to cause fish mycobacteriosis, a chronic disease characterized by the presence of numerous variable sized granulomas in tissues (Righetti et al. 2014), that can lead to high mortality rates in a variety of fish species worldwide (Stine et al. 2005; Sonda-Santos and Lara-Flores 2012) and can be pathogenic for humans due to its zoonotic potential and resistance to water disinfectants (Yanong et al. 2010). From all the literature found, there has been no report of the presence of *Mycobacterium* spp. in the GIT other than in fish with mycobacteriosis related symptomatology (Stine et al. 2005; Yanong et al. 2010; Sonda-Santos and Lara-Flores 2012; Righetti et al. 2014; Zhang et al. 2015). However, in this study, despite the presence of *Mycobacterium* spp. in the intestine of gilthead seabream fed diet MBM75, fish did not appear to show symptoms of the disease.

Some studies have also reported presence of *Staphylococcus* spp. in the GIT of fish (Ringø et al. 2006a; Ringø et al. 2006b; Bakke-McKellep et al. 2007; Askarian et al. 2012; Cantas et al. 2012) as well as *Corynebacterium* spp. (Al-Harbi and Naim Uddin 2004; Wu et al. 2010). In present study, *Staphylococcus* spp. and *Corynebacterium* spp. were present in all intestinal sections, but more predominantly in fish fed the non-MBM control diet, indicating that inclusion of MBM may reduce fish colonization by species of these genus. This might be beneficial since these genus are often associated with pathogenic species for humans and animals (Thomas et al. 2014). In particular *C. aquaticum* is considered to be pathogenic for fish, such as striped bass and rainbow trout, and mice (Baya et al. 1992).

Finally, the genus *Weissella* was present in all intestinal sections and more predominant in fish fed the non-MBM control diet, suggesting that gradual inclusion of MBM also led to its disappearance. While some strains of these genus are considered to be pathogenic for farmed rainbow trout (Figueiredo et al. 2012), other strains of *Weissella* are receiving attention as potential probiotics (Fusco et al. 2015) and Weissellin A, a protein produced by these bacteria, has shown to have antimicrobial properties, suitable for food and feed preservation (Papagianni and Papamichael 2012).

Conclusion

The future of aquaculture nutrition will rely on the search for alternative protein sources for FM replacement as current inclusion rates threaten the expansion of the industry. Results from present study indicate that MBM is a promising ingredient and that a 50% substitution did not compromise growth performance and feed utilization of gilthead seabream juveniles. However, a substitution up to 75% MBM led to a decrease in growth, lipid and energy retention and EAA digestibility. Although ADCs of EAA were high for all experimental diets (>92%), they were significantly reduced by the inclusion of MBM. The reduced performance of 75% MBM diet may be attributed to its high ash content and high levels of saturated fats that may have compromised nutrient digestibility.

Species diversity was not affected by the MBM inclusion level. However, only the 50% substitution with MBM maintained the OTUs and species richness unaltered, indicating that higher levels might compromise the GIT microbiota stability. Also, MBM

appeared to promote the development of *Bacillus* genus, a group of organisms commonly associated with beneficial effects in animal health, namely as probiotics but also *Vibrio* and *Mycobacterium* genus, often associated with pathogenic bacteria.

Overall, MBM has the potential to be included in diets for gilthead seabream juveniles but better characterization of this product is required in order to improve utilization and feeding strategies. The next step in this research could be the evaluation of the performance of a MBM intermediate inclusion level (between the 50 and 75% FM replacement level). Also the effect of dietary inclusion of MBM on general intestine health fish, fish wellbeing and immune status, as well as on flesh quality traits of gilthead seabream deserves further research.

References

- Ai, Q., Mai, K., Tan, B., Xu, W., Duan, Q., Ma, H., and Zhang, L. (2006). "Replacement of fish meal by meat and bone meal in diets for large yellow croaker, *Pseudosciaena crocea*." *Aquaculture*, 260(1-4), 255-263.
- Al-Harbi, A. H., and Naim Uddin, M. (2004). "Seasonal variation in the intestinal bacterial flora of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia." *Aquaculture*, 229(1-4), 37-44.
- Alexis, M. N. (1997). "Fish meal and fish oil replacers in Mediterranean marine fish diets", A. Tacon and B. Basurco, (eds.), *Feeding Tomorrow's Fish*. City: CIHEAM: Zaragoza, Spain, pp. 183–204.
- Allan, G. L., Stone, D. A. J., Parkinson, S., Rowland, S. J., Booth, M. A., Frances, J., and Warner-Smith, R. (2000). "Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: I. Digestibility of alternative ingredients." *Aquaculture*, 186, 293–310.
- Allan, G. L., and Rowland, S. J. (2005). "Performance and sensory evaluation of silver perch (*Bidyanus bidyanus* Mitchell) fed soybean or meat meal-based diets in earthen ponds." *Aquaculture Research*, 36(13), 1322-1332.
- Altan, O., Gamsiz, K., and Korkut, A. Y. (2010). "Soybean meal and rendered animal protein ingredients replace fishmeal in practical diets for sea bass." *The Israeli Journal of Aquaculture - Bamidgeh*, 62(2), 56-62.
- AOAC. (1990). *Official Methods of Analysis, 15th ed.*, Arlington, VA, USA: Association of Official Analytical Chemists.
- Askarian, F., Zhou, Z., Olsen, R. E., Sperstad, S., and Ringø, E. (2012). "Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens." *Aquaculture*, 326-329, 1-8.
- Atkinson, J. L., Hilton, J. W., and Slinger, S. J. (1984). "Evaluation of acid-insoluble ash as an indicator of feed digestibility in rainbow trout (*Salmo gairdneri*)." *Canadian Journal of Fisheries and Aquatic Sciences*, 41, 1384-1386.
- Baeza-Ariño, R., Martínez-Llorens, S., Nogales-Mérida, S., Jover-Cerda, M., and Tomás-Vidal, A. (2014). "Study of liver and gut alterations in sea bream, *Sparus aurata* L., fed a mixture of vegetable protein concentrates." *Aquaculture Research*, 1-12.
- Bakke-McKellep, A. M., Penn, M. H., Salas, P. M., Refstie, S., Sperstad, S., Landsverk, T., Ringo, E., and Krogdahl, A. (2007). "Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.)." *British Journal of Nutrition*, 97(4), 699-713.
- Basurco, B., Lovatelli, A., and García, B. (2011). "Chapter 1: Current Status of Sparidae Aquaculture", in M. A. Pavlidis and C. C. Mylonas, (eds.), *Sparidae: Biology and Aquaculture of Gilthead Sea Bream and Other Species*. West Sussex, UK: Blackwell Publishing Ltd.
- Baya, A. M., Lupiani, B., Bandín, I., Hetrick, F. M., Figueras, A., Carnahan, A., May, E. M., and Toranzo, A. E. (1992). "Phenotypic and pathobiological properties of

- Corynebacterium aquaticum* isolated from diseased striped bass." *Diseases of Aquatic Organisms*, 14, 115-126.
- Berge, G. E., Bakke-McKellep, A. M., and Lied, E. (1999). "In vitro uptake and interaction between arginine and lysine in the intestine of Atlantic salmon (*Salmo salar*)." *Aquaculture*, 179, 181-193.
- Bharadwaj, A. S., Brignon, W. R., Gould, N. L., and Brown, P. B. (2002). "Evaluation of meat and bone meal in practical diets fed to juvenile hybrid striped bass *Morone chrysops* x *M. saxatilis*." *Journal of the World Aquaculture Society*, 33(4), 448-457.
- BOE. (2013). "Real Decreto 53/2013 sobre protección de animales utilizados en experimentación y otros fines científicos", BOE, (ed.) 34. City: Boletín Oficial del Estado: Madrid, pp. 11370-11421.
- Booth, M. A., Allan, G. L., and Anderson, A. J. (2005). "Investigation of the nutritional requirements of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801): apparent digestibility of protein and energy sources." *Aquaculture Research*, 36(4), 378-390.
- Booth, M. A., Allan, G. L., and Anderson, A. J. (2012). "Influence of poultry meal, meat meal or soybean meal inclusion on weight gain and production characteristics of Australian snapper *Pagrus auratus*." *Aquaculture International*, 20(1), 99-115.
- Booth, M. A., Allan, G. L., and Smullen, R. P. (2013). "Digestibility of common feed ingredients by juvenile mullet *Argyrosomus japonicus*." *Aquaculture*, 414-415, 140-148.
- Brouwer, E. "Report of sub-committee on constants and factors." *Presented at Proceedings of the Third EAAP Symposium on Energy Metabolism*, London.
- Bureau, D. P., Harris, A. M., and Cho, C. Y. (1999). "Apparent digestibility of rendered animal protein ingredients for rainbow trout (*Oncorhynchus mykiss*)." *Aquaculture*, 180, 345-358.
- Bureau, D. P., Harris, A. M., Bevan, D. J., Simmons, L. A., Azevedo, P. A., and Cho, C. Y. (2000). "Feather meals and meat and bone meals from different origins as protein sources in rainbow trout (*Oncorhynchus mykiss*) diets." *Aquaculture*, 181, 281-291.
- Bureau, D. P. (2006). "Rendered Products In Fish Aquaculture Feeds", in D. L. Meeker, (ed.), *Essential Rendering: All About The Animal By-Products Industry*. Alexandria, Virginia: National Renderers Association.
- Cantas, L., Sorby, J. R., Alestrom, P., and Sorum, H. (2012). "Culturable gut microbiota diversity in zebrafish." *Zebrafish*, 9(1), 26-37.
- Cerdá, M. J. (2012). "Futuro de la alimentación de los peces en granjas marinas" *AquaTIC*. City: Universidad de Zaragoza: Zaragoza, España, pp. 78-89.
- Cerezuela, R., Fumanal, M., Tapia-Paniagua, S. T., Meseguer, J., Morinigo, M. A., and Esteban, M. A. (2012). "Histological alterations and microbial ecology of the intestine in gilthead seabream (*Sparus aurata* L.) fed dietary probiotics and microalgae." *Cell and Tissue Research*, 350(3), 477-89.
- Cerezuela, R., Fumanal, M., Tapia-Paniagua, S. T., Meseguer, J., Morinigo, M. A., and Esteban, M. A. (2013). "Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata* L.) specimens." *Fish and Shellfish Immunology*, 34(5), 1063-70.

- Cho, C. Y., Slinger, S. J., and Bayley, H. S. (1982). "Bioenergetics of salmonid fishes: Energy intake, expenditure and productivity." *Comparative Biochemistry and Physiology*, 73B(1), 25-41.
- Cho, C. Y., and Kaushik, S. J. (1990). "Nutritional energetics in fish: energy and protein utilization in rainbow trout (*Salmo gairdneri*)." *World Review of Nutrition and Dietetics Home* 61, 132-172.
- Clements, K. D., Angert, E. R., Montgomery, W. L., and Choat, J. H. (2014). "Intestinal microbiota in fishes: what's known and what's not." *Molecular Ecology*, 23(8), 1891-1898.
- Cutting, S. M. (2011). "Bacillus probiotics." *Food Microbiology*, 28(2), 214-20.
- Davies, S. J., Nengas, I., and Alexis, M. "Partial substitution of fish meal with different meat meal product in diets for seabream (*Sparus aurata*)." *Presented at Fish nutrition in practice: IVth International Symposium on Fish Nutrition and Feeding*, Biarritz, France.
- de Vos, W. M., and de Vos, E. A. (2012). "Role of the intestinal microbiome in health and disease: from correlation to causation." *Nutrition Reviews*, 70 Suppl 1, S45-56.
- Deutsch, L., Gräslund, S., Folke, C., Troell, M., Huitric, M., Kautsky, N., and Lebel, L. (2007). "Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal." *Global Environmental Change*, 17(2), 238-249.
- Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R., and Davies, S. J. (2010). "Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*)." *Aquaculture*, 300(1-4), 182-188.
- Dimitroglou, A., Merrifield, D. L., Carnevali, O., Picchiatti, S., Avella, M., Daniels, C., Guroy, D., and Davies, S. J. (2011). "Microbial manipulations to improve fish health and production - a Mediterranean perspective." *Fish and Shellfish Immunology*, 30(1), 1-16.
- El-Haroun, E. R., Azevedo, P. A., and Bureau, D. P. (2009). "High dietary incorporation levels of rendered animal protein ingredients on performance of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1972)." *Aquaculture*, 290(3-4), 269-274.
- El-Sayed, A.-F. M. (1998). "Total replacement of fish meal with animal protein sources in Nile tilapia, *Oreochromis niloticus* (L.), feeds." *Aquaculture Research*, 29, 275-280.
- Emre, Y., Sevgili, H., and Sanli, M. (2008). "Partial replacement of fishmeal with hazelnut meal in diets for juvenile gilthead sea bream (*Sparus aurata*)." *The Israeli Journal of Aquaculture - Bamidgeh*, 60(3), 198-204.
- Enes, P., Panserat, S., Kaushik, S., and Oliva-Teles, A. (2011). "Dietary carbohydrate utilization by European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) juveniles." *Reviews in Fisheries Science*, 19(3), 201-215.
- Engin, K., and Carter, C. G. (2005). "Fish meal replacement by plant and animal by-products in diets for the Australian short-finned eel, *Anguilla australis australis* (Richardson)." *Aquaculture Research*, 36, 445-454.
- Ercolini, D. (2004). "PCR-DGGE fingerprinting: novel strategies for detection of microbes in food." *Journal of Microbiological Methods*, 56(3), 297-314.
- Estruch, G., Collado, M. C., Penaranda, D. S., Tomas Vidal, A., Jover Cerda, M., Perez Martinez, G., and Martinez-Llorens, S. (2015). "Impact of fishmeal replacement

- in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene." *PLoS One*, 10(8), e0136389.
- EUMOFA. (2014). *The EU Fish Market*. European Market Observatory for Fisheries and Aquaculture Products, Brussels.
- European Union. (2001). "Regulation (EC) No 999/2001 of the European parliament and of the council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies", E. Union, (ed.) *999/2001*. City: Brussels.
- European Union. (2013). "Comission Regulation (EU) No 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies", E. Union, (ed.) *56/201*. City: Brussels.
- FAO. (2011). *Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects*. Food And Agriculture Organization Of The United Nations, Rome.
- FAO. (2012). *The State of World Fisheries and Aquaculture*. Food and Agriculture Organization of the United Nations, Rome.
- FAO. (2014). *The State of World Fisheries and Aquaculture. Opportunities and Challenges*. Food and Agriculture Organization of the United Nations, Rome.
- Feng, J.-B., Hu, C.-Q., Luo, P., Zhang, L.-P., and Chen, C. (2010). "Microbiota of yellow grouper (*Epinephelus awoora* Temminck & Schlegel, 1842) fed two different diets." *Aquaculture Research*, 41(12), 1778-1790.
- Figueiredo, H. C., Costa, F. A., Leal, C. A., Carvalho-Castro, G. A., and Leite, R. C. (2012). "Weissella sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil." *Veterinary Microbiology*, 156(3-4), 359-66.
- Flos, R., Reigh, L., Oca, J., and Ginovart, M. (2002). "Influence of marketing and different land-based systems on gilthead sea bream (*Sparus aurata*) quality." *Aquaculture International*, 10, 189–206.
- Forster, I., and Dominy, W. (2006). "Use of rendered terrestrial animal by-products in aquatic feeds", L. E. C. Suárez, D. R. Marie, M. T. Salazar, M. G. N. López, D. A. V. Cavazos, A. C. P. Cruz, and A. G. Ortega, (eds.), *Avances en Nutrición Acuicola VIII .VIII Simposium Internacional de Nutrición Acuicola*. City: Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México.
- Friedland, R. P., Petersen, R. B., and Rubenstein, R. (2009). "Bovine spongiform encephalopathy and aquaculture." *Journal of Alzheimer's Disease*, 17(2), 277-9.
- Fusco, V., Quero, G. M., Cho, G. S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., and Franz, C. M. (2015). "The genus Weissella: taxonomy, ecology and biotechnological potential." *Frontiers in Microbiology*, 6, 155.
- Gao, S., Yin, N., Zhou, F., Li, H., Zhou, J., Wang, R. J., and Shao, Q. J. (2013). "Evaluation of pea proteins and poultry protein as fish meal alternatives in the diets for juvenile black sea bream, *Acanthopagrus schlegelii*." *Aquaculture Nutrition*, 19(3), 278-288.
- García-Gallego, M., Akharbach, H., and de la Higuera, M. (1998). "Use of protein sources alternative to fish meal in diets with amino acids supplementation for the European eel (*Anguilla anguilla*)." *Animal Science*, 66(01), 285-292.

- Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R., and Wurtele, E. (2007). "Expanding the utilization of sustainable plant products in aquafeeds: a review." *Aquaculture Research*, 38(6), 551-579.
- Glencross, B. D., Booth, M., and Allan, G. L. (2007). "A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds." *Aquaculture Nutrition*, 13, 17-34.
- Goda, A. M., El-Haroun, E. R., and Kabir Chowdhury, M. A. (2007). "Effect of totally or partially replacing fish meal by alternative protein sources on growth of African catfish *Clarias gariepinus* (Burchell, 1822) reared in concrete tanks." *Aquaculture Research*, 38(3), 279-287.
- Gómez-Requeni, P., Mingarro, M., Caldach-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan, D. F., Kaushik, S., and Pérez-Sánchez, J. (2004). "Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*)." *Aquaculture*, 232(1-4), 493-510.
- Grisez, L., Reyniers, J., Verdonck, L., Swings, J., and Ollevier, F. (1997). "Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development." *Aquaculture*, 155, 387-399.
- Guo, J., Wang, Y., and Bureau, D. P. (2007). "Inclusion of rendered animal ingredients as fishmeal substitutes in practical diets for cuneate drum, *Nibea miichthioides* (Chu, Lo et Wu)." *Aquaculture Nutrition*, 13, 81-87.
- Han, S., Liu, Y., Zhou, Z., He, S., Cao, Y., Shi, P., Yao, B., and Ringø, E. (2010). "Analysis of bacterial diversity in the intestine of grass carp (*Ctenopharyngodon idellus*) based on 16S rDNA gene sequences." *Aquaculture Research*, 42(1), 47-56.
- Hardy, R. W. (2000). "New developments in aquatic feed ingredients, and potential of enzyme supplements", L. E. Cruz -Suárez, D. Ricque-Marie, M. Tapia-Salazar, M. A. Olvera-Novoa, and R. Civera-Cerecedo, (eds.), *Avances en Nutrición Acuicola V. Memorias del V Simposium Internacional de Nutrición Acuicola*. City: Mérida, Yucatán, Mexico.
- Hardy, R. W., and Barrows, F. T. (2002). "Diet Formulation and Manufacture", in J. E. Halver and R. W. Hardy, (eds.), *Fish Nutrition*. USA: Academic Press - Elsevier Science
- Haylor, G., and Bland, S. "Integrating aquaculture into rural development in coastal and inland areas." *Presented at Aquaculture in the Third Millennium*, Bangkok, Thailand.
- He, S., Liu, W., Zhou, Z., Mao, W., Ren, P., Marubashi, T., and Ringø, E. (2011). "Evaluation of probiotic strain *Bacillus subtilis* C-3102 as a feed supplement for koi carp (*Cyprinus carpio*)." *Journal of Aquatic Research Developmental*, S1, 005.
- He, S., Zhang, Y., Xu, L., Yang, Y., Marubashi, T., Zhou, Z., and Yao, B. (2013). "Effects of dietary *Bacillus subtilis* C-3102 on the production, intestinal cytokine expression and autochthonous bacteria of hybrid tilapia *Oreochromis niloticus* ♀ x *Oreochromis aureus* ♂." *Aquaculture*, 412-413, 125-130.
- Heikkinen, J., Vielma, J., Kemiläinen, O., Tirola, M., Eskelinen, P., Kiuru, T., Navia-Paldanius, D., and von Wright, A. (2006). "Effects of soybean meal based diet on

- growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*)." *Aquaculture*, 261(1), 259-268.
- Hendrick, R. L., Opoma, T. J., and Davis, D. A. (2005). "Pond production and fatty acid profiles of the fillets of channel catfish reared on diets with different protein sources." *North American Journal of Aquaculture*, 67, 304-311.
- Hernández, C., Sanchez-Gutierrez, Y., Hardy, R. W., Benitez-Hernández, A., Domínguez-Jimenez, P., González-Rodríguez, B., Osuna-Osuna, L., and Tortoledo, O. (2014). "The potential of pet-grade poultry by-product meal to replace fish meal in the diet of the juvenile spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869)." *Aquaculture Nutrition*, 20(6), 623-631.
- Hovda, M. B., Lunestad, B. T., Fontanillas, R., and Rosnes, J. T. (2007). "Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.)." *Aquaculture*, 272(1-4), 581-588.
- Hu, L., Yun, B., Xue, M., Wang, J., Wu, X., Zheng, Y., and Han, F. (2013). "Effects of fish meal quality and fish meal substitution by animal protein blend on growth performance, flesh quality and liver histology of Japanese seabass (*Lateolabrax japonicus*)." *Aquaculture*, 372-375, 52-61.
- Hu, M., Wang, Y., Luo, Z., Zhao, M., Xiong, B., Qian, X., and Zhao, Y. (2008a). "Evaluation of rendered animal protein ingredients for replacement of fish meal in practical diets for gibel carp, *Carassius auratus gibelio* (Bloch)." *Aquaculture Research*, 39(14), 1475-1482.
- Hu, M., Wang, Y., Wang, Q., Zhao, M., Xiong, B., Qian, X., Zhao, Y., and Luo, Z. (2008b). "Replacement of fish meal by rendered animal protein ingredients with lysine and methionine supplementation to practical diets for gibel carp, *Carassius auratus gibelio*." *Aquaculture*, 275(1-4), 260-265.
- Hunter, B. J., Allan, G. L., and Roberts, D. C. K. (2000). "Meat Meal Replacement in Diets for Silver Perch, *Bidyanus bidyanus*." *Journal of Applied Aquaculture*, 10(3), 51-67.
- INE. (2015). *Estatísticas da Pesca 2014*. Instituto Nacional de Estatística, I.P., Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos (DGRM), Lisboa, Portugal.
- IUCN. (2007). *Guide for the Sustainable Development of Mediterranean Aquaculture. Interaction between Aquaculture and the Environment*, Spain The World Conservation Union (IUCN), Gland, Switzerland and Malaga.
- Jamil, K., Abbas, G., Akhtar, R., Lin, H., and Li, Z. (2007). "Effects of replacing fishmeal with animal by-products meal supplementation in diets on the growth and nutrient utilization of mangrove red snapper." *Journal of Ocean University of China*, 6(3), 292-298.
- Kader, M. A., Bulbul, M., Ahmed, G. U., Hossain, M. S., Hossain, M. A., and Koshio, S. (2011a). "Effects of animal proteins in practical diets on growth and economic performance of climbing perch, *Anabas testudineus* (Bloch)." *Journal of Applied Aquaculture*, 23(2), 166-176.
- Kader, M. A., Bulbul, M., Yokoyama, S., Ishikawa, M., Koshio, S., Hossain, M. S., Ahmed, G. U., and Hossain, M. A. (2011b). "Evaluation of meat and bone meal as replacement for protein concentrate in the practical diet for sutchi catfish, *Pangasius hypophthalmus* (Sauvage 1878), reared under pond condition." *Journal of the World Aquaculture Society*, 42(3), 287-296.

- Karapanagiotidis, I. T. (2014). "The re-authorization of non-ruminant processed animal proteins in European aquafeeds." *Fisheries and Aquaculture Journal*, 5(4), e111.
- Kaushik, S. J., and Medale, F. (1994). "Energy Requirements, Utilization and Dietary Supply to Salmonids." *Aquaculture*, 124(1-4), 81-97.
- Kaushik, S. J., and Seiliez, I. (2010). "Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs." *Aquaculture Research*, 41(3), 322-332.
- Kikuchi, K., Sato, T., Furuta, T., Sakagushi, I., and Deguchi, Y. (1997). "Use of meat and bone meal as a protein source in the diet of juvenile Japanese flounder." *Fisheries Science*, 63(1), 29-32.
- Kim, D. H., and Kim, D. (2013). "Microbial diversity in the intestine of olive flounder (*Paralichthys olivaceus*)." *Aquaculture*, 414-415, 103-108.
- Kissil, G. W., Lupatsch, I., Higgs, D. A., and Hardy, R. W. (2000). "Dietary substitution of soy and rapeseed protein concentrates for fish meal, and their effects on growth and nutrient utilization in gilthead seabream *Sparus aurata* L." *Aquaculture Research*, 31, 595-601.
- Kissil, G. W., and Lupatsch, I. (2004). "Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L." *The Israeli Journal of Aquaculture - Bamidgeh*, 56(3), 188-199.
- Kokou, F., Rigos, G., Henry, M., Kentouri, M., and Alexis, M. (2012). "Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal." *Aquaculture*, 364-365, 74-81.
- Kormas, K. A., Meziti, A., Mente, E., and Frentzos, A. (2014). "Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*)." *Microbiologyopen*, 3(5), 718-28.
- Kureshy, N., Davis, D. A., and Arnold, C. R. (2000). "Partial replacement of fish meal with meat-and-bone meal, flash-dried poultry by-product meal, and enzyme-digested poultry by-product meal in practical diets for juvenile red drum." *North American Journal of Aquaculture*, 62(4), 266-272.
- Lee, K.-J., Dabrowski, K., Blom, J. H., and Bai, S. C. (2001). "Replacement of Fish Meal by a Mixture of Animal By-Products in Juvenile Rainbow Trout Diets." *North American Journal of Aquaculture*, 63(2), 109-117.
- Lee, J., Choi, I. C., Kim, K. T., Cho, S. H., and Yoo, J. Y. (2012). "Response of dietary substitution of fishmeal with various protein sources on growth, body composition and blood chemistry of olive flounder (*Paralichthys olivaceus*, Temminck & Schlegel, 1846)." *Fish Physiology and Biochemistry*, 38(3), 735-44.
- Li, K., Wang, Y., Zheng, Z.-X., Jiang, R.-L., Xie, N.-X., and Bureau, D. P. (2009). "Replacing fish meal with rendered animal protein ingredients in diets for malabar grouper, *Epinephelus malabaricus*, reared in net pens." *Journal of the World Aquaculture Society*, 40(1), 67-75.
- Li, J., Zhang, L., Mai, K., Ai, Q., Zhang, C., Li, H., Duan, Q., Ma, H., Zhang, L., and Zheng, S. (2010). "Potential of several protein sources as fish meal substitutes in diets for large yellow croaker, *Pseudosciaena crocea* R." *Journal of the World Aquaculture Society*, 41(S2).
- Libralato, S., and Solidoro, C. (2008). "A bioenergetic growth model for comparing *Sparus aurata*'s feeding experiments." *Ecological Modelling*, 214(2-4), 325-337.

- Liu, C. H., Chiu, C. H., Wang, S. W., and Cheng, W. (2012). "Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*." *Fish and Shellfish Immunology*, 33(4), 699-706.
- Lovell, T. (1998). *Nutrition and Feeding of Fish*, Norwell, Massachusetts, USA: Kluwer Academic Publishers.
- Mabrouk, H. A., and Nour, A. M. (2011). "Assessment of apparent digestibility coefficients (ADCs %) of some animal protein sources by gilthead sea bream (*Sparus aurata*)." *Egyptian Journal of Aquatic Research*, 37(2), 171-177.
- Martínez-Llorens, S., Moñino, A. V., Tomás Vidal, A., Salvador, V. J. M., Pla Torres, M., and Jover Cerdá, M. (2007). "Soybean meal as a protein source in gilthead sea bream (*Sparus aurata* L.) diets: effects on growth and nutrient utilization." *Aquaculture Research*, 38(1), 82-90.
- Martínez-Llorens, S., Vidal, A. T., Moñino, A. V., Gómez Ader, J., Torres, M. P., and Cerdá, M. J. (2008). "Blood and haemoglobin meal as protein sources in diets for gilthead sea bream (*Sparus aurata*): effects on growth, nutritive efficiency and fillet sensory differences." *Aquaculture Research*, 39(10), 1028-1037.
- Martínez-Llorens, S., Baeza-Ariño, R., Nogales-Mérida, S., Jover-Cerdá, M., and Tomás-Vidal, A. (2012). "Carob seed germ meal as a partial substitute in gilthead sea bream (*Sparus aurata*) diets: Amino acid retention, digestibility, gut and liver histology." *Aquaculture*, 338-341, 124-133.
- Masagounder, K., Hayward, R. S., and Firman, J. D. (2014). "Replacing fish meal with increasing levels of meat and bone meal, soybean meal and corn gluten meal, in diets of juvenile bluegill, *Lepomis macrochirus*." *Aquaculture Research*, 45, 1202-1211.
- Merinero, S., Martínez, S., Tomás, A., and Jover, M. (2005). "Análisis económico de alternativas de producción de Dorada en jaulas marinas en el litoral Mediterráneo español." *Revista AquaTIC*, 23, 1-19.
- Millamena, O. M., and Golez, N. V. (2001). "Evaluation of processed meat solubles as replacement for fish meal in diets for juvenile grouper *Epinephelus coioides* (Hamilton)." *Aquaculture Research*, 31(1), 281-287.
- Millamena, O. M. (2002). "Replacement of fish meal by animal by-product meals in a practical diet for grow-out culture of grouper *Epinephelus coioides*." *Aquaculture*, 204, 75-84.
- Montero, D., Lalumera, G., Izquierdo, M. S., Caballero, M. J., Saroglia, M., and Tort, L. (2009). "Establishment of dominance relationships in gilthead sea bream *Sparus aurata* juveniles during feeding: effects on feeding behaviour, feed utilization and fish health." *Journal of Fish Biology*, 74(4), 790-805.
- Moretti, A., Fernandez-Criado, M. P., Cittolin, G., and Guidastrì, R. (1999). *Manual on Hatchery Production of Seabass and Gilthead Seabream. Volume 1.*, Rome: FAO.
- Muyzer, G., Waal, E. C., and Uitterlinder, A. G. (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA." *Applied and Environmental Microbiology*, 59(3), 695-700.
- Navarrete, P., Espejo, R. T., and Romero, J. (2009). "Molecular analysis of microbiota along the digestive tract of juvenile Atlantic salmon (*Salmo salar* L.)." *Microb Ecol*, 57(3), 550-61.

- Nayak, S. K. (2010). "Role of gastrointestinal microbiota in fish." *Aquaculture Research*, 41(11), 1553-1573.
- Nengas, I., Alexis, M. N., and Davies, S. J. (1999). "High inclusion levels of poultry meals and related byproducts in diets for gilthead seabream *Sparus aurata* L." *Aquaculture*, 179, 13–23.
- NRC. (1993). *Nutrient Requirements of Fish*, Washington, D.C.: National Academy Press.
- NRC. (2011). *Nutrient Requirements of Fish and Shrimp*, Washington, D.C.: The National Academies Press.
- Oliva-Teles, A., Lupatsch, I., and Nengas, I. (2011). "Chapter 7: Nutrition and feeding of Sparidae", in M. A. Pavlidis and C. C. Mylonas, (eds.), *Sparidae: Biology and aquaculture of gilthead sea bream and other species*. West Sussex, UK: Blackwell Publishing Ltd.
- Papagianni, M., and Papamichael, E. M. (2012). "Production of the antimicrobial protein weisselin A by *Weissella paramesenteroides* DX in batch fermentations: the type of carbohydrate used as the C-source in the substrate affects the association of production with growth." *Applied Biochemistry and Biotechnology*, 168(5), 1212-22.
- Parsons, C. M., Castanon, F., and Han, Y. (1997). "Protein and amino acid quality of meat and bone meal." *Poultry Science*, 76, 361-368.
- Pereira, T. G., and Oliva-Teles, A. (2002). "Preliminary evaluation of pea seed meal in diets for gilthead sea bream (*Sparus aurata*) juveniles." *Aquaculture Research*, 33, 1183-1189.
- Pereira, T. G., and Oliva-Teles, A. (2003). "Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles." *Aquaculture Research*, 34, 1111-1117.
- Pereira, T. G., and Oliva-Teles, A. (2004). "Evaluation of micronized lupin seed meal as an alternative protein source in diets for gilthead sea bream *Sparus aurata* L. juveniles." *Aquaculture Research*, 35(9), 828-835.
- Peres, H., and Oliva-Teles, A. (2006). "Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*)." *Aquaculture*, 256, 395-402.
- Peres, H., and Oliva-Teles, A. (2007). "Effect of the dietary essential amino acid pattern on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*)." *Aquaculture*, 267, 119-128.
- Peres, H., and Oliva-Teles, A. (2008). "Lysine requirement and efficiency of lysine utilization in turbot (*Scophthalmus maximus*) juveniles." *Aquaculture*, 275(1-4), 283-290.
- Peres, H., and Oliva-Teles, A. (2009). "The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles." *Aquaculture*, 296(1-2), 81-86.
- Perez, T., Balcazar, J. L., Ruiz-Zarzuola, I., Halaihel, N., Vendrell, D., de Blas, I., and Muzquiz, J. L. (2010). "Host-microbiota interactions within the fish intestinal ecosystem." *Mucosal Immunology*, 3(4), 355-60.
- Pita, C., Gamito, S., and Erzini, K. (2002). "Feeding habits of the gilthead seabream (*Sparus aurata*) from the Ria Formosa (southern Portugal) as compared to the black seabream (*Spondylisoma cantharus*) and the annular seabream (*Diplodus annularis*)." *Journal of Applied Ichthyology*, 18, 81-86.

- Pitcher, D. G., Saunders, N. A., and Owen, R. J. (1989). "Rapid extraction of bacterial genomic DNA with guanidium thiocyanate." *Letters in Applied Microbiology*, 8, 151-156.
- Rawles, S. D., Thompson, K. R., Brady, Y. J., Metts, L. S., Aksoy, M. Y., Gannam, A. L., Twibell, R. G., Ostrand, S., and Webster, C. D. (2011). "Effects of replacing fish meal with poultry by-product meal and soybean meal and reduced protein level on the performance and immune status of pond-grown sunshine bass (*Morone chrysops* × *M. saxatilis*)." *Aquaculture Nutrition*, 17(3), e708-e721.
- Rawls, J. F., Samuel, B. S., and Gordon, J. I. (2004). "Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota." *Proceedings of the National Academy of Sciences, PNAS*, 101(13), 4596-601.
- Read, P., and Fernandes, T. (2003). "Management of environmental impacts of marine aquaculture in Europe." *Aquaculture*, 226(1-4), 139-163.
- Refstie, S., Landsverk, T., Bakke-McKellep, A. M., Ringø, E., Sundby, A., Shearer, K. D., and Krogdahl, Å. (2006). "Digestive capacity, intestinal morphology, and microflora of 1-year and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal." *Aquaculture*, 261(1), 269-284.
- Righetti, M., Favaro, L., Antuofermo, E., Caffara, M., Nuvoli, S., Scanzio, T., and Prearo, M. (2014). "Mycobacterium salmoniphilum infection in a farmed Russian sturgeon, *Acipenser gueldenstaedtii* (Brandt & Ratzeburg)." *Journal of Fish Diseases*, 37(7), 671-4.
- Ringø, E., Sperstad, S., Myklebust, R., Mayhew, T. M., and Olsen, R. E. (2006a). "The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.)." *Aquaculture Research*, 37(9), 891-897.
- Ringø, E., Sperstad, S., Myklebust, R., Refstie, S., and Krogdahl, Å. (2006b). "Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.)." *Aquaculture*, 261(3), 829-841.
- Robaina, L., Izquierdo, M. S., Moyano, F. J., Socorro, J., Vergara, J. M., Montero, D., and Fernández-Palacios, H. (1995). "Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications." *Aquaculture*, 130, 219-233.
- Robaina, L., Moyano, F. J., Izquierdo, M. S., Socorro, J., Vergara, J. M., and Montero, D. (1997). "Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (*Sparus aurata*): Nutritional and histological implications." *Aquaculture*, 157, 347-359.
- Rodríguez-Serna, M., Olvera-Novoa, M. A., and Carmona-Osalde, C. (1996). "Nutritional value of animal by-product meal in practical diets for Nile tilapia *Oreochromis niloticus* (L.) fry." *Aquaculture Research*, 27, 67-73.
- Rossi, W., and Davis, D. A. (2014). "Meat and bone meal as an alternative for fish meal in soybean meal-based diets for Florida pompano, *Trachinotus carolinus* L." *Journal of the World Aquaculture Society*, 45(6), 613-624.
- Sánchez-Lozano, N. B., Martínez-Llorens, S., Tomás-Vidal, A., and Jover Cerdá, M. (2010). "Amino acid retention of gilthead sea bream (*Sparus aurata*, L.) fed with pea protein concentrate." *Aquaculture Nutrition*, 17(2), e604-e614.
- Silva, J. G. d., and Oliva-Teles, A. (1998). "Apparent digestibility coefficients of feedstuffs in seabass (*Dicentrarchus labrax*) juveniles." *Aquatic Living Resources*, 11(3), 187-191.

- Silva, F. C., Nicoli, J. R., Zambonino-Infante, J. L., Kaushik, S., and Gatesoupe, F. J. (2011). "Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*)." *FEMS Microbiology Ecology*, 78(2), 285-96.
- Sonda-Santos, K. Y., and Lara-Flores, M. (2012). "Detection of Mycobacterium spp. by polymerase chain reaction in Nile tilapia (*Oreochromis niloticus*) in Campeche, Mexico." *African Journal of Microbiology Research*, 6(11), 2785-2787.
- Stine, C. B., Baya, A. M., Salierno, J. D., Kollner, M., and Kane, A. S. (2005). "Mycobacterial infection in laboratory-maintained Atlantic menhaden." *Journal of Aquatic Animal Health*, 17(4), 380-385.
- Stone, A. J. D., Allan, G. L., Parkinson, S., and Rowland, S. J. (2000). "Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus* Ill. Digestibility and growth using meat meal products." *Aquaculture*, 186, 311-326.
- Sullivan, J. A., and Reigh, R. C. (1995). "Apparent digestibility of selected feedstuffs in diets for hybrid striped bass (*Morone saxatilis* x *Morone chrysops*)." *Aquaculture*, 138, 313-322.
- Suloma, A., Mabroke, R. S., and El-Haroun, E. R. (2013). "Meat and bone meal as a potential source of phosphorus in plant-protein-based diets for Nile tilapia (*Oreochromis niloticus*)." *Aquaculture International*, 21(2), 375-385.
- Sun, Y. Z., Yang, H. L., Ma, R. L., and Lin, W. Y. (2010). "Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*." *Fish and Shellfish Immunology*, 29(5), 803-9.
- Sun, C. X., Xu, W. N., Li, X. F., Zhang, D. D., Qian, Y., Jiang, G. Z., and Liu, W. B. (2014). "Effects of fish meal replacement with animal protein blend on growth performance, nutrient digestibility and body composition of juvenile Chinese soft-shelled turtle *Pelodiscus sinensis*." *Aquaculture Nutrition*, 1-11.
- Takeuchi, T., Watanabe, T., and Ogino, C. (1979). "Digestibility of hydro-generated fish oil in carp and rainbow trout." *Bulletin of the Japanese Society for the Science of Fish*, 45, 1521-1525.
- Tapia-Paniagua, S. T., Chabrillon, M., Diaz-Rosales, P., de la Banda, I. G., Lobo, C., Balebona, M. C., and Morinigo, M. A. (2010). "Intestinal microbiota diversity of the flat fish *Solea senegalensis* (Kaup, 1858) following probiotic administration." *Microbiology Ecology*, 60(2), 310-9.
- Tapia-Paniagua, S. T., Reyes-Becerril, M., Ascencio-Valle, F., Esteban, M. Á., Clavijo, E., Balebona, M. C., and Morinigo, M. A. (2011). "Modulation of the intestinal microbiota and immune system of farmed *Sparus aurata* by the administration of the yeast *Debaryomyces hansenii* L2 in conjunction with Inulin." *Journal of Aquaculture Research & Development*, s1.
- Thomas, L. V., Ockhuizen, T., and Suzuki, K. (2014). "Exploring the influence of the gut microbiota and probiotics on health: a symposium report." *British Journal of Nutrition*, 112 Suppl 1, S1-18.
- Tidwell, J. H., Coyle, S. D., Bright, L. A., and Yasharian, D. (2005). "Evaluation of plant and animal source proteins for replacement of fish meal in practical diets for the largemouth bass *Micropterus salmoides*." *Journal of the World Aquaculture Society*, 36(4), 454-463.

- Venou, B., Alexis, M. N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., and Castritsi-Cathariou, I. (2003). "Effect of extrusion of wheat and corn on gilthead sea bream (*Sparus aurata*) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities." *Aquaculture*, 225(1-4), 207-223.
- Wang, Y., Li, K., Han, H., Zheng, Z.-X., and Bureau, D. P. (2008). "Potential of using a blend of rendered animal protein ingredients to replace fish meal in practical diets for malabar grouper (*Epinephelus malabricus*)." *Aquaculture*, 281(1-4), 113-117.
- Wassef, E., and Eisawy, A. (1985). "Food and feeding habits of wild and reared gilthead bream *Sparus aurata* L." *Cybium*, 9(3), 233-242.
- Webster, C. D., Tiu, L. G., Morgan, A. M., and Gannam, A. (1999). "Effect of partial and total replacement of fish meal on growth and body composition of sunshine bass *Morone chrysops* x *M. saxatilis* fed practical diets." *Journal of the World Aquaculture Society*, 30(4), 443-453.
- Wei, Z., Kangsen, M., Baigang, Z., Yangjiang, H., and Yu, Y. (2006). "A study on the meat and bone meal or poultry by-product meal as protein substitutes of fishmeal in concentrated diets for *Paralichthys olivaceus*." *Journal of Ocean University of China (Oceanic and Coastal Sea Research)*, 5(1), 63-66.
- Williams, K. C., Paterson, B. D., Barlow, C. G., Ford, A., and Roberts, R. (2003). "Potential of meat meal to replace fish meal in extruded dry diets for barramundi, *Lates calcarifer* (Bloch). II. Organoleptic characteristics and fatty acid composition" *Aquaculture Research*, 34, 33-42.
- Wilson, R. P. (2002). "Amino Acids and Proteins", in J. E. Halver and R. W. Hardy, (eds.), *Fish Nutrition*. Orlando, Florida, USA: Academic Press.
- Woodgate, S., and Veen, J. v. d. (2004). "The role of fat processing and rendering in the European Union animal production industry." *Biotechnology, Agronomy, Society and Environment*, 8(4), 283-294.
- Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y., and Wang, G. (2010). "Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*)." *Aquaculture*, 303(1-4), 1-7.
- Xavier, T. O., Michelato, M., Vidal, L. V. O., Furuya, V. R. B., and Furuya, W. M. (2014). "Apparent protein and energy digestibility and amino acid availability of commercial meat and bone meal for Nile Tilapia, *Oreochromis niloticus*." *Journal of the World Aquaculture Society*, 45(4), 439-446.
- Yang, Y., Xie, S., Cui, Y., Lei, W., Zhu, X., Yang, Y., Yu, Y. (2004). "Effect of replacement of dietary fish meal by meat and bone meal and poultry by-product meal on growth and feed utilization of gibel carp, *Carassius auratus gibelio*." *Aquaculture Nutrition*, 10, 289-294.
- Yang, Y., Xie, S., Cui, Y., Zhu, X., Lei, W., and Yang, Y. (2006). "Partial and total replacement of fishmeal with poultry by-product meal in diets for gibel carp, *Carassius auratus gibelio* Bloch." *Aquaculture Research*, 37(1), 40-48.
- Yanik, T., Dabrowski, K., and Bai, S. C. (2003). "Replacing fish meal in rainbow trout (*Oncorhynchus mykiss*) diets." *The Israeli Journal of Aquaculture - Bamidgeh*, 55(3), 179-186.
- Yanong, R. P., Pouder, D. B., and Falkinham, J. O., 3rd. (2010). "Association of mycobacteria in recirculating aquaculture systems and mycobacterial disease in fish." *Journal of Aquatic Animal Health*, 22(4), 219-23.
- Yiğit, M., Bulut, M., Ergün, S., Güroy, D., Karga, M., Kesbiç, O. S., Yılmaz, S., Acar, Ü., and Güroy, B. (2012). "Utilization of corn gluten meal as a protein source in diets

- for gilthead sea bream (*Sparus aurata* L.) juveniles." *Journal of Fisheries Sciences*, 6(1), 63-73.
- Yu, Y. (2004). "Replacement of fishmeal with poultry byproduct meal and meat and bone meal in shrimp, tilapia and trout diets", L. E. Cruz Suárez, D. Ricque Marie, M. G. Nieto López, D. Villarreal, U. Scholz, and M. González, (eds.), *Avances en Nutrición Acuícola VII. Memorias del VII Simposium Internacional de Nutrición Acuícola*. City: Hermosillo, Sonora, México.
- Zhang, D. F., Ji, C., Zhang, X. J., Li, T. T., Li, A. H., and Gong, X. N. (2015). "Mixed mycobacterial infections in farmed sturgeons." *Aquaculture Research*, 46(8), 1914-1923.
- Zhou, Z., Liu, Y., Shi, P., He, S., Yao, B., and Ringø, E. (2009). "Molecular characterization of the autochthonous microbiota in the gastrointestinal tract of adult yellow grouper (*Epinephelus awoara*) cultured in cages." *Aquaculture*, 286(3-4), 184-189.