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Validation of processed animal proteins (mono-PAPS) in experimental diets for juvenile gilthead sea bream (*Sparus aurata* L.) as primary fish meal replacers within a European perspective

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Abstract

Experimental diets were formulated to evaluate a "pure" poultry meat meal (PMM) source in diets formulated for juvenile gilthead sea bream (*Sparus aurata L.*). The digestible protein contribution of fish meal in a control diet was substituted by 25, 50 and 75% of a processed poultry meat meal (PMM) on a digestible crude protein (DCP) basis and by 5% and 10% for an enzyme treated feather meal (EFM) and also a spray-dried haemaglobin meal (SDHM) respectively. In a consecutive trial, diets were designed to assess the value of a "pure" (defatted) poultry protein substituting the fish meal (FM) protein content. Experimental diets included: a control diet, two test diets where 75% of FM was replaced by a full fat PMM (PMM75) or a defatted grade of PMM (dPMM75) and two test diets where 50% of FM was substituted for defatted PMM (dPMM50) or a 50:50 blend of soybean meal and defatted PMM (SBM/dPMM) to produce a composite product This soybean/dPMM blend was tested to enhance the nutritional value of this key plant ingredient commonly employed in sea bream diets that can be deficient in specific amino acids and minerals. In the first trial, gilthead sea bream grew effectively on diets containing up to the 75% replacement of FM attaining a mean weight of 63.6 g compared to 67.8 g for the FM control fed group. For the consecutive trial, the fishmeal based control diet yielded the highest SGR followed by dPMM50 and SBM/dPMM blend inclusion but were not significant.

Carcass FA profiles of gilthead sea bream conformed to the expected changes in relation to the dietary FA patterns, with the 18:1n-9 representative of the poultry lipid signature becoming more apparent with PMM inclusion. The ratio of n-3/n-6 fatty acids was greatly affected in sea bream fed the full fat PMM at 75% inclusion due to fish oil exclusion.

De-fatted dPMM however allowed more of the fish oil to be used in the diet and reducing this latter effect in sea bream carcass hence restoring the higher total omega-3 HUFA fatty acids namely EPA & DHA and n-3/n-6 ratio. It is concluded that poultry meat meal can be modestly incorporated into formulated diets for seabream and can be used in conjunction with soybean meal without any fundamental changes in performance and feed efficiency.

KEY WORDS*:* poultry meat meal, de-fatted meal, enzyme treated feather meal, spray-dried haemoglobin meal, gilthead sea bream, growth, feed utilization, HUFA fatty acids (n-3/n-6 ratio,

Introduction

The scope for replacing fish meal (FM) in feeds for commercially valuable fish species in aquaculture is of prime importance to meet sustainable production in many regions of the world (Hatlen, Jakobsen, Crampton, Alm, Langmyhr, Espe, & Waagbø, 2015; Moutinho, Martínez-Llorens,Tomás-Vidal, Jover-Cerdá, Oliva-Teles, & Peres, 2017). Suitable alternative proteins have been evaluated with much success, most notably those obtained from plant by-products such as soybean meals (SBM), various legumes and pulses e.g. beans and peas (Drew, Borgeson, & Thiessen, 2007; Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy, Herman, Hu, Krogdhal, Nelson, Overturf, Rust, Sealey, Skonderg, Souza, Stone, Wilson, & Wurtele, 2007; Hardy 2010; Kumar, Sándor, Nagy, Fazekas, Havasi, Sinha, De-Boeck, 2016; Rostamian, Eagderi, S., Masoudi, Salar & Asadian, 2016; Novriadi, Spangler, Rhodes, Hanson & Davis 2017).

In Europe, due to public concern and legislative control, limitations exist on exploiting animal derived proteins and fats in aquafeeds (SECA 2010). This has been the case for over a

69 decade, but specific category III sources (material derived from animals fit for human
70 consumption) are now allowed after recent approval by the European Food Safety Authority
71 (EFSA 2013). These currently include blood meal (BM) from porcine origin and now rendered
72 poultry by-products (PBM) are once again feasible in commercial fish diets within the European
73 Union (EU). Animal by-products are routinely available for use in compound diets for fish and
74 crustacean throughout the world (Bureau Harris, Bevan, Simmons, Azevedo & Cho 2000;
75 Moutinho *et al.* 2017; El-Husseiny Hassan, El-Haroun, & Suloma 2018). In the 1990's bovine
76 spongy encephalopathy (BSE) considered to be the major constraint of using animal by-product
77 in UK and Europe. The nutritional potential of by-products derived from poultry as secondary
78 protein sources in marine fish diets have been advocated in numerous studies to date. Since
79 then, there have been considerable progressions globally in the use of high quality low
80 temperature fish meal (LT FM's) and a new generation of processed animal by-products from
81 category III sources involving optimized temperature and pressure treatments with enzyme
82 hydrolysis. These have provided a prerequisite for more extensive nutritional trials involving
83 more balanced diet substitutions based on digestible protein, amino acids and energy basis.
84 Similarly, novel processes outlined by (Rebafka & Kulshrestha 2009; El-Haroun, Azevedo &
85 Bureau 2009; Abwao, Safina, Ondiba, Ogello, & Obiero 2017) such as improved cooking and
86 drying temperatures similar to those used for FMs are a better strategy to improve the quality
87 and nutrients availability of rendered animal proteins. Following the same pattern, addition of
88 exogenous enzymes to the batch cooker, associated with low-temperature and low-pressure
89 processing, has been one of the alternatives used to mitigate effects of overheating the feather
90 meal, improving the quality of the end product and saving energy (Pedersen *et al.* 2012).

91 The present investigation was performed to validate the suitability of different grades and
92 processing of PMM as a replacement for fishmeal in diets for gilthead sea bream juveniles. We
93 aimed to confirm their effects on growth performance feed utilization efficiency and changes in
94 muscle fillet lipid composition. Additionally, information about chemical composition and
95 nutritional values of specialized blood protein and feather meals as supplements in diets for such
96 species are required for feed manufacturers. Therefore, evaluation of category III premium
97 processed animal proteins (PAP's) is a necessary step towards their potential re-introduction
98 into the Aquafeed sector in Europe. More economic production of such high value marine fish
99 species such as gilthead sea bream based on a range of alternative feed ingredients and animal
100 by-products are destined to be at the forefront of these objectives.
101 It was also of interest to examine a blend of soya bean meal with PMM as a strategy to evaluate
102 any complimentary benefits of such combinations in complex formulations with lower fishmeal
103 inclusions typical in modern day formulations for marine fish species. These diets were
104 formulated under the advice of the collaborating company to extend the use of PAPS in marine
105 fish diets. It was not the aim to correct for any specific amino acid deficiencies such as
106 methionine but to test the maximum feasible inclusion of PMM. Enzyme treated feather meal
107 (EFM) and also a spray-dried hemoglobin meal (SDHM) were included as supplements to
108 evaluate their nutritional enhancement potential in terms of their protein contribution to partially
109 reduce the fish meal content of seabream diets. Additionally, the opportunity to record the fatty
110 acid composition of sea bream fed a fish meal control diet with fish oil against higher
111 substitution of Poultry Meat Meal was undertaken to assess the extent of changes in the major
112 Omega-3 (n-3) HUFA's such as EPA and DHA (eicosapentaenoic and docosahexaenoic acids
113 respectively). This is because of the high fat content in PMM and a consequent reduction in fish

oil to accommodate a consistent diet lipid content. A de-fatted PMM was also tested to allow fish oil in the formulation to maintain the optimum level of total n-3 in the diets. This is important from the retail and consumer perspective and is of topical interest.

Materials and methods

Generic fish and experimental conditions

The first feeding trial was conducted in a closed re-circulating seawater system conforming to a Recirculating aquaculture system (RAS) design for specific nutrition research. The experimental facility consisted of sixteen 110-L volume fibreglass (square) tanks connected to a biological and mechanical filtration unit (sponge filters, protein skimmer and submerged biological filter beds). Each tank was supplied with filtered seawater (salinity: 33 ± 1 ppt; temperature: 22 ± 1 ºC) at a rate of 10 L min^{-1} and continuously aerated so that oxygen levels were kept close to saturation. Besides, natural seawater was used to renew ~20% of the water system volume every week. Throughout the study, monitoring of the principal water quality parameters resulted in average values of: 7.5 for pH (Hanna pH210 benchtop meter), 0.15 mg L^{-1} for total ammonia nitrogen (Hanna chemical test kits) and 91.5% saturation for DO (YSI model 85 portable meter). Photoperiod followed a cycle of 12 h dark, 12 h light. Fish husbandry and experiments conformed to the local institutional Animal Welfare Ethics Committee Codes of Practice and were in accordance with the UK Animal Scientific Procedures Act, 1986. At the beginning of the experiment, a pooled sample of fish (25 seabream) was taken for determining chemical body composition (initial carcass sample). At the end of the experiment, 3 fish were sampled from each tank, 9 per treatment in total. Fish were killed with an excess concentration of anesthetic (MS 222, Tricaine methanesulfonate) and then individually weighed. The fish

were then pooled per tank (final carcass sample) for chemical analyses. The combined fish samples were ground in a coffee grinder and stored at −20 °C until analyzed.

Trial 1

Fish stock and feed management

The juvenile gilthead sea bream (*Sparus aurata* L.) used in Trial 1 were obtained from a commercial hatchery in France (Aquastream, Ploemeur) and acclimatized to the environmental conditions for a period of 4 weeks prior to trial commencement. During that period, they were fed a commercial marine fish diet (Skretting Salmon Nutra) twice daily to apparent satiation. At the start of the trial, fish were group weighed (initial individual weight: 22.7 ± 0.5 g, mean ± SD) and re-stocked at a density of 25 fish per tank. Fish were hand fed to 3% body weight per day twice daily. Following the one day of feed deprivation each week, the fish were weighed, and the feeding rate recalculated to correct for biomass changes and maintain accurate feeding level.

Diet preparation and experimental design

For this investigation, FM (LT94) was provided by Skretting Ltd, Longridge, Preston, Lancashire, UK and the protein sources used in this trial were obtained from Prosper De Mülder Group, Market Harborough, UK (PMM) (Now Saria Group GmbH) and from Skretting Ltd, Longridge, Preston, Lancashire, UK (Hi-Pro soybean meal, SBM). The processing method and origin of poultry derived-proteins included in the four experimental diets was as follows: the poultry meat meal (PMM) grade is derived from mixed species poultry material (i.e. chickens, turkeys, ducks and geese slaughtered fit for human consumption) minced to < 3 mm and introduced into a continuous process (Rotadisc) in the presence of natural fats to evaporate the water, and subsequently sterilized (residence time: 90 min, maximum temperature: 125 ºC). The

159 resulting material is concentrated by an expeller press to remove fat. The protein rich fraction is
160 subsequently cooled and milled. From this product, the enzyme treated feather meal (EFM) was
161 provided by Prosper de Mülder Group (now Saria Group GmbH), Market Harborough, U.K. Mixed
162 feathers are heated to 50 °C for 30 min in the presence of a commercial enzyme additive of fungal
163 source (Synergen™, Alltech Biotechnology) containing amylase, cellulose, phytase, xylanase, β-
164 glucanase, pectinase and an active protease, 12,700 HUT g^{-1} (E.C.3.4.23.18). This enzyme
165 hydrolysis step (keratinolysis), likely results in the cuticle layer being partially degraded and smaller
166 peptides produced, however there appears to be no effect on di-sulphide bonds in Keratin *per se*
167 according to Considine (2000) although the concentration of cysteine increases in the enzyme
168 treated product. The treated feathers are subsequently processed at 200 kpa for 15 min at a
169 temperature of 125 °C. The resulting ground meal is dried in a Rota-disc drier to 5% moisture and
170 ground to an average particle size of 300 microns (μ).

171 The spray-dried haemoglobin meal (SDHM; Spray-dried porcine animal blood cells-AP301®) was
172 supplied by APC Inc. Europe S.A., Barcelona, Spain. The specifications of the different test
173 ingredients utilized in both trials are given in Table 1. A FM based diet served as the control dietary
174 formulation (Table 2). Using pre-established digestibility coefficients (Davies Gouveia, Laporte,
175 Woodgate & Nates 2009), five experimental diets (PMM25, PMM50, PMM75, SDHM10 and
176 EFM5) were derived from the basal formulation to achieve specific replacement of the protein
177 component of FM replacement levels with various animal by-products (25, 50 and 75%) for PMM;
178 5% for EFM and 10% for SDHM while maintaining digestible protein and lipid levels constant at
179 40% and 15% respectively across all dietary treatments in compliance with the nutritional
180 requirements for gilthead sea bream NRC (2011). During feed preparation, all macro-ingredients,
181 vitamins and minerals premixes were uniformly mixed together before the addition of marine fish oil

and de-ionised water. The resulting mixture was extruded through a 3 mm aperture die of a California pellet mill. Pellets were air-dried by convection in a warm air cabinet (37 ºC) and stored in plastic sealed containers throughout the duration of the study. Each diet was fed to 3 replicate groups of fish for a period of 9 weeks.

Trial 2

Fish stock and feed management

The juvenile gilthead sea bream (*Sparus aurata* L.) used in Trial 2 were obtained from a commercial hatchery in France (Aquastream, Ploemeur) and acclimatized to the environmental conditions for 4 weeks before the start of the experiment. During that period, they were fed a propriety commercial marine fish diet (Skretting Salmon Nutra) as in trial 1. At the start of the trial fish were group weighed (initial individual weigh: 10.07 ± 0.05 g, mean ± SD) and re-stocked at a density of 50 fish per tank. Fish were hand fed to 4% body weight per day twice daily and corrected at each weekly weighing as described in trial 1.

In Trial 2, five semi-purified diets were designed to attain a target of 400 gkg^{-1} digestible protein, 150 gkg^{-1} lipids and formulated to meet current known nutritional requirements for gilthead sea bream juveniles (Table 3). For the 4 experimental diets, the FM in the control diet was partially replaced by the following ingredients: PMM (75% FM substitution level), a defatted grade of the same PMM (50 and 75% FM substitution level) and a 50:50 mixture of defatted PMM (dPPM) provided by Prosper de Mülder Group, Market Harborough, UK and SBM (Hi-Pro SBM; 50% FM replacement level) provided by Skretting Ltd, Longridge, Preston, Lancashire, UK. In this study, a further defatting of the material was achieved. Deffated PMM (dPMM) was obtained following hexane extraction: PMM was soaked and mixed for 24 h and filtered through a 100 µ sieve to remove the fat and solvent mixture; the defatted sample was

205 then air dried to remove traces of solvent. Each diet was fed to 3 replicate groups of fish for a
206 period of 6 weeks.

207 *Water quality*

208 The water temperature was maintained at 25±1 ^{0}C for the sea bream for best growth) with a
209 salinity of 33-34 ppt. The photoperiod was maintained at12-h light: 12-h dark by means of
210 artificial daylight simulation. All fish were held in 65 L fiberglass tanks (40 cm length, 17.5 cm
211 width and 27-38 cm depth) on the basis of the Guelph model (tanks were made with a sloping
212 floor so that faecal material could be voided and recovered in external conical transparent
213 separation chambers fitted with a valve). Within the system, the flow rates applied enabled a
214 complete exchange of three to five volumes per hour. All principal water quality parameters
215 were controlled on a regular basis during the course of the study to remain within satisfactory
216 limits.

217 *Analytical methods of feeds and body composition*

218 Proximate analysis of ingredients, experimental diets and fish conformed to standard AOAC
219 methods (AOAC 2003). Essential amino acids were determined by Eclipse Scientific Group
220 (Chatteris, Cambrideshire, England) using standard protocols. Samples were first digested using
221 6N HCl and tryptophan treated separately with 4 N Methane sulphonic acid. Digested samples
222 were subsequently diluted with HPLC grade water. All samples were subjected to pre-column
223 derivatisation with o-Phthaldialdehyde OPA with gradient HPLC using a Nucleosil C18 5 um,
224 60 x 4 mm, Knauer column at ambient temperature with subsequent fluorescence detection at
225 330-365 nm excitations and 440-530 nm emission. A gradient elusion was employed with the
226 mobile phase being; A, 0.1 M sodium acetate, pH 6.95 and methanol: tetrahydrofuran (92.5: 5:
227 2.5) and B, methanol: tetrahydrofuran (97.5: 2.5) with a flow rate of 1.2 ml min^{-1}. In Trial 2, the

fatty acid profile of the experimental diets was determined (FM, PMM75, dPMM75) along with the corresponding final carcass samples by Eclipse Scientific group; Cambridgeshire; UK; (Table 4) following FAME preparation and subsequent GLC separation and quantification with standard FAME fatty acids (Table 7). Table 1 shows the nutritional composition including the essential amino acid profiles of the test ingredient sources. Both tables 2 and 3 present the diet formulations, their nutritional analysis and also essential amino acid profiles for trials 1 and 2 respectively.

Statistical treatments

Statistical analysis of data was performed using one-way analysis of variance (ANOVA) at the 5% level of significance. Tukey's *post hoc* analysis was applied to mean values where appropriate (Minitab 13 for windows, Minitab Inc., State College, USA).

Results

Trial 1

Growth performance and feed utilisation

Growth performance and feed utilization for gilthead sea bream fed the experimental diets are presented in Table 4. Fish showed on average a 200% increase in weight gain with all treatments producing specific growth rates (SGR's) comparable to the control diet, there were no significant differences (P = 0.71) in SGR between (FM) diet 1.7 % day^{-1} and the highest PMM level of 75% dietary protein replacement (1.6 % day^{-1}), however a significant (P<0.05) reduction of feed intake with increasing PMM substitution apparent at the highest level (0.9 g $fish^{-1}$ day^{-1}) compared to (1.0 g $fish^{-1}$ day^{-1}) for the FM fed group.

However, a significant (P<0.05) decrease of feed conversion ratios (FCR) values were obtained for SDHM10, EFM5 and PMM25 groups (1.30-1.37), also, fish fed higher inclusion

level of PMM showed improving in FCR (1.37) compared to the FM group (1.43) consistent with the other parameters but not deemed significant (P<0.05).

A weight gain (g) and weight gain (%) of gilthead sea bream fed the SDHM10 and PMM25 diets were apparently more efficient (P≥0.05) than fish fed FM, following the same pattern converting dietary protein to live weight gain (protein efficiency ratio (PER) were 1.62 and 1.60 vs. 1.5 for fish fed SDHM10 and PMM25 diets vs. fish fed FM (Table 4). On the other hand, the PER of fish fed PMM75 was significantly (P<0.05) lower (1.4) compared to all other experimental groups (1.5-1.6, Table 6). However, there were not reflected by the direct calibration of apparent net protein utilization (aNPU) for a group of fish which ranged from 21.4% to 23.6%. No statistical differences (P>0.05) were found in the proximate composition of whole fish carcass (Table 4). Considering the pattern of protein retention relative to the amount of protein fed, aNPU observed across treatments were found not to be statistically different (P>0.05).

Health related parameters

Following the 9-week period in trial 1, the different diets tested did not significantly (P>0.05) influence the condition factor (K) or hepato-somatic index (HSI) of the fish. No significant (P>0.05) differences were found in the hematocrit value, haemoglobin concentration or Red Blood cell count (RBCC) among the blood samples analysed. Values ranged from 36.5 to 42.0 (Hct, %), 7.2 to 7.8 (Hb, g dL^{-1}), 2.2 to 2.7 (RBCC 10^6 mm^{-3}) respectively within the test groups, against 39.00 (Hct, %), 7.7 (Hb, g dL^{-1}) and 2.4 (RBCC 10^6 mm^{-3}) for the control diet (Table 5).

Trial 2

Growth performance and feed utilisation

274 After 42 days of feeding, significant (P<0.05) differences were found in live weight gain and
275 SGR of gilthead sea bream juveniles receiving PMM and defatted PMM (dPMM; Table 6). Fish
276 fed with the control diet had a mean weight gain that was significantly (P<0.05) higher than
277 those fed with PMM75 and dPMM75. The same pattern was observed with SGR (FM: 3.6% /
278 day; dPMM50: 3.5% / day; SBM/dPMM: 3.4% / day; PMM75: 3.2 % / day and dPMM75: 3.2% /
279 day).
280 Feed conversion ratios were significantly (P<0.05) improved for diets including the
281 alternative protein sources in comparison with the control diet. A similar trend was observed for
282 PER: fish fed the blend of SBM/dPMM and dPMM75 were more efficient at converting protein
283 into live weight gain with PERs of 1.5 and 1.3 respectively. Likewise, superior aNPU values
284 were obtained form the PMM75 and dPMM75 levels with the PMM75 (aNPU ~25.4%)
285 significantly (P<0.05) better than the FM fed gilthead sea bream (21.6%). The SBM/dPMM
286 blend also resulted in a significantly (P<0.05) higher aNPU (27.76%) compared to the dPMM50
287 and FM groups (22.7% and 21.6% respectively). The PER from gilthead sea bream fed the
288 blend of dPMM75 and SBM/dPMM was significantly (P<0.05) higher (1.3 and 1.5)
289 respectively compared to the FM group (1.1).
290 No major significant differences (P>0.05) were observed in gross nutrient composition of
291 fish carcasses analyzed at the end of this trial (Table 6). The FA analysis of the PMM diets
292 demonstrated the expected trend associated with diet lipid composition (i.e. lipid sources; Table
293 7). In the control diet, where lipid was primarily of marine origin, the ratio of n-3/n-6 fatty acids
294 was highest (2.53). In PMM75, where poultry fat accounted for ~50% of the total lipid content,
295 this ratio decreased to 0.41. This was largely a consequence of a reduction in 20:5n-3 (from
296 1.4% to 0.8%) and 22:6n-3 (from 1.9% to 0.9%) as well as an augmentation of 18:2n-6 (from

297 1% to 6.7%). The amount of animal fat present in PMM 75 diet was also reflected by an
298 increase in 16:0 and 18:1n-9 when compared to the control diet. Finally, the utilization of a
299 defatted source of PMM in dPMM75 allowed restoration of the n-3/n-6 FA's ratio at 1.14. For
300 this diet, compared to the one where 75% of FM was replaced with full fat PMM, the amount of
301 18:1n-9 decreased from 32.9% to 24.3% while the level of 18:2n-6 varied from 6.7% to 4.4% in
302 the tissues of gilthead sea bream.

303 **Discussion**

304 This evaluation of a premium grade PMM and refined blood and feather meal proteins in
305 diets for gilthead sea bream follows the previous foundation studies of Davies *et al.* (2009).
306 Consequently, the trials acted as a prerequisite for more accurate substitution of processed
307 animal proteins (PAP's) into balanced diet formulations for this species in contemporary aqua
308 feeds. On this basis, the substitution of FM with PMM and EFM as well as SDHM was effective
309 due to prior knowledge of the protein and energy digestibility data compared to the other
310 previous studies using gross nutrient levels.

311 Trial 1 validates the efficacy of using up to 75% of the dietary protein as PMM (57% of diet)
312 with performance of juvenile gilthead sea bream attaining the same criteria measured as those
313 fed a control diet. There was only a slight indication of a reduced palatability encountered at this
314 level for this ingredient, with seabream adapting to its inclusion. Growth and feed utilization
315 indicators supported the use of PMM and strategic use of EFM and SDHM as reported for this
316 fish species (Serwata 2007; Yones & Metwalli 2016; El-Husseiny *et al.* 2018). The overall EAA
317 profile of the PMM75 diet was similar to a high-quality FM protein control. In our
318 investigation, we replaced LT FM with the test ingredient at commercially acceptable levels and
319 found that a majority of EAA's exceeded requirement levels as expressed as percent of protein

for all diets, except for 75% inclusion of PMM where both methionine and histidine was below the reported requirement for sea bream by Peres & Olivia-Teles (2009). It should be noted that these workers used a mixture of whole protein and crystalline amino acids in semi-purified diets for sea bream. Given the inefficiency of crystalline amino acids utilization in some species this could elevate the apparent requirements of essential amino acids and is not strictly comparable with the present study with whole protein sources. A better comparison arises from the data found for sea bream and related species in the NRC 2011 Nutrient Requirements of Fish and Shrimp.

The trend in decreasing SGR for gilthead sea bream, although deemed not significant, may have reflected these shortages of EAA's. Thus, specific deficiencies of these amino acids may have caused reduction in growth performance; furthermore, the lower growth performance observed in gilthead seabream may be due to a slightly reduced palatability of the PMM compared to fishmeal. The other factor that could be the major reason for declining the growth may be due to the varying quality of tested PMM, which are significantly influenced by their processing methods (Shapawi, Ng & Mostafa 2007; Rostamian *et al.* 2016). It was interesting that diets only supplemented with either a SDHM and and EFM showed as good a performance as the FM group and superior to the higher levels of PMM inclusion (50 and 75%). These diets complied with the amino acid pattern of the fish meal diet meeting all EAA requirements for this species. These results could be explained due to the use of the commercial enzyme Synergen^{TM} that can help to enhance the degradation of the keratin structure in feather meal to small peptides and increase the overall cysteine amino acid concentration in the feather meal; however we based our EFM inclusion on a previous protein digestibility coefficient of 25% for gilthead sea bream (Davies *et al.* 2009) although individual EAA digestibility may be higher for

this species. In the latter study, higher inclusions of feather meal were used for digestibility determination and this may have explained the much-reduced DC for this ingredient. Only 5% feather meal was included in the current investigation and may not be strictly comparable to the conditions of the study by Davies *et al* 2009.

The FCR and PER values were significantly improved indicating enhanced nutritional value of SDHM10. Indeed, combinations of animal proteins may show complementary amino acid profiles. Such synergistic characteristics of complementary proteins need to be examined for further FM replacement by exploring various protein blends. For example, the histidine level in SDHM is appreciably higher than those found in FM and PMM. Although isoleucine concentration is lower in SDHM, it is nonetheless a valuable source of leucine (12% of total protein) which is an EAA for gilthead sea bream (4.5% of dietary protein).

In Trial 2, the control diet produced the overall best growth performance of juvenile gilthead sea bream compared to other treatments containing poultry meat meals (PMM's) although the defatted PMM and blended PMM with SBM produced favorable results although not deemed to be significantly different to FM alone. However, improved protein utilization efficiency was also seen in terms of the aNPU reported for gilthead sea bream fed PMM and dPMM at 75% total protein replacement. These values are in accordance with data reported for this species by Nengas Alexis & Davies (1999) and Laporte (2007). Consequently, fish fed the SBM/dPMM blend as a partial FM replacement grew as well as the fish fed the FM and exhibited the best productivity values in terms of FCR, PER and aNPU. Although both protein sources are said to be deficient in methionine (Nengas *et al.* 1999; Hertrampf & Piedad-Pascual 2000), combining SBM and PMM might result in a partial improvement of the EAA compared to the use of SBM alone as soybean meal is a major plant ingredient in marine fish diets within low fishmeal

366 formulations. This blend may mitigate the effects of the lower methionine, arginine and lysine
367 content in SBM in gilthead sea bream diets. This result tends to confirm an optimal substitution
368 rate of FM by PMM between 25 and 50% for this particular marine species. For most
369 carnivorous fish, the recommended substitution rates of FM by PBM (studies with sub adult fish
370 mainly) would generally range from 25 to 50% (Nengas *et al.* 1999; Turker, Yigit, Ergün,
371 Karaali & Erteken 2005; Yigit, Erdem, Koshio, Ergün,Türker & Karaali 2006; Wang, Han,
372 Zheng & Bureau 2008; Yu 2008; Li, Wang, Zheng, Jiang & Xie 2009; Booth, Allan &
373 Anderson 2011; Metts, Rawles, Brady, Thompson, Gannam, Twibell & Webster 2011;
374 Moutinho *et al.* 2017) but the feasibility of even higher or total replacement without amino acid
375 supplementation was reported by some authors (Takagi, Hosokawa, Shimeno & Ukawa 2000;
376 Saadiah, Abol-Munafi & Utama 2011). Removing the poultry fat component of PMM to test a
377 75% FM replacement with a relatively "pure" protein source did not yield any improvement
378 compared with the same inclusion level of the original full fat PMM (indicating the minimal
379 influence of dietary lipid on production performance). PMM75 and dPMM75 diets appeared to
380 be equally palatable to the fish since exactly the same amount of feed were consumed (FI = 0.98
381 g $fish^{-1}$ day^{-1}). By comparison with dPMM50, the SBM/dPMM blend did not lead to a
382 significant reduction of feed intake in sea bream. While the efficacy of SBM to replace FM in
383 diets for gilthead sea bream was also examined by several researchers (El-Haroun & Bureau.
384 2007), limited information is available on the use of blends of SBM and animal protein
385 concentrates in this species (De Francesco, Parisi, Pérez-Sánchez, Gómez-Réqueni, Médale,
386 Kaushik, Mecatti & Poli 2007; Dias *et al.* 2009). Palatability and EFA profile of PMM are
387 presumed to be the main factors limiting the growth of gilthead sea bream, when full fat grades
388 of PMM are included at a high level, as seen in trial 1. The blending of SBM with dPMM

appeared to raise the amino acid levels towards those observed in the dPMM50 diet and only methionine seemed to remain below the reported requirement levels for sea bream by Peres & Olivia-Teles (2009). Separate short-term and long-term palatability trials are to be encouraged to test limitation on feed intake for gilthead sea bream before practical use of such ingredients can be applied in feed manufacture. Carcass FA profiles of gilthead sea bream conformed to the expected changes in relation to the dietary FA patterns, with the 18:1n-9 (oleic) representative of the poultry lipid signature becoming apparent. Agreeing with what is usually described in wild or farmed gilthead sea bream (Mnari, Bouhlel, Chraief, Hammami, Romdhane, El Cafsi & Chaouch 2007), 16:0, 18:1n-9 were the principal saturated fatty acid (SFA) and mono unsaturated fatty acid (MUFA) regardless the dietary regime. 22:6n-3 (was the dominant highly unsaturated fatty acids (HUFA) within the carcass of fish fed FM and defatted (dPMM75), whereas 18:2n-6 appeared to be the primary HUFA in the carcass of fish fed PMM75. Marine fish are usually not known to have the ability to elongate and desaturate C18n-3 HPUFA (linolenic) to effectively generate the long chain C20:5n-3 and C22:6n-3 (eicosapentaenoic and docosahexaenoic) FA's respectively (Greene 1990). Within the context of total FO replacement, the lack of a well-balanced FA profile (Sargent, Henderson & Tocher 2002) and a lower palatability (Regost, Arzel, Robin, Rosenlund & Kaushik 2003) or digestibility (Caballero, Obach, Rosenlund, Montero, Gisvol, Izquierdo 2002) are likely to limit the success of marine fish production when other lipid sources are utilized at the expense of fish oils. In this study, sea bream requirements for EFA's were likely met since diet manipulation did not result in a reduction of FO below 50% of the total dietary lipid content for this species, and a total n-3 in the diet of 3.1 % of the oil (0.4% of the diet) was retained in a PMM level of 75% inclusion (trial II). The minimum requirement for the Gilthead sea bream was found to be 0.4% of the diet

by Ibeas, Cejas, Fores, Badia, Gomez & Hernández (1997) for EPA: DHA. No pathological signs of essential fatty acid deficiencies such as skin hemorrhaging or fin erosion were observed in our study and fish were in excellent condition throughout.

It is well established from the literature that the nature of dietary oil influences carcass quality and FA pattern in fish tissues and organs for the gilthead sea bream (Izquierdo, Montero, Robaina, Caballero, Rosenlund & Ginés 2005; Caballero, Torstensen, Robaina, Montero & Izquierdo 2006; De Francesco *et al.* 2007; Piedad-Pascual *et al.* 2007). However, Aoki Shimazu, Kukushige, Akano, Yamagata & Watanabe (1996); Wang *et al.* (2008); Li *et al.* (2009); Booth *et al.* (2011) did not find any noticeable difference in the flesh quality between adult red sea bream (*Pagrus major*), Malabar grouper *(Epinephelus malabaricus)* and Australian snapper *(Pagrus auratus, Sparidae)* respectively fed with or without FM as a dietary protein source. In terms of human consumption and consumer acceptance high levels of HUFA in fish muscle that can be obtained with proper diet manipulation would be a desirable benefit (Kaushik 1997; Trushenski & Boesenberg 2009). The varying ratios of n-3 to n-6 and n-9 ratios resulting from the dietary changes within the current study may have such implications for gilthead sea bream; especially if the fish are fed diets containing a standard PMM over a longer time course and particularly gilthead sea bream attaining harvestable weight. It may also be possible to enhance marine fish diets containing poultry meat meals with selected algal products like Schizochytrium sp containing high levels of DHA (docosahexaenoic acid) constituting the bulk of their n-3 fatty acids and around 25% of the dry biomass. This could be provided during the final phase of production in a 'finisher' diet. The algal meal and extracted oil would complement such diets for sea bream and sea bass allowing for optimization of the n-3 profile in the flesh of fish for the consumer. This concept

434 has been explored for Atlantic salmon (*Salmo salar*) by Kousoulaki, Nengas Sweetman & Berge
435 (2016) with promising results.

436 Future processing of PMM to remove residual fat could be employed at the finishing phase of
437 production to mitigate the changing of n-3 to n-6 & n-9 ratios in compliance to consumer demands
438 for a defined product with high omega-3 highly un-saturated fatty acid (HUFA) lipids notably EPA
439 and DHA. The trend towards a reduced n-3/n-6 ratio in the fillets of farmed salmon has been well
440 documented recently with much concern that the combined EPA and DHA levels have been
441 reduced by as much as 50% over the last 15 years mainly due to the increased utilization of
442 vegetable oils like Canola and soybean oils in salmonid feeds (Sprague, Dick & Tocher 2016).
443 Indeed, we see this potential here with sea bream, if diets with higher animal fat levels are
444 constructed. Evidence for correction by using defatted PMM is shown in our study to allow more
445 formulation space for alternative oil sources richer in both EPA and DHA hence restoring the same
446 profile of fatty acids in the control fishmeal diet for seabream.

447 The present investigation confirms that PMM is an effective protein concentrate supporting
448 growth and development of juvenile gilthead sea bream replacing up to 75% of FM protein.
449 There may be additional benefits by the inclusion of supplementary levels of 5 and 10%
450 respectively of EFM and SDHM as premium grade ingredients to provide enhanced EAA
451 contribution and enhanced palatability of diets with reduced fishmeal levels. However, despite a
452 trend in the technical improvement of rendered animal by-products over the last two decades,
453 the threshold for maximizing dietary inclusion has not been realized compared to the earlier
454 findings of Nengas *et al.* (1996 & 1999). The cost benefit analysis of further technological
455 processing must be re-assessed as well as more work to using supplementary crystalline amino
456 acid in conjunction with these protein sources.

Clearly there is still much scope in developing feeds for gilthead sea bream and other marine species based on a new generation of by-products, contributing towards a bio-secure and sustainable agenda for the aquafeed sector within a European context and beyond. As a result of this research, EU dependency on imported alternative protein sources for use in aquaculture feeds such as soya bean meal could be adding a measured contribution to global food security by reducing plant ingredient imports. The research has also contributed to the Common Fisheries Policy of aligning sustainable wild fisheries with sustainable aquaculture development by considering alternative strategies. The scientific evidence leading to regulatory change at the EU level involved significant industry investment in research and development leading to improved competitiveness of the EU aquaculture industry, a reduction in the environmental impact of fish farming and improved fish health and welfare.

New scientific information concerning the safety and efficacy of inclusion of mono-PAPs in farmed fish diets was established in the last decade. This has now led to regulatory change at the EU level (Regulation introduced Feb 2013), permitting re-authorization of the use of mono-PAPs in aquaculture diets. However, in the UK and some EU countries it is the retailer that restricts their use due to the sensitivity of the consumer with regard to animal protein products in the food chain.

Conclusion

The results from this study showed that processed animal proteins have good nutritive value and can be a valuable protein source for gilthead sea bream diets. Modest levels of these ingredients can be used in gilthead sea bream feeds without detriment in a balanced formulation. Novel techniques for producing processed animal protein and coupling with exogenous enzymes associated with low-temperature and low-pressure processing, has been one of the alternatives used to improving the quality of processed animal protein. Key opportunities may

arise from the use of specific exogenous enzymes such as proteases and solid state fermentation products to achieve superior digestibility of rendered animal material in aquafeeds. Also, various ensiling methods to stabilize the protein component and fats could be applied as well as natural ant-oxidants. The use of various feed additives such lactobacillus and probiotics with the addition of organic carbohydrates is a relatively economic approach to achieve effective ensiling and protein hydrolysis.

It is evident that further characterization of processed animal proteins (PAPS) and some refinements of the diet formulation for seabream are required to obtain comparable levels of performance with conventional higher fish meal-based diets for marine fish species. More work will be needed to support the aqua-feed industry in addressing both the retailers and consumer confidence for fish fed animal by-products in the UK and Europe although widely accepted in other parts of the world. These must also enable production of marine fish without altering the amount of invaluable HUFA lipids in the fillets of fish to ensure maintaining the healthy benefits to the consumer.

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726

Table 1 Proximate composition and essential amino acid profile of the test ingredients was used in Trials 1 and 2 g kg^{-1} based on dry matter

	Experimental ingridients					
	FM LT94 [1]	EFM[2]	SDHM[3]	PMM[2]	dPMM[2]	SBM[1]
Proximate composition						
Dry matter	926.0	899.0	908.0	941.0	947.0	878.0
Crude protein	730.0	811.0	909.0	620.0	700.0	500.0
Crude lipid	119.0	63.0	28.0	166.0	57.0	8.0
Gross energy (MJ Kg^{-1})	21.2	22.9	22.2	20.9	20.2	19.6
Ash	133.0	22.0	31.0	170.0	157.0	73.0
*EAA composition**						
Arginine	41.40	36.60	36.90	41.70	39.30	36.50
Histidine	17.30	7.70	69.00	11.40	10.70	7.70
Isoleucine	25.60	19.50	5.50	18.20	17.10	21.30
Leucine	50.40	39.10	123.30	43.50	41.00	36.40
Lysine	52.50	26.00	82.80	38.30	36.10	30.90
Threonine	31.00	23.70	33.10	25.60	24.10	19.00
Tryptophan	6.90	7.90	11.00	5.50	5.10	7.00
Valine	31.60	28.00	84.70	28.60	27.00	25.40
Methionine	19.50	8.20	7.40	10.00	9.40	6.90
Phenylalanine	27.70	22.20	65.40	23.10	21.70	24.40
aDCP‡ (%)	87.50	21.70	82.80	79.20	79.20	87.00

[1] Skretting Ltd, Longridge, Preston, Lancashire, UK
[2] Prosper De Mülder Group, Market Harborough, UK
[3] American Protein Corporation (APC), Ankeny, Iowa, USA
* Manufacturer specifications
aDCP‡ = Apparent digestibility crude protein

10 **Table 2** Formulation, proximate composition (g kg^{-1}), essential amino acid profile of the experimental diets (g 16 g^{-1}
11 N) and essential amino acid requirements (g 16 g^{-1} N) in Trial #1.

	Experimental diets						Requirement[10]
	FM	PMM25	PMM50	PMM75	EFM5	SDHM10	
Fish meal LT94[1]	640.0	480.0	320.0	160.0	608.0	576.0	
Poultry meat meal[2]	0.0	190.0	380.0	570.0	0.0	0.0	
Enzyme treated feather meal[2]	0.0	0.0	0.0	0.0	108.0	0.0	
Spray-dried haemoglobin meal[3]	0.0	0.0	0.0	0.0	0.0	68.0	
Marine fish oil	74.0	67.7	62.2	56.7	70.0	79.5	
Starch[4]	113.3	113.3	113.3	113.3	113.3	113.3	
Dextrin[5]	56.7	56.7	56.7	56.7	56.7	56.7	
Vitamin[6]	5.0	5.0	5.0	5.0	5.0	5.0	
Mineral[7]	5.0	5.0	5.0	5.0	5.0	5.0	
Cellulose[8]	106.0	82.3	57.8	33.3	34.0	96.5	
Proximate composition							
Dry matter	965.70	961.80	952.10	953.70	961.80	963.30	
Crude protein	460.80	467.70	486.20	530.50	489.70	474.40	
Crude lipid	121.50	114.10	126.50	140.60	144.00	180.10	
Gross energy (MJ Kg^{-1})	20.44	20.57	20.61	20.82	21.80	20.92	
Ash	94.80	102.40	108.10	97.30	112.60	92.60	
Essential Amino acid profile[9]							
Arginine	2.91* (6.31)[+]	2.97 (6.35)	3.04 (6.25)	3.10 (5.85)	3.16 (6.44)	3.04 (6.41)	5.55
Histidine	1.06 (2.29)	1.01 (2.16)	0.96 (1.98)	0.91 (1.72)	1.09 (2.22)	1.38 (2.90)	1.98
Isoleucine	2.00 (4.35)	1.85 (3.95)	1.69 (3.48)	1.54 (2.90)	2.11 (4.32)	1.84 (3.87)	2.55
Leucine	3.32 (7.21)	3.32 (7.09)	3.31 (6.82)	3.31 (6.24)	3.58 (7.31)	3.75 (7.91)	4.75
Lysine	3.56 (7.74)	3.40 (7.27)	3.24 (6.66)	3.07 (5.80)	3.67 (7.49)	3.72 (7.84)	5.13
Threonine	1.86 (4.03)	1.88 (4.02)	1.90 (3.91)	1.92 (3.63)	2.02 (4.12)	1.87 (3.95)	2.89
Tryptophan	0.49 (1.07)	0.47 (1.01)	0.46 (0.94)	0.44 (0.82)	0.55 (1.13)	0.51 (5.36)	0.75
Valine	2.75 (5.97)	2.61 (5.57)	2.46 (5.07)	2.32 (4.37)	2.92 (5.96)	3.00 (3.63)	3.21
Methionine	1.33 (2.89)	1.19 (2.54)	1.05 (2.15)	0.90 (1.70)	1.35 (2.76)	1.24 (2.62)	2.60
Phenylalanine	1.73 (3.76)	1.74 (3.72)	1.75 (3.59)	1.75 (3.30)	1.89 (3.85)	1.97 (4.14)	5.76[11]

[1] Skretting Ltd. Longridge, Preston, Lancashire, UK
[2] Prosper De Mülder Group, Market Harborough. UK
[3] American Protein Corporation (APC), Ankeny, Iowa, USA
[4] Starch from corn (Sigma S4126)
[5] Dextrin type II from corn (Sigma D2130)
[6] Sigma-Aldrich Chemical.
[7] Skretting Aquaculture, Longridge Preston, UK.
[8] Sigma (C8002)
[9] Calculated
[10] Peres and Oliva-Teles (2007)
[11] Phenylalanine + tyrosine.
* Percentage of the diet.
[+] Percentage of the protein.

Table 3 Formulation, proximate composition (g kg^{-1}), essential amino acid profile of the experimental diets (g 16 g^{-1} N) and essential amino acid requirements (g 16 g^{-1} N) in in Trial #2.

	Experimental diets					Requirement[10]
	FM	PMM75	dPMM50	dPMM75	SBM/dPMM	
Fish meal LT94[1]	640.0	160.0	320.0	160.0	320.0	
Poultry meat meal[2]	0.0	570.0	0.0	0.0	0.0	
Defatted poultry meat meal[2]	0.0	0.0	333.0	495.0	194.0	
Soybean meal (de-hulled)[3]	0.0	0.0	0.0	0.0	194.0	
Marine fish oil	73.0	57.0	100.0	110.0	100.0	
Starch[4]	113.0	113.0	113.0	113.0	113.0	
Dextrin[5]	57.0	57.0	57.0	57.0	57.0	
Vitamin[6]	5.0	5.0	5.0	5.0	5.0	
Mineral[7]	5.0	5.0	5.0	5.0	5.0	
Additive (Vitamin C)	1.0	1.0	1.0	1.0	1.0	
Cellulose[8]	106.0	32.0	66.0	54.0	11.0	
Proximate composition						
Moisture	865.80	886.50	860.70	873.10	877.70	
Crude protein	456.70	476.50	460.80	454.40	454.80	
Crude lipid	114.70	148.20	114.10	131.30	133.00	
Gross energy (MJ Kg^{-1})	16.86	17.54	16.60	17.14	17.32	
Ash	83.70	110.30	97.40	100.80	87.80	
Essential Amino acid profile[9]						
Arginine	2.91*(6.31)$^{+}$	3.10 (6.51)	2.76 (5.99)	2.67 (5.88)	2.92 (6.43)	5.55
Histidine	1.06 (2.29)	0.91 (2.00)	0.88 (1.92)	0.79 (1.75)	0.98 (2.15)	1.98
Leucine	3.32 (7.21)	3.31 (7.25)	3.03 (6.57)	2.86 (6.29)	3.16 (6.95)	4.75
Lysine	3.56 (7.74)	3.07 (6.73)	2.98 (6.48)	2.68 (5.89)	3.08 (6.78)	5.13
Threonine	1.86 (4.03)	1.92 (4.21)	1.73 (3.76)	1.66 (3.65)	1.76 (3.88)	2.89
Tryptophan	0.49 (1.07)	0.44 (0.96)	0.42 (0.90)	0.38 (0.83)	0.48 (1.06)	0.75
Valine	2.75 (5.97)	2.32 (5.08)	2.28 (4.94)	2.02 (4.46)	2.39 (5.26)	3.21
Methionine	1.33 (2.89)	0.90 (1.98)	0.98 (2.12)	0.80 (1.76)	0.98 (2.16)	2.60
Phenylalanine	1.73 (3.76)	1.75 (3.83)	1.59 (3.45)	1.51 (3.32)	1.76 (3.87)	5.76[11]

[1] Skretting Ltd. Longridge, Preston, Lancashire, UK.
[2] Prosper De Mülder Group, Market Harborough, UK.
[3] American Protein Corporation (APC), Ankeny, Iowa, USA.
[4] Starch from corn (Sigma S41126).
[5] Dextrin type II from corn (Sigma D2130).
[6] Sigma-Aldrich Chemical.
[7] Skretting Aquaculture, Longridge Preston, UK.
[8] Sigma (C8002).
[9] Calculated.
[10] Peres and Oliva-Teles (2007).
[11] Phenylalanine + tyrosine.
* Percentage of the diet.
[+] Percentage of the protein.

Table 4 Growth performances, feed utilization parameters and proximate composition of gilthead sea bream fed the experimental diets of Trial 1 (means ± SE).

	Experimental diets						±SEM
	FM	PMM25	PMM50	PMM75	EFM5	SDHM10	
Growth performance							
Initial weight (g)	22.85 ± 0.54	22.42 ± 0.28	22.67 ± 0.29	22.91 ± 0.21	22.67 ± 0.32	22.66 ± 0.53	0.17
Final weight (g)	67.75 ± 0.55^{abc}	68.99 ± 1.16^{abc}	63.87 ± 0.14^{ab}	63.58 ± 2.18^{a}	69.77 ± 0.40^{bc}	70.69 ± 1.47^{c}	3.04
Weight gain (g)	44.88 ± 0.02^{abc}	46.57 ± 1.44^{abc}	41.20 ± 0.15^{ab}	40.67 ± 2.05^{a}	47.10 ± 0.26^{bc}	48.03 ± 1.52^{c}	3.13
Weight gain (%) [1]	195.5 ± 4.57^{ab}	207.8 ± 9.02^{ab}	181.9 ± 3.00^{ab}	177.4 ± 8.10^{a}	207.9 ± 3.24^{ab}	212.2 ± 9.37^{b}	14.70
Feed intake (g fish^{-1} day^{-1})	1.02 ± 0.02^{b}	0.98 ± 0.03^{ab}	0.91 ± 0.01^{ab}	0.89 ± 0.04^{a}	0.98 ± 0.01^{ab}	0.99 ± 0.02^{ab}	0.05
SGR (% day^{-1})[2]	1.72 ± 0.02^{ab}	1.78 ± 0.05^{ab}	1.64 ± 0.02^{ab}	1.62 ± 0.05^{a}	1.78 ± 0.02^{ab}	1.80 ± 0.05^{b}	0.08
FCR [3]	1.43 ± 0.02^{c}	1.33 ± 0.01^{ab}	1.39 ± 0.02^{bc}	1.37 ± 0.01^{bc}	1.32 ± 0.02^{ab}	1.30 ± 0.02^{a}	0.05
PER [4]	1.52 ± 0.02^{b}	1.60 ± 0.01^{bc}	1.48 ± 0.02^{b}	1.37 ± 0.01^{a}	1.55 ± 0.02^{bc}	1.62 ± 0.02^{c}	0.09
aNPU (%)[5]	21.40 ± 0.65^{a}	22.19 ± 0.63^{a}	21.42 ± 2.89^{a}	22.43 ± 3.62^{a}	22.78 ± 1.14^{a}	23.62 ± 4.17^{a}	0.85

	Initial	FM	PMM25	PMM50	PMM75	EFM5	SDHM10	±SEM
Carcass composition g kg^{-1}								
Moisture	686.0 ± 0.24	676.2 ± 0.13	663.2 ± 0.54	670.8 ± 1.84	662.4 ± 1.77	676.5 ± 0.05	668.5 ± 1.85	0.83
Crude protein	522.4 ± 0.08	420.1 ± 0.84	397.6 ± 1.35	409.5 ± 1.11	412.3 ± 1.55	415.4 ± 1.11	407.4 ± 2.43	4.29
Crude lipid	336.4 ± 0.65	285.9 ± 0.96	304.7 ± 0.39	293.2 ± 1.48	290.3 ± 0.50	274.5 ± 0.48	290.8 ± 0.66	1.97
Ash	105.0 ± 0.14	83.7 ± 0.03	82.7 ± 0.21	84.2 ± 0.30	86.5 ± 0.47	83.9 ± 0.40	84.1 ± 0.53	0.80
Gross energy (MJ Kg^{-1})	25.03 ± 0.00	20.04 ± 0.99	20.92 ± 0.34	20.44 ± 0.43	19.95 ± 0.23	19.99 ± 0.38	20.34 ± 0.70	1.83

Values are presented as means of three replicates ± SE. One-way Anova with Tukey's pair wise comparison test (†) or Kruskal Wallis's test with post hoc multiple comparison testing (‡) in the case of a lake of normality in the data set were utilized to reveal significant differences between treatments. In each row, values with the same superscripts are not significantly different (P>0.05).

[1] Weight gain (%) = 100 × (mean final weight - mean initial weight) / men initial weight.

[2] FCR: feed intake / weight gain.

[3] SGR: 100 × [(ln mean final weight-ln mean initial weight) / days].

[4] PER: mean weight gain / mean protein intake.

[5] aNPU: 100 × (protein deposition / digestible protein intake).

Table 5 Morphometric measurements and general haematological indices of gilthead sea bream assessed on termination of Trial 1 (means ± SE).

	Experimental diets						±SEM
	FM	PMM25	PMM50	PMM75	EFM5	SDHM10	
General morphometry							
Condition factor (K) [1]	2.06 ± 0.02	2.08 ± 0.00	2.09 ± 0.03	2.11 ± 0.03	2.08 ± 0.02	2.10 ± 0.03	0.02
Hepatosomatic index (%)[2]	1.32 ± 0.28	1.41 ± 0.03	1.35 ± 0.03	1.36 ± 0.13	1.30 ± 0.08	1.35 ± 0.04	0.04
Haematocrit (%)	39.00 ± 1.80	36.50 ± 1.90	38.53 ± 3.71	41.97 ± 1.30	39.33 ± 2.76	37.05 ± 2.05	1.94
Haemoglobin (g dL^{-1})	7.65 ± 0.60	7.24 ± 0.11	7.72 ± 0.99	7.63 ± 0.24	7.74 ± 0.12	7.81 ± 0.12	0.20
RBCC ($\times 10^6$ mm^{-3})	2.40 ± 0.20	2.20 ± 0.13	2.73 ± 0.44	2.59 ± 0.13	2.71 ± 0.14	2.34 ± 0.15	0.22

Values are presented as means of three replicates ± SE. One-way Anova with Tukey's pairwise comparison test or Kruskal Wallis's test with post hoc multiple comparison testing in the case of a lake of normality in the data set were utilized to reveal significant differences between treatments. In each row, values with the same superscripts are not significantly different ($P > 0.05$).

[1] K: (fish weight (g) × 100) / (fish length (cm))^3.

[2] Hepatosomatic index: 100 × (liver weight / whole body weight).

Table 6 Growth performances, feed utilization parameters and proximate composition of gilthead sea bream fed the experimental diets of Trial 2 (means ± SE).

	Experimental diets					±SEM
	FM	PMM75	dPMM50	dPMM75	SBM/Dpmm	
Growth performance						
Initial weight (g) †	10.07 ± 0.06	10.08 ± 0.05	9.92 ± 0.14	10.14 ± 0.07	10.19 ± 0.10	0.10
Final weight (g) †	35.31 ± 1.23^{b}	31.32 ± 0.50^{a}	33.35 ± 0.16^{ab}	30.95 ± 0.46^{a}	33.67 ± 0.38^{ab}	1.80
Weight gain (%)[1] †	250.6 ± 13.2^{b}	210.7 ± 6.6^{a}	236.5 ± 6.2^{ab}	205.4 ± 5.2^{a}	230.3 ± 1.9^{b}	18.61
Feed intake (g fish^{-1} day^{-1})‡	1.39 ± 0.01^{a}	0.98 ± 0.02^{b}	1.14 ± 0.03^{ab}	0.98 ± 0.03^{b}	1.01 ± 0.03^{ab}	0.18
SGR (% day^{-1})[2] †	3.58 ± 0.10^{b}	3.24 ± 0.06^{a}	3.47 ± 0.05^{ab}	3.19 ± 0.05^{a}	3.41 ± 0.01^{ab}	0.16
FCR [3]†	1.93 ± 0.07^{b}	1.62 ± 0.01^{a}	1.71 ± 0.06^{ab}	1.65 ± 0.03^{a}	1.50 ± 0.05^{a}	0.30
PER[4] †	1.12 ± 0.04^{a}	1.28 ± 0.01^{abc}	1.26 ± 0.04^{ab}	1.31 ± 0.03^{bc}	1.45 ± 0.05^{c}	0.12
aNPU (%)[5] †	21.64 ± 0.88^{a}	25.43 ± 0.16^{bc}	22.71 ± 0.59^{ab}	24.77 ± 0.45^{abc}	27.76 ± 1.24^{c}	2.61

	Initial	FM	PMM75	dPMM50	dPMM75	SBM/dPMM	±SEM
Carcass composition g kg^{-1}							
Moisture	706.0 ± 0.4	693.0 ± 0.5	700.0 ± 0.5	708.0 ± 0.9	696.0 ± 0.1	689.0 ± 0.2	0.74
Crude protein	486.0 ± 0.2	521.0 ± 0.7	526.0 ± 0.8	521.0 ± 0.4	514.0 ± 0.1	510.0 ±0 .3	1.44
Crude lipid	217.0 ± 1.3	296.0 ± 1.6	293.0 ± 1.2	310.0 ± 0.2	308.0 ± 0.1	310.0 ± 0.8	3.60
Ash	133.0 ± 0.1	108.0 ± 0.2^{b}	122.0 ± 0.4^{a}	118.0 ± 0.1^{ab}	121.0 ± 0.3^{a}	112.0 ± 0.1^{ab}	0.78
Gross energy (MJ Kg^{-1})	22.1 ± 0.0	25.4 ± 0.2	24.8 ± 0.2	25.1 ± 0.1	25.1 ± 0.1	25.4 ± 0.1	1.27

Values are presented as means of three replicates ± SE. One-way Anova with Tukey's pairwise comparison test (†) or Kruskal Wallis's test with post hoc multiple comparison testing (‡) in the case of a lake of normality in the data set were utilized to reveal significant differences between treatments. In each row, values with the same superscripts are not significantly different (P>0.05).

[1] Weight gain (%) = 100 × (mean final weight - mean initial weight) / men initial weight.

[2] FCR: feed intake / weight gain.

[3] SGR: 100 × [(ln mean final weight-ln mean initial weight) / days].

[4] PER: mean weight gain / mean protein intake.

[5] aNPU: 100 × (protein deposition / digestible protein intake).

73 **Table 7** Fatty acid composition of experimental diets and resulting carcasses (expressed as weight percent of total fatty
74 acid) of gilthead sea bream fed the experimental diets of Trial 2 (means ± SE).

	Diets			±SEM	Carcasses			±SEM
	FM	PMM75	dPMM75		FM	PMM75	dPMM75	
14:0	9.0	4.3	7.6	2.41	5.2 ± 0.1	3.4 ± 0.1	5.0 ± 0.1	0.99
16:0	23.4	24.6	24.3	0.62	18.7 ± 0.2	19.4 ± 0.6	19.0 ± 0.2	5.46
18:0	3.7	6.1	5.5	1.25	3.6 ± 0.1	4.3 ± 0.2	3.5 ± 0.6	0.44
Total SFA	40.4	38.1	41.8	1.87	31.1	30.0	31.2	0.67
16:1n-7	11.4	8.2	10.5	1.65	9.4 ± 0.1	8.6 ± 0.1	9.8 ± 0.1	0.61
18:1n-9	18.7	32.9	24.3	7.16	20.5 ± 0.4	30.1 ± 0.9	23.1 ± 0.2	4.97
20:1n-9	9.2	3.4	3.7	3.27	5.3 ± 0.1	2.7 ± 0.1	2.7 ± 0.0	1.50
22:1n-11	9.3	2.4	2.9	3.85	4.9 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	1.56
Total MUFA	49.5	47.5	42.5	3.61	41.4	44.4	39.1	2.66
18:2n-6	1.0	6.7	4.4	2.87	2.4 ± 0.2	8.8 ± 0.1	5.2 ± 0.1	3.21
20:4n-6	0.1	0.2	0.3	0.10	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.06
Total n-6	1.7	7.6	5.6	3.00	4.8	11.0	7.7	3.10
18:3n-3	0.4	0.7	0.8	0.21	0.8 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	0.35
18:4n-3	0.3	0.2	0.6	0.20	1.5 ± 0.1	1.0 ± 0.0	1.4 ± 0.1	0.27
20:5n-3	1.4	0.8	2.3	0.75	7.5 ± 0.2	4.0 ± 0.1	6.0 ± 0.2	1.76
22:5n-3	0.1	0.3	0.5	0.20	2.1 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	0.40
22:6n-3	1.9	0.9	1.9	0.58	10.5 ± 0.4	5.4 ± 0.2	6.8 ± 0.1	2.64
Total n-3	4.3	3.1	6.4	1.67	23.3	13.7	17.7	4.82
Total PUFA	6.0	10.7	12.0	3.16	28.1	24.7	25.4	1.80
Ratio n-3/n-6	2.53	0.41	1.14	1.08	4.8	1.2	2.3	0.78

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