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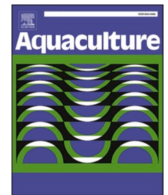

Insect and single-cell protein meals as replacers of fish meal in low fish meal and fish oil diets for gilthead sea bream (*Sparus aurata*) juveniles

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A R T I C L E I N F O

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A B S T R A C T

In recent years, the aquaculture research and industry has undertaken intensive efforts to identify novel alternatives to fish meal, in an attempt to convert fish feeds to more effective and sustainable, in response to the continuous growth of this sector. Among the several potential candidates, insect meals and single-cell proteins from bacteria have shown some of the most promising results, but they have not been thoroughly validated in gilthead sea bream (*Sparus aurata*). Therefore, the aim of the present study was to evaluate how performance, protein digestibility *in vitro*, fish body composition and the expression of some gut health-related genes were affected by the replacement of 33% and 66% of fish meal by either the black soldier fly (*Hermetia illucens*) insect meal (INS5, INS10 diets), or the single-cell protein from the bacteria *Methylococcus capsulatus* (SCP5, SCP10 diets) in practical diets for gilthead sea bream juveniles, after a nutritional trial of 112 days. The single-cell protein product supported gilthead sea bream growth and feed utilization and allowed up to 66% of replacement of the dietary fish meal. In contrast, the insect meal product led to a reduced growth and worsened feed utilization when included at the highest dietary level (10%), making possible to replace fish meal only up to 33%. Proximate composition and amino acid profile were not majorly affected by the experimental diets, but moderate inclusions of the single cell protein from *M. capsulatus* (5%) significantly increased the fish fillet contents of important fatty acids for human nutrition, like 20:5n-3 and 22:6n-3, possibly related with a higher n-3 LC-PUFA sparing effect resulting from and optimized ratio of saturated and monounsaturated fatty acids. The relative expressions of some molecular makers related with fish gut health (*hsp90, hsp70, mchii, cox-2, tnfa, il-1b*) were also similar, irrespective of the dietary treatment.

1. Introduction

During the last decades, the overexploitation of fish wild stocks has limited the availability and price of fish meals (FM), traditionally used as major protein source in aquaculture feeds. Furthermore, gaining awareness in how dependency on forage fisheries affects aquaculture sustainability has forced for a replacement of FM derived from wild fisheries in fish feeds. For this purpose, a significant part of aquaculture research effort in the last two decades was focused on the replacement of FM by more constantly available, economic and sustainable raw materials. Nowadays, aquaculture producers undoubtably recognise that reducing FM in fish feeds is mandatory for aquaculture progression and expansion, not only from an economic point of view, but also to increase sustainability and responsibility of the sector. Most of the research effort that aimed to test substitutes for FM has focused on plant/vegetable meals (VM). However, and despite current aquafeeds incorporate a high

Abbreviations: DHA, docosahexaenoic acid; EAA, essential amino acids; EPA, eicosapentaenoic acid; FA, fatty acids; FM, fish meal; INS, insect meal; LC-PUFA, long-chain polyunsaturated fatty acids; NEAA, non-essential amino acids; SCP, single-cell protein;; VM, vegetable meal.

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amount of vegetable raw materials, one of the constraints associated to their high level of inclusion in fish diets remains the presence of anti-nutritional factors, which are known to cause inflammation problems in the digestive tract, especially in carnivorous fish. At the same time, vegetable-based diets often lead to low palatable feeds due to the high content of non-soluble carbohydrates, fibres and starch (Naylor et al., 2021). Furthermore, depending on the source, some VM does not provide the desirable protein content and balanced amino acid profile as FM for covering fish nutritional requirements. Therefore, the successful inclusion of these ingredients in aquafeeds depends on fish species and feed habits, being more challenging for carnivorous species like gilthead sea bream (*Sparus aurata*), and sometimes being associated to a reduction in fish productive parameters and/or poorer fish health (Torrecillas et al., 2017a; Simó-Mirabet et al., 2018). Consequently, novel raw materials have been emerging in the market more recently, with high potential for protein source, replacing FM and decreasing the dependency on VM, to maximize fish production while guarantee more sustainable and responsible feeds. Insect meals and bacterial single-cell proteins are among the most interesting novel proteins recently considered (Salter and Lopez-Viso, 2021).

Insect meals are rich in protein (60–70% on a dry matter basis (DM)), with well-balanced essential amino acid (EAA) profile, for instance containing high contents of methionine, which is often a limiting EAA in animal or plant meals (Henry et al., 2015; Basto et al., 2020). Their potential is not limited to the protein content, but it can also contain high contents of lipids (10–50% DM), and, although they are not naturally rich in the essential n-3 long-chain polyunsaturated fatty acids (LC-PUFA), their FA profile can be modulated through the rearing substrates and technological processes (Magalhães et al., 2017). Moreover, insect meals can be also source vitamins (especially B_{12}) and minerals (iron and zinc) (Barroso et al., 2014). Considering their potent nutrient composition, insect meals may modulate fish immune system response due to the presence of antioxidant peptides, chitin and antimicrobial peptides, thus potentially promoting animal health, particularly at local level by triggering gut associated lymphoid tissue response, improving fish health and welfare (Henry et al., 2015; Komi et al., 2018; Stenberg et al., 2019; Zarantoniello et al., 2022a, 2022b). The inclusion of novel insect meals in fish feeds was allowed in the European Union in 2017 (EC Regulation 893/2017), and a some research has been carried out to investigate their potential for aquaculture feeds, which depends not only on their composition but also on their nutrient bioavailability. Several insect species have been studied in the last years and tested in several fish species, including in rainbow trout (*Orcorhynchus mykiss*) (St-Hilaire et al., 2007; Sealey et al., 2011; Zarantoniello et al., 2022a, 2022b), Nile tilapia (*Oreochromis niloticus*) (Ogunji et al., 2006, 2008; Sánchez-Muros et al., 2016), turbot (*Scophthalmus maximus)* (Kroeckel et al., 2012) and European sea bass (*Dicentrarchus labrax*) (Basto et al., 2020, 2021). Meals from Diptera insects, for instance black soldier fly (*Hermetia illucens*), are apparently the most similar to FM in terms of protein quality (Barroso et al., 2014). However, the few studies that have evaluated this meal on gilthead sea bream used usually high FM inclusion (Karapanagiotidis et al., 2014; Fabrikov et al., 2020, 2021; Randazzo et al., 2021) being worthy validating the replacement of FM by insect meals in the current context of modern feeds with low dietary FM *a priori*.

Single-cell protein meals can be obtained from cultures of different microbial sources, including microalgae, fungi (yeast and others) and bacteria, all of them generally rich in protein and vitamins, and capable to be reared in waste substrates, positioning them as highly-profitable and sustainable raw materials (Jones et al., 2020). In particular, meals coming from bacterial single-cells stands out for their very low footprint and their fast growth in different conditions (Øverland et al., 2010). Furthermore, these single-cell protein meals have a very high protein content (50–80% in DM), with a well-balanced EAA profile, as well can be rich in vitamins, phospholipids and other functional compounds. Several bacterial sources are being currently tested in fish feeds and many commercial products have been emerging in the market. *Methylococcus capsulatus* is one of the most promising methylotrophs that are being currently produced at commercial scale (Jones et al., 2020). The inclusion of this microbial source can constitute 52% and 38% of the dietary protein in diets for Atlantic Salmon (*Salmo salar*) and rainbow trout, respectively, without compromising growth performance (Øverland et al., 2010). Furthermore, *M. capsulatus* was shown to improve gut health in salmon, reducing the enteritis associated with high soybean feeds (Øverland et al., 2010). However, to our knowledge, the inclusion of *M. capsulatus* as potential FM replacer in diets for gilthead sea bream has not been yet investigated.

The aim of the present study was thus to evaluate the potential of commercially-available novel products from insect meal (*H. illucens*) and bacterial single-cell protein (*M. capsulatus*), as FM replacers in diets for gilthead sea bream (*Sparus aurata*) juveniles using the current context of ocean-friendly aquaculture feeds. *In vitro* digestibility of the alternative feeds containing these novel proteins was measured, as well as their *in vivo* effects on fish growth performance and tissue composition. Some parameters related to fish health, namely the expressions of health-related genes in posterior gut, were also monitored.

2. Material and methods

2.1. Ethical statement

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA_ULPGC_12/2020).

2.2. Experimental diets

A total of five experimental diets were formulated with similar proximate composition (Table 1). A control diet was formulated to contain 15% FM that was 33 and 66% replaced by 5 and 10%, respectively, with one of the two selected alternative novel proteins: black soldier fly *H. illucens* (InnovaFeed, France; INS5 and INS10) and the single-cell protein meal obtained from a by-product resulting from the fermentation of the bacteria *Methylococcus capsulatus* (FeedKind, Calysta, Menlo Park, CA; SCP5 and SCP10). All diets were supplemented with lysine whereas INS diets were further supplemented with methionine to approximately reach similar contents of these EAA in all diets to cover gilthead sea bream nutritional requirements (NRC, 2011). Feeds were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) and shipped to the ECOAQUA Institute laboratories (Canary Islands, Spain), where they were analysed for proximate (Table 1), amino acid (Table 2) and fatty acid (Table 3) composition.

2.3. In vitro digestibility of the feeds

Protein digestibility *in vitro* (degree of hydrolysis, DH) was determined by pH-stat titration according to the method developed by Dimes and Haard (1994) and modified by Alarcon et al. (2002). DH is defined as the percentage of peptide bonds cleaved and is based on the titration of the amino groups generated by the cleavage of the peptide bonds. Crude enzyme extract previously isolated from the pyloric caeca of adult gilthead sea bream was used to determine total dietary protein digestibility at three feed quantity/enzyme ratios (5, 15 and 30 mg protein/U trypsin activity) in order to simulate variations both in enzyme activity and feed intake occurring in the digestive tract *in vivo*. Autohydrolysis rates of each feed are measured with crude enzyme extracts being replaced by distilled water. Autohydrolysis rates reflect the intrinsic capacity of the feed to release peptide particles without the action of digestive enzymes. Each determination was performed at 23 °C

Table 1
Formulation and proximate composition of the experimental diets.

	Experimental diets				
Raw material (%)	Control	INS5	INS10	SCP5	SCP10
Fish meal [1]	15.00	10.00	5.00	10.00	5.00
Insect Meal[2]		5.00	10.00		
Single cell[3]				5.00	10.00
Corn gluten[4]	9.00	9.00	9.00	9.00	9.00
Wheat gluten[5]	16.74	16.37	16.01	17.00	16.86
Sunflower meal[6]	5.00	5.00	5.00	5.00	5.00
Faba beans[7]	10.00	10.00	10.00	10.00	10.00
Wheat[8]	6.58	6.39	6.15	6.04	6.51
Soy protein concentrate[9]	22.00	22.00	22.00	22.00	22.00
Fish oil[10]	7.93	8.51	9.14	8.51	8.50
Rapeseed oil[11]	4.93	4.40	3.82	4.44	3.49
Phosphate[12]	1.08	1.26	1.45	1.13	1.53
DL-Methionine[13]		0.05	0.13		
Choline	0.27	0.30	0.34	0.31	0.31
Lysine HCl[14][15,16]	0.07	0.32	0.56	0.17	0.50
Mineral mix[15]	0.10	0.10	0.10	0.10	0.10
Vitamin premix[16]	0.30	0.30	0.30	0.30	0.30
Lecithin[17]	1.00	1.00	1.00	1.00	1.00
Proximate composition (% DM)					
Protein	54.82	53.08	51.94	52.97	53.69
Lipids	17.46	18.08	17.75	18.42	17.68
Ash	5.69	5.19	5.06	5.20	5.23

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement).
[1] FF SKAGEN A/S (Denmark).
[2] InnovaFeed (France) (Protein: 57–62%; Lipids: 8–11; Ash: 8–10; Moisture: 2.5).
[3] Calysta (USA) (Protein: 70.6%; Lipids: 9.8; Ash: 7.1; Moisture: 6).
[4] CARGILL (The Netherlands).
[5] CARGILL (The Netherlands).
[6] BUNGE (Hungary).
[7] Cefetra BV (The Netherlands.
[8] Lantmnnen Ek For (Sweeden).
[9] IMCOPA (Brazil).
[10] Norsildmel AS (Norway).
[11] AAK, AB (Sweeden).
[12] Yara (Norway).
[13] Evonik (Germany).
[14] Ajinomoto (France).
[15,16] Trouw Nutrition (The Netherlands).
[17] Berg Schmidt (Germany).

in triplicate.

Additionally, the two predominant protease inhibitor groups, namely the Kunitz trypsin inhibitor (KTI) and Bowman−Birk protease inhibitor (BBI) were determined in each experimental diet. For that, protease inhibitors were extracted by grounding feeds and mixed with distiller water (50 mg/mL) followed by extraction with chloroform and isolated by centrifugation. KTI was assayed against trypsin activity (Erlanger et al., 1961), whereas BBI was assayed against chymotrypsin activity (Ásgeirsson and Bjarnason, 1991). Each extract was assayed at three different inclusion rates. All reactions were performed in triplicate.

2.4. Experimental fish and conditions of the in vivo trial

Triplicate groups of gilthead sea bream juveniles with an initial body weight of 65.37 ± 3.36 g (mean ± SD) and an average total length of 15.64 ± 0.37 cm were randomly distributed in 15 cylindroconical tanks of 500 L capacity, at a density of 25 fish per tank. Tanks were equipped with microbubble aeration, in an open waterflow system. Water temperature during the trial ranged between 19.3 and 22.6 °C, dissolved oxygen between 5.7 and 6.4 mg/L, and salinity was 37 mg/L.

Fish were fed under natural photoperiod, 3 times a day until apparent satiety for 112 days. After each meal, the uneaten pellets were collected with a strainer through the tank outlet, that were dried for 24 h and weighed to estimate feed intake. Fish weighed and length were monitored every 30 days to estimate growth performance parameters as follows:

Weight gain, WG (g): final weight – initial weight;
Feed intake, FI (g/feed fish/day): feed intake/days/number of fish;
Feed conversion ratio, FCR: dry feed intake (g)/weight gain (g);
Specific growth rate, SGR (%/day): (ln final mean weight - ln initial mean weight)/days x 100;
Protein efficiency ratio, PER: weight gain/protein consumed.
Lipid efficiency ratio, LER: weight gain/lipid consumed.

Table 2
Amino acid composition (mg/ g protein) of the experimental diets.

Experimental diets					
	Control	INS5	INS10	SCP5	SCP10
EAA					
Arg	60.7	52.7	52.6	53.1	50.6
His	16.7	18.3	27.1	18.4	14.3
Ile	12.2	11.0	8.0	8.9	11.5
Leu	25.9	20.2	17.4	19.9	22.3
Lys	19.4	20.8	22.9	20.3	19.2
Met	33.3	31.1	28.7	28.9	31.2
Phe	21.6	30.8	35.0	27.5	27.1
Thr	35.0	32.2	31.8	30.7	30.9
Val	4.8	3.4	5.3	4.0	3.9
NEAA					
Ala	7.4	4.4	5.4	5.5	5.7
Asp	51.8	56.3	46.7	33.4	33.2
Cys	26.6	19.5	20.7	19.9	17.1
Glu	31.2	43.4	79.2	29.0	67.1
Gly	17.7	16.3	15.8	16.4	15.1
Pro	26.4	25.0	25.1	25.5	24.8
Ser	45.2	45.1	51.7	35.3	45.6
Tau	5.0	4.7	4.4	2.6	4.5
Tyr	39.8	50.1	74.6	56.9	35.0

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement).
EAA (essential aminoacids): Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, metionine; Phe, phenilalanine; Thr, threonine; Val, valine; NEAA (non-eseential aminoacids): Ala, alanine; Asp, aspartame; Cys, cysteine; Glu, glutamine; Gly, glycine; Pro, poline; Ser, serine; Tau, taurine; Tyr, tyrosine.

2.5. Sampling procedures

Before each sampling, fish were fasted for 24 h. In handling procedures for weight estimation, fish were anesthetized with clove oil (0.2 mL/L; Guinama S.L; Spain, Ref. Mg83168) diluted in alcohol 100% (1:2), and individually weighed and measured. At the end of the trial, twelve fish per tank were euthanized with an excess of anesthesia and samples of whole-body (six fish), and fillets (another 6 fish) were collected for proximate composition analysis. Additionally, a portion of the posterior gut was collected from 5 fish per tank, fixed in RNAlater and stored for molecular analysis. All samples were frozen at −80 °C until the respective analysis.

2.6. Biochemical composition

Samples of diets, whole-body and fillets were homogenized (T25 Digital Ultra-turraX, IKA®, Germany) before analysis. Protein, ash and moisture contents were determined (AOAC, 2019). Protein quantification was determined according to the Kjeldahl technique. Amino acid profiles of the samples were estimated after acid hydrolysis with HCl 6 M

Table 3
Fatty acid composition (% total identified fatty acids) of the experimental diets.

Experimental diets	Control	INS5	INS10	SCP5	SCP10
14:0	1.87	1.51	1.15	1.30	0.33
14:1n-7	0.18	0.06	0.02	0.06	0.02
14:1n-5	0.30	0.15	0.09	0.10	0.02
15:0	0.30	0.19	0.20	0.15	0.06
15:1n-5	0.24	0.08	0.01	0.03	0.03
16:0ISO	0.23	0.08	0.01	0.07	0.02
16:0	8.55	9.86	12.45	8.41	5.18
16:1n-7	1.65	1.93	2.81	1.85	1.45
16:1n-5	0.29	0.09	0.10	0.10	0.06
16:2n-6	0.00	0.00	0.00	0.00	0.00
16:2n-4	0.19	0.15	0.16	0.12	0.11
17:0	0.17	0.10	0.12	0.10	0.10
16:3n-4	0.28	0.21	0.26	0.22	0.20
16:3n-3	0.27	0.15	0.11	0.09	0.09
16:3n-1	0.28	0.10	0.05	0.06	0.05
16:4n-3	0.38	0.19	0.22	0.15	0.18
16:4n-1	0.00	0.00	0.00	0.00	0.00
18:0	1.83	2.08	2.36	2.07	2.36
18:1n-9	22.85	26.26	28.73	26.70	31.76
18:1n-7	2.05	2.41	2.40	2.18	2.68
18:1n-5	0.35	0.22	0.19	0.17	0.21
18:2n-9	0.17	0.11	0.02	0.06	0.03
18:2n-6	13.81	15.51	17.79	16.46	20.14
18:2n-4	0.32	0.09	0.08	0.08	0.08
18:3n-6	0.53	0.28	0.13	0.17	0.14
18:3n-4	0.24	0.09	0.07	0.06	0.09
18:3n-3	3.60	3.85	4.15	4.10	5.06
18:3n-1	0.37	0.06	0.02	0.05	0.02
18:4n-3	1.26	1.24	1.49	1.35	1.60
18:4n-1	0.39	0.09	0.03	0.05	0.05
20:0	0.73	0.48	0.38	0.46	0.47
20:1n-9	0.49	0.49	0.46	0.44	0.45
20:1n-7	4.08	4.11	3.48	4.10	3.93
20:1n-5	0.41	0.17	0.14	0.19	0.16
20:2n-9	0.38	0.09	0.02	0.09	0.05
20:2n-6	0.61	0.31	0.26	0.32	0.30
20:3n-9	0.18	0.09	0.03	0.09	0.06
20:3n-6	0.15	0.09	0.04	0.11	0.06
20:4n-6 (ARA)	0.54	0.41	0.34	0.32	0.35
20:3n-3	0.35	0.17	0.11	0.10	0.13
20:4n-3	0.50	0.43	0.35	0.42	0.40
20:5n-3 (EPA)	5.00	5.45	4.98	5.40	5.35
22:1n-11	7.93	7.47	5.16	7.29	5.73
22:1n-9	0.86	0.71	0.55	0.61	0.67
22:4n-6	0.95	0.22	0.04	0.13	0.06
22:5n-6	0.69	0.29	0.14	0.31	0.18
22:5n-3	1.06	0.72	0.53	0.88	0.63
22:6n-3 (DHA)	12.15	11.17	7.81	12.44	8.89
SFA	13.46	14.22	16.66	12.49	8.50
MUFA	41.68	44.14	44.12	43.82	47.16
n-3 PUFA	24.55	23.37	19.74	24.93	22.32
n-6 PUFA	17.27	17.11	18.74	17.82	21.24

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement); ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

and phenol 0.01% at 110 °C for 24 h. Then, samples were dried under N_2 atmosphere and reconstituted with 1000 μL ultra-pure water and then filtered by a 0.22 μm filter. Aliquots of the filtrate were stored at −20 °C until use. Samples were then derivatized using 4-dimethylaminoazobenzene-4′-sulfonyl chloride (Chang et al., 1982). The amino acid separation was carried out in an HPLC (YOUNG LIN YL 9100; YL Instruments, Korea) using a Kromasil Classic® C18 4 μm 4.0*250 mm column equipped with a column guard Nova-Pak® C18 4 μm, WAT 044380. Acetonitrile: ultra-pure water (60:40) and phosphate buffer solution (NaH2PO4, 9 mM) will be used as eluents. Quantification was performed using a UV/Vis detector YL 9120 (YL Instruments). A mixture of 18 amino acids were injected at three or more different concentrations to obtain the standard curve for each amino acid. The concentration of each amino acid was expressed as a percentage of the sum of amino acid analysed.

Total lipids were extracted with a mixture of chloroform:methanol (2:1) with 0.01% BHT (Folch et al., 1957). Fatty acid methyl esters were extracted from total lipids (Christie, 1989), then separated with gas-liquid chromatography under the conditions described by Izquierdo et al. (1990), quantified by a flame ionizator detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified by comparison with previous characterized standards.

2.7. Expression of health-related genes

RNA from the posterior gut samples was extracted and purified under iced conditions. The samples were homogenized with Tissuelyser II (Qiagen) with Tri Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and RNA was separated by adding 250 μL of chloroform and centrifuged at 12000 *g*, for 30 min at 4 °C. After centrifugation, the upper aqueous phase that contained the RNA was mixed with EtOH 70% and passed through a RNeasy spin column of the kit-RNeasy. To purify the RNA, RW1 and RPE buffers were added to the column that retained the pure RNA bonded inside its membrane. Finally, 35 μL of RNase-free water was added to the column to draw the final purified RNA retained in the membrane. All these steps followed according the established protocol described by the manufacturer. RNA quantity was determined by the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the RNA integrity was determined by Gel Red™ staining (Biotium Inc., Hayward, CA) using a 1.4% agarose electrophoresis gel. The synthesis of cDNA was carried out using the iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA).

The mRNA levels of heat shock protein 70 (*hsp70*), *heat shock protein 90* (*hsp90*), *cyclooxygenase-2 (cox2)*, *tumour necrosis factor alpha* (*tnfa*), *major histocompatibility complex class II* (*mhcII*), and *interleukine 1b* (*il-1b*) were determined by RT-PCR (iQ5 Multicolour Real-Time PCR detection system, Bio-Rad), with cytoplasmic β-actin 1 (β-actin) used as a housekeeping gene. Primer sequences, and the respective annealing temperatures as well as concentrations used are described in Table 4. For RT-PCR, a mix with 7.5 μL of the Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA) (ref 1,708,886), 0.6 μL of each primer at a concentration of 10 mM, 5 μL of cDNA at dilution 1:10 and 1.3 μL MiliQ water was prepared and dispensed in each well. cDNA and RT-PCR control blanks were including replacing cDNA by water. RT-PCR conditions were as follows: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at the respective annealing temperature (T_m, Table 4), 30 s at 72 °C, 1 min at 95 °C, and a final 81 cycles of 10s from 55 °C to 95 °C. The resulting data was used to calculate the relative gene expression according to the $2^{-\Delta\Delta}$CT method (Livak and SCHMITTGEN, 2001).

2.8. Statistical analysis

Data are presented as mean ± SD. Data normality and homogeneity of variances were tested with Shapiro–Wilk and Levene's tests, respectively. Data were analysed with orthogonal contrasts to compare the experimental diets among them. Significant differences were detected when *P*-value was below 0.05 ($P < 0.05$). Statistical treatment of the data was carried out using SPSS 21.0 software.

3. Results

3.1. In vitro digestibility of the experimental diets

All experimental diets exhibited higher autohydrolysis and digestibility rates *in vitro* than Control diet at the level of 5 mg dietary protein/U trypsin (Table 5). SCP feeds exhibited consistently high

Table 4
Primer sequences, concentrations and annealing temperatures of genes assessed in posterior gut of gilthead sea bream fed the experimental diets.

Gene	Primer sequence (5′-3′)	Conc. (μM)	T_m(°C)	Accesion number[a]
hsp70	F: 5′-TTGACCATTGAGGATGGCATC-3′ R: 5′-TCCTTCTTGTACTTGCGCTTG-3′	0.6	60	EU805481.1
hsp90	F: 5′-GTCATCCTGCTG TTCGAGACC-3′ R:5′-CTCCTCTACGGGAACGTCGTC-3′	0.6	60	DQ012949.1
cox2	F: 5′-FGAGTACTGGAAGCCGAGCAC-3′ R: 5′-GATATCACTGCCGCCTGAGT-3′	0.6	60	AM296029
mhcII	F: 5′-GAGTTCCTCCCCAACCACGATG-3′ R: 5′-GCCGTCGTGTTAAGTTTCTCGTCA-3′	0.6	62	DQ211541.1
tnfa	F: 5′-CTCACACCTCTCAGCCACAG-3′ R: 5′-TTCCGTCTCCAGTTTGTCG-3′	0.6	62	AJ413189.2
il-1b	F: 5′-AGCGACATGGCACGATTTC-3′ R: 5′-GCACTCTCCTGGCACATATCC-3′	0.6	62	AJ277166.2
β-actin	F: 5′-TCTGTCTGGATCGGAGGCTC-3′ R: 5′-AAGCATTTGCGGTGGACG-3′	0.6	60	KY388508.1

Tm, annealing temperature; *hsp70, heat shock protein 70; hsp90, heat shock protein 90; cox2, cyclooxygenase-2; mhcII, major histocompatibility complex class II; tnfa, tumour necrosis factor alpha; il-1b, interleukin 1-beta.*

[a] *Gen Bank: http://www.ncbi.nlm.nih.gov/*

Table 5
Protein autohydrolysis and digestibility rates *in vitro* of the experimental diets related with three increasing dietary protein quantities/U trypsin.

Autohydrolysis rates (%)			
mg dietary protein/ U digestive capacity	**5**	**15**	**30**
Control	67.31 ± 1.88	33.56 ± 1.06	25.41 ± 0.81
INS5	91.31 ± 4.31	26.25 ± 3.75	16.56 ± 0.31
INS10	91.13 ± 8.63	31.16 ± 0.09	10.94 ± 0.31
SCP5	75.00 ± 7.50	31.25 ± 2.50	19.19 ± 1.56
SCP10	78.19 ± 3.19	32.59 ± 1.09	19.03 ± 1.44
Digestibility rates (%)			
mg dietary protein/ U digestive capacity	**5**	**15**	**30**
Control	75.66 ± 2.34	56.94 ± 4.87	43.55 ± 2.30
INS5	94.50 ± 4.87	73.84 ± 5.97	39.95 ± 1.61
INS10	99.84 ± 0.66	41.53 ± 2.09	24.06 ± 0.72
SCP5	94.41 ± 6.28	74.50 ± 1.25	49.38 ± 0.22
SCP10	96.84 ± 1.97	72.84 ± 0.34	37.63 ± 1.84

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement).

digestibility rates at all ratios of mg dietary protein/U digestive capacity tested as a result of a high enzymatic hydrolysis (Table 5). In contrast, INS feeds, particularly INS10 showed lower digestibility rates with the increasing of the dietary protein/U trypsin (Table 5). The main characteristic of the Control diet was sustained autohydrolysis and digestibility rates *in vitro* at all levels of mg dietary protein/U trypsin tested (Table 5).

In addition, all diets were found to contain both KTIs and BBIs, coming predominantly from the vegetable sources used, and which were similar among all the experimental diets (Table 6).

3.2. Growth performance and feed utilization

After 112 days of feeding, SCP diets, either at 5 or 10% of dietary inclusion (SCP5 or SCP10) led to similar fish growth (final weight and length, weight gain or SGR) as Control diet (Table 7). In contrast, fish fed INS diets, particularly at 10% of inclusion level (INS10) showed the lowest final weight compared to all the other experimental diets, and lower weight gain or SGR compared with SCP5 ($P < 0.05$; Table 7). Furthermore, INS diets, both at 5 and 10% of dietary inclusions (INS5 and INS10) led to a reduced feed intake in fish compared to SCP5 diet ($P < 0.05$; Table 7). Although no significant statistical differences were noted in FCR or PER, a similar worsening tendency was also observed in sea bream fed INS10 diet, as well as a significantly lower ($P < 0.05$) LER when compared with fish fed the Control diet (Table 7).

3.3. Whole-body and flesh composition

The proximate composition of whole-body and fillet did not majorly differ among fish fed the experimental diets (Table 8). Only ash content of whole-body was significantly affected by the experimental diets, with SCP5 diet leading to a higher content compared with Control or INS5 ($P < 0.05$; Table 8). Similarly, most of amino acid contents in fish whole-body were similar, irrespective of the diet (Table 9), with INS5 diet leading to a higher lysine and proline contents compared with SCP or INS at 10% (SCP10 and INS10) of dietary inclusion, respectively ($P < 0.05$; Table 9).

In addition, fatty acid profile of fish whole-body were similar among gilthead sea bream fed the different experimental diets and generally reflected the dietary pattern (Table 10). Fillet fatty acid composition was clearly more affected by the experimental diets than whole-body fatty acid profile (Table 10 *vs* Table 11). Therefore, fish fed SCP diets showed higher 16:1n-7 or 18:1n-7 than those fed control diet in agreement with the dietary profile ($P < 0.05$; Tables 10 and 11). Similarly, SCP diets, particularly at moderate inclusion at 5% (SCP5), led also to a higher content of 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3, as well as a higher n-3 PUFA and n-3/n-6 ration in fillets, when compared with the Control diet ($P < 0.05$; Table 11). Furthermore, in fillets of fish fed SCP10 diet, 22:6n-3 was also lower when compared to those fed SCP5 ($P < 0.05$; Table 11).

Table 6
KTI and BBI content (Units/mg feed) of the experimental diets.

	Experimental diets				
	Control	INS5	INS10	SCP5	SCP10
KTI (U/ mg feed)	0.54 ± 0.15	0.58 ± 0.32	0.68 ± 0.20	0.50 ± 0.17	0.34 ± 0.09
BBI (U/ mg feed)	0.63 ± 0.09	0.53 ± 0.05	0.69 ± 0.12	0.83 ± 0.12	1.31 ± 0.40

Data expressed as mean ± SD. Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement).

Table 7
Growth performance and feed utilization of gilthead sea bream (*Sparus aurata*) fed the experimental diets for 112 days.

	Experimental diets				
	Control	INS5	INS10	SCP5	SCP10
Initial body weight (g)	65.33 ± 0.11	65.37 ± 0.31	65.33 ± 0.29	65.25 ± 0.98	65.69 ± 0.09
Final body weight (g)	161.38 ± 3.09^{a}	158.38 ± 8.04^{a}	152.21 ± 3.94^{b}	163.51 ± 3.69^{a}	161.22 ± 4.42^{a}
Weight gain (g)	96.05 ± 3.16^{ab}	93.01 ± 8.00^{ab}	86.88 ± 3.76^{b}	98.26 ± 4.67^{a}	95.53 ± 4.51^{ab}
SGR (g fish^{-1} day^{-1})	0.81 ± 0.02^{ab}	0.79 ± 0.05^{ab}	0.75 ± 0.02^{b}	0.82 ± 0.03^{a}	0.80 ± 0.03^{ab}
Feed intake (g feed fish^{-1} day^{-1})	1.32 ± 0.04^{ab}	1.31 ± 0.04^{b}	1.32 ± 0.01^{b}	1.38 ± 0.01^{a}	1.31 ± 0.01^{ab}
FCR	1.54 ± 0.08	1.59 ± 0.13	1.69 ± 0.11	1.57 ± 0.07	1.54 ± 0.11
PER	1.17 ± 0.06	1.15 ± 0.09	1.10 ± 0.02	1.16 ± 0.06	1.16 ± 0.08
LER	3.72 ± 0.19^{a}	3.49 ± 0.19^{ab}	3.34 ± 0.17^{b}	3.46 ± 0.24^{ab}	3.69 ± 0.29^{ab}

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement).
Values (mean ± SD, $n = 3$) with different superscript letters in the same row are significantly different ($P < 0.05$).

Table 8
Proximate composition of whole-body and fillets of gilthead sea bream (*Sparus aurata*) fed the experimental diets for 112 days.

	Experimental diets				
	Control	INS5	INS10	SCP5	SCP10
Whole-body					
Protein	15.59 ± 0.55	15.66 ± 0.12	15.78 ± 0.29	15.54 ± 0.31	15.78 ± 0.51
Lipids	14.08 ± 1.59	14.85 ± 0.29	14.68 ± 0.59	15.44 ± 0.37	13.65 ± 1.20
Moisture	67.51 ± 2.31	66.96 ± 0.31	67.17 ± 0.58	66.44 ± 0.43	68.42 ± 1.49
Ash	1.11 ± 0.03^{b}	1.11 ± 0.04^{b}	1.15 ± 0.07^{ab}	1.23 ± 0.07^{a}	1.17 ± 0.01^{ab}
Fillet					
Protein	20.69 ± 0.41	20.88 ± 0.18	20.60 ± 0.05	21.15 ± 0.27	20.95 ± 0.41
Lipids	7.68 ± 0.84	8.02 ± 0.07	8.18 ± 0.11	8.06 ± 0.04	8.20 ± 0.16
Moisture	70.60 ± 0.71	70.11 ± 0.09	70.15 ± 0.05	69.93 ± 0.19	70.16 ± 0.10
Ash	1.40 ± 0.06	1.37 ± 0.13	1.40 ± 0.13	1.39 ± 0.09	1.34 ± 0.03

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement). Values (mean ± SD, $n = 3$).

3.4. *Posterior gut expression of health-related genes*

Despite de lack of significant differences in the relative expressions of *hsp90*, *hsp70*, *cox2*, *mhcii, tnfa*, *il-1b*, among fish fed the different diets, a tendency to a slight downregulation of those genes could be noted in fish fed INS diets (Fig. 1). In contrast, Control and SCP diets showed very similar expression levels of all the genes (Fig. 1).

4. Discussion

Protease inhibitors have been recognized as potent antinutritional factors in animals, including in fish (Krogdahl et al., 2010), impairing protein digestibility and the bioavailability of the amino acids. The two predominant protease inhibitor groups that are present predominantly in vegetable raw materials are KTIs and BBIs, that inhibit trypsin and chymotrypsin activity, respectively. Since all the diets tested contained similar plant-based ingredients inclusion, KTIs and BBIs were consequently similar in all the diets. The enzymatic hydrolysis values obtained are an indication that the feed is compatible with the palette of the digestive enzymes of gilthead sea bream that act to make its contents bioavailable. The high enzymatic hydrolysis values of SCP feeds of the present study is particularly important as it indicates sustained digestibility even at high feed consumption rates, which suggest the potential of *M. capsulatus* as a highly digestible protein source. Indeed, the moderate dietary inclusion of the SCP at 5%, and equivalent to a moderate replacement of the dietary FM of 33%, apparently stimulated fish feed intake, also denoting the high palatability of this SCP product and the good acceptability of these feeds by gilthead sea bream. These results agreed well with the good growth, feed and nutrient utilization showed by fish fed these diets *in vivo*, even at the highest dietary inclusion (10%), which was similar to those fed the control diet, and thus

Table 9
Whole-body amino acid composition (μg/ mg protein) of gilthead sea bream (*Sparus aurata*) fed the experimental diets.

Experimental diets					
	Control	INS5	INS10	SCP5	SCP10
EAA					
Arg	18.4 ± 6.40	20.5 ± 6.15	21.3 ± 8.90	18.4 ± 7.88	22.2 ± 8.12
His	3.4 ± 0.48	3.8 ± 0.73	3.8 ± 0.99	3.4 ± 0.94	3.6 ± 0.22
Ile	10.1 ± 2.55	12.5 ± 3.99	12.4 ± 5.49	9.9 ± 2.36	11.8 ± 1.60
Leu	21.4 ± 3.79	22.5 ± 4.08	23.7 ± 7.66	17.4 ± 4.54	22.4 ± 3.96
Lys	17.2 ± 0.67^{ab}	18.7 ± 1.60^{a}	18.9 ± 3.79^{ab}	17.5 ± 2.16^{ab}	16.8 ± 0.87^{b}
Met	31.1 ± 5.01	33.8 ± 5.19	36.1 ± 7.27	28.7 ± 5.13	32.8 ± 3.35
Phe	11.1 ± 3.76	10.6 ± 2.79	10.5 ± 3.03	7.9 ± 1.12	8.5 ± 1.38
Thr	68.0 ± 11.08	77.2 ± 11.63	66.8 ± 6.33	75.8 ± 13.85	74.2 ± 11.49
Val	30.8 ± 6.58	32.6 ± 6.16	30.8 ± 9.81	26.5 ± 4.92	25.8 ± 3.59
NEAA					
Ala	23.5 ± 4.45	25.7 ± 3.76	27.6 ± 6.62	22.7 ± 7.23	25.1 ± 4.25
Asp	8.6 ± 1.12	8.9 ± 2.83	9.4 ± 5.09	8.1 ± 0.80	9.7 ± 3.01
Cys	29.9 ± 4.53	32.7 ± 4.96	33.0 ± 8.77	26.2 ± 5.25	31.2 ± 3.43
Glu	28.1 ± 5.43	30.2 ± 7.34	32.4 ± 11.69	28.4 ± 8.02	32.0 ± 11.06
Gly	7.8 ± 0.72	8.3 ± 0.95	7.8 ± 1.61	7.9 ± 0.45	7.3 ± 0.24
Pro	14.0 ± 4.57^{ab}	15.8 ± 7.07^{a}	9.9 ± 5.48^{b}	11.1 ± 5.27^{ab}	10.3 ± 3.33^{ab}
Ser	16.3 ± 2.54	17.1 ± 3.37	16.9 ± 5.27	13.4 ± 1.05	14.9 ± 2.27
Tau	12.8 ± 2.14	14.1 ± 2.84	16.2 ± 3.20	12.9 ± 3.85	14.9 ± 2.19
Tyr	15.0 ± 1.67	17.4 ± 3.96	19.1 ± 6.53	15.2 ± 3.79	15.5 ± 2.74

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement). EAA (essential aminoacids): Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, metionine; Phe, phenilalanine; Thr, threonine; Val, valine; NEAA (noneseential aminoacids): Ala, alanine; Asp, aspartame; Cys, cysteine; Glu, glutamine; Gly, glycine; Pro, poline; Ser, serine; Tau, taurine; Tyr, tyrosine. Values (mean ± SD, $n = 3$).

Table 10
Whole-body fatty acid composition (% total fatty acids) of gilthead sea bream (*Sparus aurata*) fed the experimental diets.

	Experimental diets				
	Control	INS5	INS10	SCP5	SCP10
14:0	2.50 ± 0.21	2.74 ± 0.32	2.93 ± 0.11	2.51 ± 0.41	2.42 ± 0.12
14:1n-7	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
14:1n-5	0.11 ± 0.01	0.12 ± 0.02	0.12 ± 0.00	0.12 ± 0.03	0.12 ± 0.00
15:0	0.29 ± 0.03	0.31 ± 0.04	0.32 ± 0.01	0.31 ± 0.06	0.30 ± 0.02
15:1n-5	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
16:0ISO	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00
16:0	16.31 ± 1.26	17.14 ± 2.03	17.19 ± 0.45	17.33 ± 2.91	16.43 ± 1.26
16:1n-7	3.98 ± 0.19[b]	4.17 ± 0.18[ab]	4.35 ± 0.09[ab]	4.43 ± 0.37[a]	4.51 ± 0.15[a]
16:1n-5	0.14 ± 0.03	0.14 ± 0.03	0.13 ± 0.00	0.13 ± 0.01	0.12 ± 0.01
16:2n-6	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
16:2n-4	0.17 ± 0.00	0.16 ± 0.02	0.18 ± 0.00	0.16 ± 0.02	0.17 ± 0.00
17:0	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
16:3n-4	0.28 ± 0.01	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.01	0.28 ± 0.01
16:3n-3	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.00	0.09 ± 0.01	0.09 ± 0.01
16:3n-1	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01
16:4n-3	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.02
16:4n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
18:0	3.74 ± 0.25	3.83 ± 0.56	3.85 ± 0.22	4.04 ± 0.73	3.86 ± 0.32
18:1n-9	34.96 ± 1.17	35.03 ± 1.72	34.56 ± 0.52	34.80 ± 2.34	34.18 ± 1.80
18:1n-7	2.89 ± 0.11	2.91 ± 0.03	2.97 ± 0.04	3.00 ± 0.15	2.95 ± 0.17
18:1n-5	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.00	0.19 ± 0.01	0.18 ± 0.01
18:2n-9	0.19 ± 0.02	0.22 ± 0.02	0.21 ± 0.04	0.24 ± 0.02	0.25 ± 0.05
18:2n-6	14.98 ± 0.48	14.51 ± 1.19	14.99 ± 0.39	14.30 ± 1.16	15.12 ± 0.42
18:2n-4	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.00	0.09 ± 0.01	0.09 ± 0.00
18:3n-6	0.25 ± 0.04[ab]	0.25 ± 0.05[ab]	0.24 ± 0.01[b]	0.26 ± 0.05[ab]	0.28 ± 0.00[a]
18:3n-4	0.10 ± 0.03	0.09 ± 0.01	0.09 ± 0.00	0.08 ± 0.02	0.09 ± 0.01
18:3n-3	2.59 ± 0.38	2.38 ± 0.55	2.36 ± 0.20	2.30 ± 0.72	2.64 ± 0.46
18:3n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:4n-3	0.48 ± 0.14	0.45 ± 0.19	0.45 ± 0.07	0.44 ± 0.25	0.54 ± 0.18
18:4n-1	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.01
20:0	0.34 ± 0.04	0.36 ± 0.05	0.36 ± 0.02	0.37 ± 0.05	0.34 ± 0.04
20:1n-9	0.53 ± 0.03	0.53 ± 0.03	0.52 ± 0.02	0.52 ± 0.00	0.50 ± 0.01
20:1n-7	2.96 ± 0.13[a]	2.86 ± 0.14[a]	2.79 ± 0.08[a]	2.75 ± 0.04[ab]	2.55 ± 0.11[b]
20:1n-5	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
20:2n-9	0.30 ± 0.04	0.27 ± 0.02	0.27 ± 0.02	0.28 ± 0.05	0.28 ± 0.02
20:2n-6	0.49 ± 0.01	0.45 ± 0.03	0.47 ± 0.01	0.45 ± 0.06	0.45 ± 0.02
20:3n-9	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20:3n-6	0.20 ± 0.04	0.17 ± 0.03	0.18 ± 0.02	0.19 ± 0.05	0.19 ± 0.03
20:4n-6 (ARA)	0.26 ± 0.04	0.24 ± 0.07	0.25 ± 0.02	0.24 ± 0.10	0.27 ± 0.05
20:3n-3	0.17 ± 0.01	0.15 ± 0.03	0.16 ± 0.00	0.15 ± 0.04	0.16 ± 0.02
20:4n-3	0.40 ± 0.10	0.36 ± 0.14	0.36 ± 0.03	0.36 ± 0.21	0.41 ± 0.13
20:5n-3 (EPA)	1.73 ± 0.60	1.61 ± 0.82	1.57 ± 0.25	1.63 ± 1.13	1.94 ± 0.75
22:1n-11	2.78 ± 0.22	2.78 ± 0.16	2.66 ± 0.09	2.63 ± 0.14	2.36 ± 0.12
22:1n-9	0.71 ± 0.05	0.71 ± 0.03	0.69 ± 0.03	0.70 ± 0.01	0.65 ± 0.03
22:4n-6	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
22:5n-6	0.11 ± 0.03	0.10 ± 0.04	0.11 ± 0.01	0.12 ± 0.04	0.11 ± 0.03
22:5n-3	0.80 ± 0.30	0.68 ± 0.38	0.66 ± 0.08	0.73 ± 0.55	0.85 ± 0.36
22:6n-3 (DHA)	3.20 ± 1.31	2.93 ± 1.75	2.65 ± 0.42	3.04 ± 2.45	3.60 ± 1.73
SFA	23.35 ± 1.76	24.55 ± 2.99	24.83 ± 0.74	24.74 ± 4.16	23.52 ± 1.76
MUFA	49.47 ± 1.76	49.67 ± 2.31	49.20 ± 0.75	49.50 ± 2.72	48.34 ± 2.42
n-3 PUFA	9.53 ± 2.86	8.72 ± 3.87	8.37 ± 1.06	8.83 ± 5.36	10.29 ± 3.65
n-6 PUFA	16.40 ± 0.62	15.83 ± 1.40	16.34 ± 0.42	15.67 ± 1.45	16.55 ± 0.56

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement); ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values (mean ± SD, n = 3).

indicating that FM can be replaced up to 66% by this *M. capsulatus* product. In agreement with the present results, the same SCP also supported a good growth rate in rainbow trout (Aas et al., 2006), and in Atlantic salmon when FM was replaced by this SCP (Romarheim et al., 2013) or by a mixture of bacteria containing *M. capsulatus* (Berge et al., 2005). The replacement of FM by *M. capsulatus* biomass was also possible up to 30 and 100% in diets for Japanese yellowtail (*Seriola quinqueradiata*) (Biswas et al., 2020) or Florida pompano (*Trachinotus carolinus*) (Rhodes et al., 2015), respectively, without consequences for fish growth, when supplemented with lysine (like the present diets) and taurine (Rhodes et al., 2015).

In contrast, the present results suggest that FM can only be replaced with moderate dietary inclusion levels of this insect meal product (up to 33% of replacement), without negative consequences for gilthead sea bream productive parameters. In agreement, previous studies also showed that *H. illucens* prepupae meal could successfully replace up to 30% of the dietary FM in fish diets, including gilthead sea bream (Karapanagiotidis et al., 2014), European sea bass (Magalhães et al., 2017) or turbot (Kroeckel et al., 2012), with higher replacements threatening feed palatability, fish intake, nutrient utilization and/or fish growth. Another study with turbot fed a diet including *H. illucens* meal at 30% of the total dietary protein led to a lower *in vivo* protein digestibility coefficient and reduced feed intake, with the authors attributing this effect to the excessive content of chitin present in the insect meal (Kroeckel et al., 2012). Despite the inclusion of the insect meal did not affect fish feed intake (compared with Control diet), the potential lower digestibility of this feed with high insect meal content, probably added to

Table 11
Fillet fatty acid composition (% total fatty acids) of gilthead sea bream (*Sparus aurata*) fed the experimental diets.

	Experimental diets				
	Control	INS5	INS10	SCP5	SCP10
14:0	2.46 ± 0.14	2.47 ± 0.24	2.47 ± 0.16	2.25 ± 0.02	2.36 ± 0.01
14:1n-7	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
14:1n-5	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.12 ± 0.00
15:0	0.28 ± 0.03	0.27 ± 0.03	0.27 ± 0.01	0.26 ± 0.00	0.28 ± 0.01
15:1n-5	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
16:0ISO	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
16:0	16.28 ± 0.73	15.93 ± 1.18	15.54 ± 0.58	15.11 ± 0.15	16.62 ± 0.06
16:1n-7	3.88 ± 0.13^{b}	3.86 ± 0.16^{b}	3.96 ± 0.19^{b}	4.07 ± 0.03^{ab}	4.39 ± 0.01^{a}
16:1n-5	0.12 ± 0.03	0.11 ± 0.02	0.11 ± 0.01	0.11 ± 0.00	0.12 ± 0.01
16:2n-6	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
16:2n-4	0.16 ± 0.00	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.00	0.16 ± 0.00
17:0	0.10 ± 0.01^{b}	0.12 ± 0.02^{ab}	0.12 ± 0.00^{ab}	0.13 ± 0.00^{a}	0.12 ± 0.00^{ab}
16:3n-4	0.28 ± 0.01	0.28 ± 0.01	0.28 ± 0.00	0.27 ± 0.01	0.29 ± 0.01
16:3n-3	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.08 ± 0.00
16:3n-1	0.05 ± 0.02	0.06 ± 0.00	0.06 ± 0.02	0.05 ± 0.00	0.06 ± 0.00
16:4n-3	0.06 ± 0.02^{b}	0.08 ± 0.01^{ab}	0.08 ± 0.01^{ab}	0.10 ± 0.00^{a}	0.07 ± 0.00^{ab}
16:4n-1	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:0	3.75 ± 0.23	3.74 ± 0.31	3.63 ± 0.13	3.44 ± 0.00	3.94 ± 0.05
18:1n-9	34.37 ± 1.24^{a}	33.34 ± 1.37^{ab}	32.78 ± 0.56^{ab}	31.95 ± 0.12^{b}	33.87 ± 0.09^{ab}
18:1n-7	2.61 ± 0.13^{ab}	2.67 ± 0.04^{ab}	2.71 ± 0.03^{a}	2.50 ± 0.14^{b}	2.71 ± 0.03^{a}
18:1n-5	0.18 ± 0.01^{ab}	0.17 ± 0.01^{b}	0.17 ± 0.00^{b}	0.17 ± 0.00^{b}	0.19 ± 0.00^{a}
18:2n-9	0.19 ± 0.04	0.21 ± 0.04	0.21 ± 0.04	0.21 ± 0.02	0.23 ± 0.00
18:2n-6	15.55 ± 0.10	15.46 ± 0.45	15.75 ± 0.13	15.31 ± 0.07	15.33 ± 0.12
18:2n-4	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.00	0.09 ± 0.01	0.09 ± 0.00
18:3n-6	0.24 ± 0.04	0.27 ± 0.06	0.27 ± 0.04	0.30 ± 0.03	0.27 ± 0.00
18:3n-4	0.08 ± 0.01	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.00
18:3n-3	2.69 ± 0.26	2.79 ± 0.22	2.77 ± 0.09	3.04 ± 0.03	2.74 ± 0.02
18:3n-1	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:4n-3	0.49 ± 0.11^{b}	0.59 ± 0.13^{ab}	0.63 ± 0.02^{ab}	0.74 ± 0.04^{a}	0.53 ± 0.01^{ab}
18:4n-1	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
20:0	0.33 ± 0.05	0.31 ± 0.02	0.32 ± 0.01	0.28 ± 0.02	0.31 ± 0.00
20:1n-9	0.50 ± 0.05	0.49 ± 0.01	0.48 ± 0.00	0.45 ± 0.02	0.45 ± 0.01
20:1n-7	2.92 ± 0.24^{a}	2.72 ± 0.23^{ab}	2.63 ± 0.08^{ab}	2.51 ± 0.11^{b}	2.56 ± 0.06^{ab}
20:1n-5	0.15 ± 0.01	0.14 ± 0.00	0.15 ± 0.00	0.13 ± 0.01	0.14 ± 0.00
20:2n-9	0.29 ± 0.03	0.28 ± 0.03	0.29 ± 0.03	0.31 ± 0.01	0.34 ± 0.06
20:2n-6	0.51 ± 0.02	0.48 ± 0.03	0.49 ± 0.01	0.46 ± 0.04	0.48 ± 0.02
20:3n-9	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20:3n-6	0.22 ± 0.03	0.22 ± 0.02	0.23 ± 0.02	0.23 ± 0.01	0.24 ± 0.03
20:4n-6 (ARA)	0.30 ± 0.05	0.33 ± 0.05	0.35 ± 0.02	0.36 ± 0.01	0.30 ± 0.00
20:3n-3	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
20:4n-3	0.43 ± 0.09^{b}	0.47 ± 0.08^{ab}	0.51 ± 0.03^{ab}	0.56 ± 0.01^{a}	0.45 ± 0.02^{ab}
20:5n-3 (EPA)	1.97 ± 0.60^{b}	2.37 ± 0.64^{ab}	2.56 ± 0.25^{ab}	3.08 ± 0.15^{a}	2.10 ± 0.02^{ab}
22:1n-11	2.55 ± 0.26	2.45 ± 0.16	2.41 ± 0.18	2.22 ± 0.13	2.17 ± 0.01
22:1n-9	0.64 ± 0.05	0.63 ± 0.04	0.61 ± 0.03	0.57 ± 0.04	0.60 ± 0.01
22:4n-6	0.10 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.00
22:5n-6	0.12 ± 0.02	0.14 ± 0.03	0.15 ± 0.01	0.16 ± 0.00	0.12 ± 0.00
22:5n-3	0.84 ± 0.23^{b}	0.97 ± 0.28^{ab}	1.07 ± 0.12^{ab}	1.25 ± 0.03^{a}	0.87 ± 0.08^{ab}
22:6n-3 (DHA)	3.66 ± 1.20^{b}	4.55 ± 1.67^{ab}	4.90 ± 0.73^{ab}	6.37 ± 0.58^{a}	3.73 ± 0.19^{b}
SFA	23.26 ± 1.11	22.90 ± 1.72	22.40 ± 0.86	21.53 ± 0.10	23.70 ± 0.09
MUFA	48.08 ± 1.74^{a}	46.76 ± 1.86^{ab}	46.19 ± 0.49^{ab}	44.86 ± 0.54^{b}	47.37 ± 0.11^{ab}
n-3 PUFA	10.40 ± 2.51^{b}	12.07 ± 3.02^{ab}	12.78 ± 1.23^{ab}	15.39 ± 0.76^{a}	10.74 ± 0.29^{ab}
n-6 PUFA	17.05 ± 0.23^{ab}	17.01 ± 0.54^{ab}	17.36 ± 0.14^{a}	16.93 ± 0.05^{ab}	16.85 ± 0.11^{b}
n-3/n-6	0.61 ± 0.14^{b}	0.71 ± 0.16^{ab}	0.74 ± 0.07^{ab}	0.91 ± 0.05^{a}	0.64 ± 0.02^{ab}

Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement); ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values (mean ± SD, n = 3).

the slightly lower protein content of that diet (52% in INS10 *vs* 53.6% in the other diets), might explain the observed reduced trend in feed and nutrient utilization *in vivo* of gilthead sea bream, and, ultimately, in growth, suggesting a lower acceptability of this raw material when present at high dietary inclusions. Indeed, chitin present in insect meals is known to be indigestible for carnivorous fish since chitinase activity is absent in carnivorous guts or not proven to efficiently break down chitin molecules (Kroeckel et al., 2012). Although we did not measure the chitin content of insect diets of the present experiment, the insect meal tested in our study was reported to have a chitin content of 70 g/kg (Heuel et al., 2021), a value that is lower than the ones found for other commercial products from the same insect species (Kroeckel et al., 2012). Although chitin content of the meals can be influenced by the technological treatment and therefore can vary depending on the product, the chitin content of the insect meal used here was also likely to be excessively high for gilthead sea bream, when it was included at high dietary inclusion levels (10%) and when tested under the current context of low FM dietary contents. Consequently, chitin might have contributed to the potential lower *in vitro* digestibility of this feed that affected feed acceptability by gilthead sea bream and fish performance. Low dietary chitin contents of 1 and 1.6% were sufficient to reduce feed intake and growth in carp (*Cyprinus carpio* var. Jian) and turbot, respectively (Gopalakannan and Arul, 2006; Kroeckel et al., 2012). In the present study, 10% of dietary inclusion of this insect meal would approximately correspond to 7 g/kg (0.7%) of chitin in the diet, which suggests that gilthead sea bream might be a highly-sensitive species to

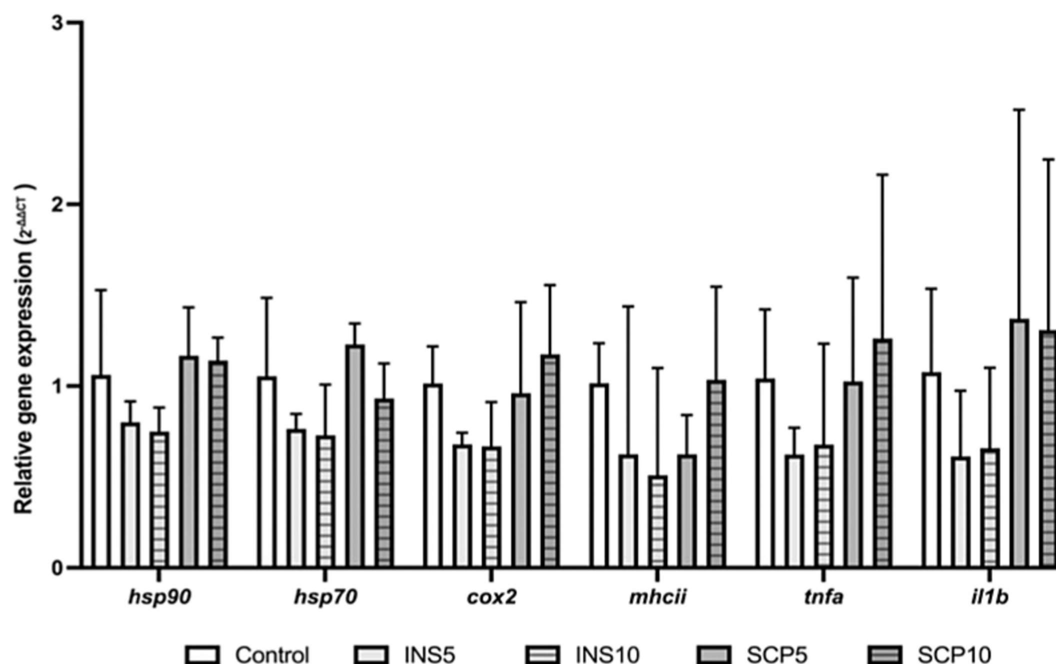

Fig. 1. Relative expression of health-related genes assessed in posterior gut of gilthead sea bream (*Sparus aurata*) fed the experimental diets for 112 days. Data analysed with orthogonal constrasts (95% of confidence level) and presented as mean ± SD; $n = 5$ fish × 3 tank for each diet; Control: control diet with 15% FM; INS5: 5% inclusion of insect meal (33% of FM replacement); INS10: 10% inclusion of insect meal (66% of FM replacement); SCP5: 5% inclusion of single-cell protein (33% of FM replacement); SCP10: 10% inclusion of single-cell protein (66% of FM replacement). *hsp90, heat shock protein 90; hsp70, heat shock protein 70; cox2, cyclooxygenase-2; mhcII, major histocompatibility complex class II; tnfa, tumour necrosis factor alpha).*

the chitin content present in the diet. In contrast to the present results, the studies carried out with Atlantic Salmon indicate that 100% of FM replacement with *H. illucens* larvae meal is possible in practical diets without negative influence on growth and enhancing FCR (Lock et al., 2016; Belghit et al., 2019). These differences could be attributed to several factors, including the meal quality and its technological treatment, or the different fish species and their digestive capacities (Krogdahl et al., 2015).

Little effect was observed in fish whole-body proximate composition and amino acid profile related with the dietary treatment. Similar contents were thus observed in the essential amino acid profile of the whole-body of fish fed the different novel protein diets, except for lysine, which was lowered by the highest dietary inclusion of single cell protein feed (10%) compared with insect meal at 5%, but which was according with the dietary pattern and did not affect fish performance, indicating that these dietary levels in all the diets were still sufficient to meet sea bream requirements for all the EAA. In addition, sea bream fed high inclusion levels of insect meal (10%) showed lower proline, a conditionally essential amino acid, compared with those fed the same novel protein at half of inclusion level (5%), despite the similar dietary levels among all diets. Although the digestibility of the specific amino acids were not determined, these results might suggest a potential lower digestibility of this non-essential amino acid in diets with high insect meal content, which is also in agreement with the potential sligthly lower *in vitro* protein digestibility noted in this diet. Furthermore, this lower proline content in fish whole-body is also in concordance with reduced growth observed in sea bream fed this diet, since proline is the precursor to produce important metabolites involved in fish growth, like hydroxyproline (Aksnes et al., 2008; Li and Wu, 2018). Additionally, more than the amino acid profile, the fatty acid profile of fish fillets usually reflects better the dietary fatty acid composition, and most of the differences found in the fatty acid contents of fish tissues were in concordance with the dietary trend. Furthermore, whereas whole-body fatty acid profile was little affected by the experimental diets, fish fillets, which are the most interesting fish products concerning consumers acceptance, showed different results, suggesting a potential difference in the mobilization and/or deposition of some fatty acids from the diet in fish muscle. For instance, C16:1 fatty acids were one of the few fatty acids that were significantly affected by the dietary treatment, being the highest in fish fed SCP diets, in agreement with other study that aimed to analyse the fatty acid composition of pig meat fed diets including *M. capsulatus* meal (Øverland et al., 2005). Interestingly, fish fed moderate levels of the single cell protein (5%) showed the highest contents of 20:5n-3, 22:5n-3 as well as 22:6n-3 in fish fillets, the most important fatty acids concerning human cardiovascular health, that were significantly higher compared with those fed the Control diet despite their similar dietary contents. These results might suggest a difference in the mobilization and/or deposition of those fatty acids between fish fed the different diets, that, ultimately affects their deposition on fish flesh. It might also be related with the slightly higher SFA + MUFA dietary content of SCP5 diet compared with the Control diet, which are known to exert a sparing effect of n-3 PUFA, allowing their retention and incorporation in tissues with high polar lipid content like fish muscle (Rombenso et al., 2021). In fact, except for fillets of fish fed SCP10 diet (also with a lower dietary SFA), the fillet contents on those fatty acids (particularly in DHA) of fish fed the other experimental diets (INS5, INS10), with higher SFA + MUFA, were also numerically higher than those found for fish fed Control diet. Therefore, this result is particularly important since it suggests a better nutritional quality of fish products that were fed with this *M. capsulatus* product, that is important for consumers, at least in which concerns lipid profile. Indeed, except for fillets of fish fed SCP10 diet, the fillet contents on those fatty acids (particularly in DHA) of fish fed the other experimental diets (INS5, INS10) were also numerically higher than those found for fish fed Control diet, in agreement with previous studies that compared insect meal incorporation in the feeds with actual commercial feeds with high contents of vegetable ingredients (Pulido et al., 2022).

Furthermore, irrespective of the different antinutritional factor contents of the novel diets, that are known to contribute for increasing the inflammatory processes in fish, the relative expressions of the

immune genes (*hsp70*, *hsp90*, *mhcII*, *cox-2*, *tnfa*, *il1b*) determined in posterior gut of gilthead sea bream were not significantly altered in none of the novel diets, compared with the control diet. Indeed, posterior gut is an important intestinal region for the digestion and nutrient absorption, including that of peptides, as well as valuable indicator of fish health. For instance, TNFa and IL1*b* act as pro-inflammatory mediators that were shown to be overexpressed in fish posterior gut in response to the replacement of the dietary FM, mainly by plant ingredients, and particularly after a long-term feeding (Torrecillas et al., 2017b; Kumar et al., 2020). In addition, COX2 is an enzyme that mediates the production of other pro-inflammatory molecules like prostaglandins, from ARA and is thus activated in response to inflammation (Calder, 2012). Similarly, different HSP families, including *hsp90* and *hsp70*, were shown to be responsive to the exposure to toxic substances or anti-nutritional factors in feeds, that may lead to nutritional stress (Roberts et al., 2010). The lack of differences in the relative expression of inflammatory genes in the present study, might indicate that the inclusion of *H. illucens* and *M. capsulatus* meals at the tested doses and for the present feeding period, were not inducing an inflammatory-like process in gilthead sea bream intestine compared to a practical control diet and considering the modern approach of the aquaculture feed industry (with high contents on plant proteins), in agreement with previous studies in other fish species (Zarantoniello et al., 2022a, 2022b). It is widely reported that an increase on the relative expression of pro-inflammatory cytokines are commonly reported in fish fed vegetable diets for instance, particularly based on soybean meal (Venold et al., 2012; Miao et al., 2018). Therefore, considering that all diets contained similar dietary vegetable proteins, the different pattern of gut health related genes expression observed (in absolute values) between fish fed INS (down-regulated) and SCP (up-regulated) meals, might be potentially related to their composition itself, in terms of Pattern Recognition Receptors (PRR) that are activated and microbiota modulation. Indeed, *H. illucens* is rich in chitin and lauric acid, which can act as prebiotics and were also shown to positively modulate rainbow trout microbiota (Terova et al., 2019). Other previous studies with rainbow trout fed insect diets relate its supplementation with reduced intestinal inflammation (and the down-regulation of pro-inflammatory genes) (Kumar et al., 2021). However, the up-regulation of pro-inflammatory cytokines, observed in gut of other fish species fed this single-cell protein product from *M. capsulatus*, have been also related with an ameliorated enteritis and an improved intestinal health (Zhang et al., 2022). Indeed, an increase in these pro-inflammatory cytokines is known to contribute also for cell proliferation, mucosal architecture and consequently, contributing for gut immunity, rather than increasing inflammation (Leonel et al., 2013). Further studies, particularly related wit morphological analyses of fish intestines, are necessary to fully validate the potential of these two protein sources (insect and single-cell proteins) for fish health.

5. Conclusions

The single-cell protein supported gilthead sea bream growth and feed utilization and allowed up to 66% of replacement of the dietary FM content in a practical diet. In contrast, the insect meal product led to a reduced growth and worsened feed utilization when included at the highest dietary level (10%). Therefore, the replacement of the dietary FM was only possible up to 33% with this insect meal product. In addition, the novel protein sources had little impact on proximate and amino acid and fatty acid profiles of the fish tissues, nor in the relative expression of some molecular makers related with fish gut health, suggesting their suitability as protein sources for aquafeeds and for sea bream. However, moderate inclusions of the single cell protein from *M. capsulatus* (5%) significantly increased the fish fillet contents of important fatty acids for human nutrition, like 20:5n-3 and 22:6n-3, possibly related with a higher n-3 LC-PUFA sparing effect resulting from the slightly higher SFA and MUFA contents, and potentially suggesting a higher nutritional quality (at least in which concerns lipid quality), when this novel protein is included in the feed.

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CRediT authorship contribution statement

Marta Carvalho: Investigation, Formal analysis, Writing – original draft. **Silvia Torrecillas:** Formal analysis, Writing – original draft. **Daniel Montero:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Antonio Sanmartín:** Investigation, Formal analysis. **Ramon Fontanillas:** Conceptualization, Formal analysis, Resources, Writing – review & editing. **Ana Farías:** Formal analysis, Resources, Writing – review & editing. **Katerina Moutou:** Formal analysis, Resources, Funding acquisition, Writing – review & editing. **Jorge Hernández Velásquez:** Formal analysis. **Marisol Izquierdo:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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