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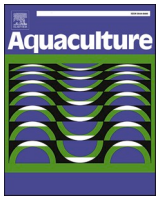

Combining *Hermetia illucens* and *Tenebrio molitor* meals in diets for European seabass: Effects on growth, nutrient utilisation, intestinal morphology and muscle quality

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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

This study explored the potential of an insect meal (IM) mixture of black soldier fly larvae (BSFL) and yellow mealworms (YMs) to substitute 3% (diet IM3), 25% (diet IM25) and 50% (diet IM50) of the fishmeal (FM) protein in a control diet (CTRL; 15% FM) for European seabass (*Dicentrarchus labrax*) juveniles. The four diets were isoproteic and isolipidic and were tested in triplicate in a recirculating aquaculture system with water at 22 °C and 35 ppt salinity, under a 12 h light/12 h dark photoperiod. After a 75-day feeding period, the fish growth performance, nutrient utilisation, intestinal morphology, muscle fatty acid profile and antioxidant capacity were evaluated. The diets containing IM ensured similar growth (DGI = 2.0) and feed efficiency (FCR = 1.0–1.1) to CTRL. Moreover, they promoted comparable nitrogen and energy retention efficiencies (38–40% and 44–47%). However, fish fed with the IM diets presented 20–27% lower faecal phosphorus losses than the control group. Anterior intestine integrity was maintained in all fish, but those fed IM50 displayed longer *villi* than the control. The muscle fatty acid profile of IM-fed fish also resembled that of the control, with 364–405 mg EPA + DHA per 100 g of fillet. IM25 and IM50 promoted higher lauric acid deposition in the muscle, and the muscle of fish fed with IM50 presented the highest oxygen radical absorbance capacity, suggesting higher antioxidant capacity. Overall, the study shows that the IM mixture can be an adequate protein source for seabass juveniles, also offering functional benefits.

1. Introduction

Fishmeal (FM) has been conventionally used as a protein source in aquafeeds due to its high palatability and digestibility, high biological value and well-balanced amino acid (AA) profile (IFFO, 2024; Oliva-Teles et al., 2015). However, its limited availability has led to decreased utilisation over the years (Naylor et al., 2021). Diets for European seabass (*Dicentrarchus labrax*) – a carnivorous marine fish of commercial importance in European and Mediterranean aquaculture (European Commission and EUMOFA, 2023) – contained 50–70% FM in the 1990s (Alexis, 1997; Kaushik et al., 2004), but now typically include 15–20% FM, depending on the developmental stage (Filipa-Silva et al., 2023; Montero et al., 2023; Vasilaki et al., 2023). Plant proteins and processed animal proteins derived from terrestrial animal by-products (e.g., poultry, blood, and feather meals) have been successfully used as FM alternatives, as they are widely abundant and cost-effective protein sources (Glencross et al., 2024; Serra et al., 2024). Nonetheless, these ingredients still present limitations. So, future aquafeed formulations are expected to include other emerging protein sources, such as single-cell proteins and insect meals (IMs), with each ingredient being used in a strategic quantity considering availability, price, nutritional quality, and environmental footprint (Glencross et al., 2023, 2024;

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Turchini et al., 2019).

Insects reared in controlled environments have raised interest for inclusion in aquafeeds due to their high protein content (up to 68% of dry matter, DM) (Gasco et al., 2020a), high feed efficiency (van Raamsdonk et al., 2017) and, in the case of some species, rapid growth and reproduction (Oonincx et al., 2015). To date, eight species have been authorised for inclusion in aquafeeds in the European Union (EU) [Regulation (EU) 2017/893; Regulation (EU) 2021/1925]. Among those, black soldier fly larvae (BSFL, *Hermetia illucens*) and yellow mealworms (YMs, *Tenebrio molitor*) have been the most explored (Tran et al., 2022b). BSFL and YMs are already produced at an industrial scale worldwide (Niyonsaba et al., 2021) and can be reared on vegetal by-products of the agri-food sector, converting low-value substrates into high-value biomass, thus contributing to a circular bioeconomy (Fantatto et al., 2024; Fowles and Nansen, 2020; Gasco et al., 2020b). Besides protein, BSFL are rich in saturated fatty acids (SFAs) (Barragán-Fonseca et al., 2017; Smets et al., 2020), especially lauric acid (C12:0), which has antimicrobial properties (Franco et al., 2024; Nitbani et al., 2022; Saviane et al., 2021; Ullah et al., 2022). In its turn, the fat of YMs is mainly composed of mono- and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) (Adámková et al., 2020). Other bioactive compounds found in BSFL and YMs include chitin and antioxidants (del Hierro et al., 2020; Muñoz-Seijas et al., 2025; Navajas-Porras et al., 2024; Ravi et al., 2020), which may provide functional benefits to the feed (Veldkamp et al., 2022).

One limitation of both BSFL and YM meals is their lower content of essential AAs (EAAs), namely lysine (Lys) and methionine (Met), compared to FM (Belghit et al., 2019; Mastoraki et al., 2020). Hence, replacing high levels of FM with these IMs may require EAA supplementation to meet the nutritional requirements of a carnivorous species like the European seabass and support optimal growth. For instance, Magalhães et al. (2017) demonstrated that a diet containing 19.5% BSFL meal, replacing 50% of FM, met the EAA requirements of seabass juveniles and maintained growth. However, higher substitution levels would require EAA supplementation. Similarly, Basto et al. (2021, 2023) showed that diets with 40–60% YM meal, fully replacing FM, maintained seabass growth, but AA supplementation was required.

Another limitation associated with IM incorporation in aquafeeds is its high price compared to FM and soybean meal (Gasco et al., 2023). Moreover, despite the forecasted growth of the European insect protein sector (IPIFF, 2023), the current supply of IM is still low (Biteau et al., 2024; Gasco et al., 2023; Selvaraj and Won, 2024). In 2022, approximately four thousand tonnes of IM were available in the European market for inclusion in animal feed (IPIFF, 2023), whereas 450 thousand tonnes of FM were used in the EU in the same year (European Commission and EUMOFA, 2023). This may not only pose challenges to the incorporation of high levels of IM into aquafeeds but also to the consistent supply of IM derived from a specific species. Hence, incorporating a mixture of IMs derived from more than one species into aquafeeds could reduce feed manufacturers' reliance on a single source, increasing formulation flexibility. In addition, BSFL and YM meals offer complementary advantages for inclusion in aquafeeds, which relate to their protein content, fatty acid (FA) profiles (Gasco et al., 2020b; Tognocchi et al., 2023), digestibility (Basto et al., 2020; Gasco et al., 2022), and composition in bioactive compounds (Veldkamp et al., 2022). A life cycle analysis also estimated that a diet for rainbow trout (*Oncorhynchus mykiss*) with BSFL, YM and other emerging ingredients was associated with lower greenhouse gas emissions than a conventional diet (Langeland et al., 2023).

This study aimed to evaluate the potential of a 50:50 mixture of defatted BSFL and YM meals to partially substitute FM and provide functional benefits in practical diets for European seabass. The IM mixture was included at 0.48%, 4.2%, and 8.4%, replacing 3% (diet IM3), 25% (diet IM25) and 50% (diet IM50) of the FM protein in a commercial-like control diet (CTRL). These inclusion levels were selected to reflect current market constraints, namely the limited availability and high cost of IMs, allowing for evaluation under industry-relevant conditions. After 75 days of feeding, growth performance, nutrient utilisation, intestinal morphology, muscle FA profile, and muscle antioxidant capacity were evaluated.

2. Materials and methods

2.1. Ethical statement

The experimental trials and sampling procedures described in this article were approved by CIIMAR's ethical committee for managing animal welfare (ORBEA) and by the Portuguese General Directorate of Food and Veterinary Affairs (DGAV) (ORBEA_CIIMAR-37-2023).

2.2. Ingredients and diets

The black soldier fly larva (BSFL) and yellow mealworm (YM) meals used in the study were obtained from Ingredient Odyssey S.A. - Ento-Green (Santarém, Portugal) and ThunderFoods S.A. (Santarém, Portugal), respectively. Throughout the production cycle, the larvae were fed diets mainly containing local by-products of the agri-food sector, such as olive pomace and wheat bran in the case of BSFL, and wheat bran in the case of YMs. The BSFL and YMs were harvested within 12–14 days and 11–12 weeks of the production cycle, respectively, and were then processed into defatted meals using mechanical pressure and heat.

To evaluate the effects of including a mixture of these insect meals (IMs) in diets for European seabass juveniles, four isoproteic (46% dry matter, DM), isolipidic (19% DM), and isoenergetic (22.5 kJ/g DM) diets were formulated (Table 1). The control diet (CTRL) was formulated to have a similar ingredient composition to commercial diets for European seabass. It contained moderate levels of fishmeal (FM) (15%), as well as plant proteins (55%) and poultry by-products meal (9%) as the main protein sources. Fish oils (12%) and rapeseed oil (3%) were the main lipid sources. The other three diets were obtained by replacing 3%, 25% and 50% of the FM protein in CTRL with a 50:50 mixture of the BSFL and YM meals (diets IM3, IM25 and IM50, respectively). Similar levels of the essential n-3 long-chain polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were maintained across diets by adding an extra 0.4% and 1.1% of salmon oil to IM25 and IM50, respectively, at the expense of rapeseed oil. The diets containing IM were also supplemented with an extra 0.05–0.85% monocalcium phosphate (MCP) to ensure phosphorus (P) requirements were met. Supplementation with crystalline amino acids (AAs) was not necessary to meet the requirements of European seabass. The diets were extruded by Sparos Lda (Olhão, Portugal) with a pellet diameter of 2 mm. The chemical composition and AA and fatty acid (FA) profiles of the IMs and diets are listed in Tables 1, 2 and 3, respectively.

2.3. Growth trial

2.3.1. Experimental conditions

European seabass juveniles were obtained from Aquicultura Balear S.A.U. (Palma De Mallorca, Spain) and transported to CIIMAR's Bioterium of Aquatic Organisms (Matosinhos, Portugal). Immediately after arrival, the fish were subjected to a 15-day quarantine period in a saltwater recirculating aquaculture system (RAS), during which they were fed with a commercial diet [55% crude protein (CP) and 9.5% crude fat (CF), on a DM basis; Aquasoja, Soja de Portugal, Portugal] and acclimated to the abiotic conditions of the growth trial (22 °C and salinity of 35 ppt). After this period, the fish fasted for 24 h and were lightly anaesthetised with 0.006% (*v*/v) 2-phenoxyethanol (Sigma-Aldrich, U.S.A.) for individual weight (12.9 ± 1.3 g) and total length (10.9 ± 0.4 cm) determination. Then, homogeneous groups of 75 fish were established and distributed among twelve 250-L fibreglass tanks within a RAS. The diets were randomly assigned to triplicate tanks, and the fish

Table 1
Ingredients (%) and chemical composition [% dry matter (DM), unless stated otherwise] of the black soldier fly larvae (BSFL) meal, yellow mealworm (YM) meal, and experimental diets.

	Insect meals (IMs)		Experimental diets			
	BSFL	YM	CTRL	IM3	IM25	IM50
Ingredients						
BSFL meal[1]	100	–	–	0.24	2.15	4.20
YM meal[2]	–	100	–	0.24	2.15	4.20
Fishmeal Super Prime[3]	–	–	15.00	14.55	11.25	7.50
Fish protein hydrolysate[4]	–	–	3	3	3	3
Poultry meal[5]	–	–	6	6	6	6
Poultry blood meal[6]	–	–	3	3	3	3
Soy protein concentrate[7]	–	–	10	10	10	10
Wheat gluten[8]	–	–	5.7	5.7	5.7	5.7
Corn gluten meal[9]	–	–	12	12	12	12
Soybean meal[10]	–	–	12.5	12.5	12.5	12.5
Sunflower meal 40[11]	–	–	4	4	4	4
Wheat meal[12]	–	–	6.10	5.97	5.25	4.45
Whole peas[13]	–	–	5	5	5	5
Vitamins and minerals[14]	–	–	1	1	1	1
Choline chloride 50	–	–	0.2	0.2	0.2	0.2
Antioxidant	–	–	0.2	0.2	0.2	0.2
Sodium propionate	–	–	0.1	0.1	0.1	0.1
Monocalcium phosphate	–	–	1.00	1.05	1.40	1.85
Sardine oil[15]	–	–	4	4	4	4
Salmon oil[16]	–	–	8.2	8.2	8.6	9.3
Rapeseed oil[17]	–	–	3.0	3.0	2.5	1.8
Chemical composition						
Dry matter (%)	93.9	90.5	94.7	94.4	93.9	93.0
Crude protein (N x 6.25)	55.1	76.1	45.6	46.1	45.7	46.3
Crude protein (N x 5.33)	47.0	64.9	–	–	–	–
True protein (ΣAAs)	48.5	63.3	44.1	44.0	43.5	43.8
Crude fat	8.98	6.17	18.7	18.8	18.5	18.7
Ash	10.3	5.93	8.32	8.19	8.06	7.52
Phosphorus	0.72	0.98	0.93	0.94	0.94	0.94
Hexosamines	6.28	5.59	0.71	0.74	0.82	0.95
Hexosamines from IMs[18]	6.28	5.59	–	0.04	0.11	0.25
Gross energy (kJ/g DM)	19.0	20.0	22.5	22.3	22.5	22.7
DP (mg/g DM)	–	–	421	427	421	432
DE (kJ/g DM)	–	–	20.6	20.5	20.6	20.8
DP/DE (mg/kJ DM)	–	–	20.5	20.8	20.5	20.8

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of BSFL meal and YM meal. AAs – amino acids; DE – digestible energy; DP – digestible protein. [1] Ingredient Odyssey S.A. - EntoGreen, Portugal, [2] ThunderFoods S.A., Portugal, [3] Diamante, Pesquera Diamante, Peru (Crude protein, CP: 66.3%; Crude fat, CF: 11.5%), [4] CPSP90, Sopropêche, France (CP: 82.6%; CF: 9.6%), [5] Savinor UTS, Portugal (CP: 62.4%; CF: 12.5%), [6] Savinor UTS, Portugal, [7] Soycomil P, ADM, The Netherlands (CP: 62.2%; CF: 0.7%), [8] Vital, Roquette, France (CP: 80.4%; CF: 5.8%), [9] Copam, Portugal (CP: 61.2%; CF: 5.2%), [10] Fiskå Mølle, Norway, [11] AGP Slovakia, s.r.o., Slovakia (CP: 42.9%; CF: 3.8%), [12] Molisur, Spain (CP: 11.7%; CF: 1.6%), [13] Ribeiro e Sousa Lda, Portugal, [14] Premix Lda, Portugal, [15, 16] Sopropêche, France, [17] JC Coimbra, Portugal. [18] Dietary chitin levels of IM3, IM25 and IM50 were determined by subtracting the hexosamine content in CTRL from the hexosamine content of the respective diet.

were hand-fed until apparent satiation three times daily (8:30, 12:00, and 16:00) for 75 days. The photoperiod was set to 12 h light/12 h dark, the water flow rate was 16 L/min, and abiotic parameters were maintained at adequate levels for the species. The measured water temperature was 22.1 ± 0.3 °C, salinity was 35.2 ± 0.6 ppt, pH was 7.4 ± 0.2, and dissolved oxygen levels were 7.7 ± 0.3 mg/L. Ammonium and nitrite levels were kept below the recommended values (Blancheton, 2000). The feed intake per tank was monitored throughout the trial, and the weight of any uneaten pellets was subtracted from the total amount of feed provided per tank.

2.3.2. Sampling

For analysis of the initial whole-body composition, 15 fish from the initial stock were sacrificed by anaesthetic overdose with 0.05% (*v*/v) 2-phenoxyethanol and stored at −80 °C. Then, after 75 days of feeding and following a 24-h fasting period, the fish in each tank were lightly anaesthetised for individual weight (g) and total length determination (cm). Five fish per tank were euthanised with a sharp blow on the head, and the liver and viscera were removed and weighed for calculation of the viscerosomatic and hepatosomatic indices (VSI and HSI, respectively). The liver was then fixed in isopentane (chilled with dry ice) and stored at −80 °C for posterior histological processing. A 1-cm portion of anterior intestine (collected 1 cm after the pyloric caeca) was collected from the same fish and fixed in phosphate-buffered 4% (*w*/*v*) formaldehyde (pH 7) for 24 h, with agitation, at room temperature. In addition, 300 mg of dorsal muscle were collected, snap-frozen in liquid nitrogen, and stored at −80 °C for posterior quantification of total lipids, FAs, the oxygen radical absorbance capacity (ORAC) and lipid peroxidation (LPO) levels. Lastly, six fish from each tank were sacrificed with 0.05% (v/v) 2-phenoxyethanol, and stored at −80 °C for final whole-body composition determination.

Table 2
Amino acid (AA) profile (% total AAs) of the black soldier fly larvae (BSFL) meal, yellow mealworm (YM) meal, and experimental diets.

	Insect meals		Experimental diets			
	BSFL	YM	CTRL	IM3	IM25	IM50
Essential AA (EAA)						
Arginine	5.50	5.59	5.86	5.93	5.97	5.94
Histidine	3.13	3.35	2.56	2.59	2.64	2.73
Lysine	6.60	5.93	5.99	6.17	5.98	5.86
Threonine	4.47	4.43	4.13	4.15	4.23	4.18
Isoleucine	5.02	4.84	4.91	4.95	4.99	4.96
Leucine	7.13	7.41	9.13	8.93	8.89	8.86
Valine	6.54	6.88	5.13	5.15	5.21	5.29
Methionine	2.37	1.81	2.54	2.50	2.56	2.59
Phenylalanine	5.05	4.30	5.22	5.12	5.23	5.26
ΣEAA	45.8	44.6	45.5	45.5	45.7	45.7
Non-essential AA (NEAA)						
Cystine	1.69	1.47	2.21	2.17	2.12	2.33
Tyrosine	8.49	8.54	4.38	4.25	4.49	4.69
Aspartic acid + Asparagine	10.1	8.60	9.00	9.29	9.16	9.12
Glutamic acid + Glutamine	11.9	11.9	17.1	17.1	16.7	16.4
Alanine	6.15	7.64	5.88	5.87	5.84	5.86
Glycine	5.35	5.56	4.88	4.94	4.91	4.85
Proline	5.69	6.38	5.94	5.85	5.94	5.94
Serine	4.83	5.39	5.13	5.08	5.13	5.12
ΣNEAA	54.2	55.4	54.5	54.5	54.3	54.3
ΣEAA/ΣNEAA	0.85	0.80	0.83	0.83	0.84	0.84
Total	100	100	100	100	100	100

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of BSFL meal and YM meal.

2.4. Digestibility trial

After the growth trial, a subset of the fish remaining in each tank was transferred to a Guelph system of 55-L fibreglass tanks equipped with sedimentation columns, designed according to Cho and Slinger (1979). Twelve homogeneous groups of fish (85.1 ± 16.5 g of body weight; 20 kg/m^3) were established, and the same diet of the growth trial, now containing 1% chromium oxide (Cr_2O_3) as an inert digestibility marker, was assigned to the same three groups. The fish were submitted to the same abiotic conditions of the growth trial, and, for an acclimation period of ten days, they were hand-fed with the experimental diets three times daily until apparent satiation (9:00, 12:00, and 15:30). After this period, a fixed quantity of feed was established according to the group with the lowest average feed intake, and hand-fed to the fish throughout the digestibility trial. Faeces were collected before the morning meal, centrifuged (10 min, 3000 *g*, 4 °C), and stored at −20 °C (pooled per tank).

Table 3
Fatty acid (FA) profile [% total FAs or % dry matter (DM)] of the black soldier fly larvae (BSFL) meal, yellow mealworm (YM) meal, and experimental diets.

	Insect meals		Experimental diets			
	BSFL	YM	CTRL	IM3	IM25	IM50
Saturated FA (SFA) (% FA)						
C12:0	21.7	0.25	0.06	0.07	0.24	0.42
C14:0	3.78	2.19	2.81	2.82	2.89	2.93
C16:0	15.3	15.1	13.1	13.1	13.2	13.2
C18:0	2.47	5.39	3.09	3.11	3.09	3.08
ΣSFA[a]	44.5	23.9	20.5	20.5	20.8	21.0
Monounsaturated FA (MUFA) (% FA)						
C16:1 n-7	2.04	1.02	3.85	3.84	3.90	3.90
C18:1 n-9	32.0	35.0	34.0	34.0	33.6	33.1
C18:1 n-7	1.11	0.28	2.89	2.89	2.86	2.80
C20:1 n-9	0.09	0.11	1.97	1.97	2.02	2.09
C22:1 n-11	–	–	1.34	1.33	1.38	1.46
ΣMUFA[b]	35.3	36.4	44.6	44.6	44.3	43.9
Polyunsaturated FA (PUFA) (% FA)						
C18:2 n-6	14.7	37.4	14.4	14.1	14.4	14.5
C18:3 n-3	1.55	0.87	3.51	3.51	3.46	3.41
C20:5 n-3 (EPA)	–	–	6.07	6.09	6.04	5.99
C22:5 n-3	–	–	1.04	1.05	1.08	1.10
C22:6 n-3 (DHA)	–	–	3.60	3.56	3.54	3.52
ΣPUFA[c]	17.4	38.5	32.1	31.9	32.1	32.2
Σn-3	1.55	0.87	15.6	15.6	15.5	15.5
Σn-6	15.5	37.6	15.5	15.3	15.5	15.7
Σn-6/Σn-3	10.0	43.2	1.00	0.98	1.00	1.02
EPA + DHA	–	–	9.67	9.65	9.58	9.51
DHA/EPA	–	–	0.59	0.58	0.59	0.59
Non-identified FA (% FA)	2.79	1.21	2.86	2.91	2.84	2.84
FA (% DM)						
C12:0	2.01	0.02	0.01	0.01	0.04	0.07
EPA	–	–	0.89	0.89	0.88	0.89
DHA	–	–	0.52	0.51	0.51	0.51
EPA + DHA	–	–	1.41	1.40	1.39	1.40

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of BSFL meal and YM meal. DHA – docosahexaenoic acid; EPA – eicosapentaenoic acid.

[a] SFAs sum (C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, including C10:0 in the BSFL meal and C24:0 in the diets).

[b] MUFAs sum (C14:1, C16:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9, C22:1 n-9, including C17:1 n-7, C22:1 n-11 and C24:1 n-9 in the diets).

[c] PUFAs sum (C16:4 n-1, C18:2 n-6, C18:3 n-3, C20:2 n-6, including C16:2 n-4, C16:3 n-4, C18:3 n-6, C18:4 n-3, C20:3 n-6, C20:3 n-3, C20:4 n-6, C20:4 n-3, C20:5 n-3, C22:2 n-6, C22:5 n-3 and C22:6 n-3 in the diets).

2.5. Chemical analyses

2.5.1. Proximate composition, chitin, phosphorus and chromium

Before whole-body composition analysis, the fish were pooled per tank and ground. DM content was determined after drying in an oven at 105 °C for 24 h. Then, the samples were freeze-dried for proximate composition analysis, as were the faeces collected during the digestibility trial.

The BSFL and YM meals, diets, freeze-dried whole body, and freeze-dried faeces were analysed for proximate composition in conformity with the AOAC (2006) methodology. DM was determined after drying at 105 °C for 24 h; ash was quantified after combustion in a muffle furnace for 6 h (550 °C); P levels were quantified in ash samples, after digestion with 6 M HCl (150 °C) in Kjeldahl digestion tubes, and following the molybdate blue/ascorbic acid spectrophotometric method (820 nm) (Marques et al., 2023); gross energy was determined using an adiabatic bomb calorimeter (C2000, IKA-Werke GmbH & Co. KG, Germany); CP was assessed by nitrogen (N) quantification through the Dumas combustion method (FP-528, Leco Corporation, U.S.A.) and posterior multiplication by a nitrogen-to-protein conversion factor (K_p). In the case of the experimental diets, a K_p of 6.25 was considered, based on the general assumption that proteins contain 16% N. For the IMs, besides a K_p of 6.25, a K_p of 5.33 was considered, as suggested by Boulos et al. (2020) for CP determination in insect larvae.

CF was determined by extraction with petroleum ether at 40–60 °C (Soxtec™ 2055, Foss, Sweden). Before CF extraction of the IMs, the samples were hydrolysed (2.5 g of sample in 100 mL of 3 M HCl at 100 °C for 1 h), filtered using filter paper (pore diameter of 5–8 μm), and the retentate dried overnight (50 °C). Chitin levels of the IMs and diets were estimated as described by Guerreiro et al. (2020). The IMs were solely deproteinised, whereas the diets were sequentially defatted and deproteinised for spectrophotometric quantification of hexosamines at 650 nm. Chitin levels of the IMs were expressed as %DM. For the diets IM3, IM25 and IM50, the chitin levels were obtained through hexosamine quantification and posterior subtraction of the hexosamine content in CTRL. The chromium (Cr) levels in the diets used in the digestibility trial and faeces were quantified according to Bolin et al. (1952).

2.5.2. Total lipids and fatty acids

Before determination of the total lipid levels and FA profile, the muscle samples were freeze-dried, pooled per tank, and homogenised (A 11 basic analytical mill, IKA-Werke GmbH & Co. KG, Germany). The total lipids of the fish dorsal muscle were extracted and quantified according to Folch et al. (1957), using 2:1 (*v*/v) dichloromethane:methanol with 0.01% BHT. The FA composition of the IMs, diets, and muscle samples was determined through gas chromatography (Nexis GC-2030 gas chromatograph with AOC-20i auto-injector flame-ionisation detector, Shimadzu, Japan) after direct transmethylation and using the internal standard C23:0 as reference, as described by Filipa-Silva et al. (2023). Each FA methyl ester (FAME) was identified by comparison of its retention peak with that obtained with a known FA mixture (Sigma 47,885-U Supelco 37 Component FAME Mix, U.S.A.). FA levels were obtained using the conversion factors determined by Visentainer (2012) and were expressed as % DM, % total FAs, or mg/100 g wet muscle.

2.5.3. Amino acids

The AA composition of the IMs and diets was determined by ultra-high-performance liquid chromatography on a Waters Reversed-Phase Amino Acid Analysis System, in conformity with Teodósio et al. (2021), after freeze-drying and hydrolysis with 6 M HCl for 72 h (116 °C) in nitrogen-flushed glass vials. The chromatographic peaks were analysed using the EMPOWER software (Waters, U.S.A.), and AA levels were expressed as % total AAs. Values for tryptophan are not presented due to partial loss during hydrolysis.

2.6. Histological analysis

2.6.1. Liver

Frozen liver sections of nine fish per dietary treatment were obtained using a cryostat and then stained with Oil Red O (010303, Diapath, Italy) for lipid droplet evidentiation. The quantity of lipid droplets in each section was analysed using a light microscope (Olympus BX51, 194 GmbH, Germany) with magnification set to 200×, alongside a camera (Olympus DP50, GmbH, Germany). Following a semi-quantitative approach, one of the following scores was attributed to each analysed fish: 0 – no lipid droplets; 1 (low) – on average, < 1/3 of the hepatocytes' cytoplasm presented lipid droplets; 2 (moderate) – on average, 1/3 to 2/3 of the hepatocytes' cytoplasm presented lipid droplets; 3 (high) – on average, > 2/3 of the hepatocytes' cytoplasm presented lipid droplets; 4 (extreme) – 3/3 of the hepatocytes' cytoplasm presented lipid droplets.

2.6.2. Anterior intestine

After fixation, intestinal samples of nine fish per dietary treatment were kept in 70% (*v*/v) ethanol until further classical histological processing and embedding in paraffin. Then, cross-sections of 3 μm were obtained using a semi-automated rotary microtome (Leica RM 2245, Leica Biosystems, Nussloch, Germany) and stained with Alcian Blue/Periodic Acid Schiff (AB/PAS, pH 2.5). Morphometric analysis of the cross-sectional perimeter (mm), absorption area (mm^2), *muscularis* thickness (μm), submucosa thickness (μm), *villi* length (mm), *lamina*

propria thickness (μm), goblet cell number (acid and neutral) and goblet cell average area were performed in one scanned cross-section per fish (Pannoramic 1000 Flash DX, 3DHistech, Hungary). For this, imaging software was used (cellSens Standard 2.2, Olympus, Japan) and the analysis was conducted as described by Ferreira et al. (2023), with minor modifications: *muscularis* thickness was determined by subtracting the radius of the cross-section without *muscularis* from the radius of the whole cross-section; for *villi* length determination, the eight longest *villi* of each section were measured from the tip to the base (defined as indicated in Fig. 1) and the average *villi* length per section was calculated; for submucosa thickness determination, two measurements were performed per *villi* selected for *villi* length determination, from the base of the *villi* to the *muscularis*, and an average of sixteen measurements per section was obtained (Fig. 1).

2.7. Muscle antioxidant capacity and lipid peroxidation levels

The antioxidant capacity of muscle samples was determined through the ORAC assay, using fluorescein as a fluorescent probe, according to Filipa-Silva et al. (2023). Fluorescence measurements were performed every minute for 80 min, using excitation and emission wavelengths of 485 nm and 528 nm, respectively (BioTek Synergy HTX, U.S.A.). ORAC values were expressed as μmol of Trolox equivalents/g of wet muscle. Muscle LPO levels were estimated through the quantification of thiobarbituric acid-reactive substances, including malondialdehyde, as by-products of LPO. Muscle samples were homogenised in ice-cold phosphate buffer (0.1 M, pH 7.4) in a ratio of 1:8 (*w/v*), using a mechanical tissue homogeniser (T25 easy clean digital Ultra Turrax, IKA Werke GmbH & Co. KG, Germany). Then, homogenate aliquots containing 1.7% (*v*/v) of 4% (w/v) BHT (prepared in ethanol) were stored at −80 °C until the analysis, performed as described by Filipa-Silva et al. (2023). Malondialdehyde equivalents were quantified spectrophotometrically at 535 nm, in microplates (BioTek Synergy HTX, U.S.A.). The molar extinction coefficient of 156,000 $M^{-1}cm^{-1}$ was used for calculations, so LPO levels were expressed as nmol of malondialdehyde equivalents/g of wet muscle.

2.8. Calculations

Growth performance and feed efficiency calculations were conducted as follows, considering W_i and W_f the fish's initial and final weights (g), respectively, and ABW the average body weight (g) [$(W_f + W_i) / 2$]: Weight gain (g) = $W_f - W_i$; final condition factor = W_f / length^3 × 100; voluntary feed intake (VFI, %/day) = feed intake / ABW / days × 100; daily growth index (DGI) = 100 × ($W_f^{1/3} - W_i^{1/3}$) / days; feed conversion ratio (FCR) = dry feed intake / ($W_f - W_i$). Somatic indices were calculated as: hepatosomatic index (HSI, %) = liver weight / W_f × 100; viscerosomatic index (VSI, %) = viscera weight / W_f × 100.

The apparent digestibility coefficient (ADC) of the nutrients or energy in the diets were calculated as: nutrient or energy ADC (%) = 1 – [dietary Cr content / faecal Cr content × faecal nutrient (or energy) content / dietary nutrient (or energy) content] × 100. Calculations concerning nutrient and energy utilisation were conducted as: faecal nutrient losses (mg / 100 g ABW / day) = crude nutrient intake (mg / 100 g ABW / day) × (1 – nutrient ADC/100); faecal energy losses (kJ / kg ABW / day) = crude energy intake (kJ / kg ABW / day) × (1 – energy ADC/100); digestible nutrient intake (mg / 100 g ABW / day) = crude nutrient intake × nutrient ADC; digestible energy intake (kJ / kg ABW / day) = crude energy intake – faecal energy losses; metabolic nutrient losses (or branchial and urinary, in the case of N and P) (mg / 100 g ABW / day) = digestible nutrient intake – nutrient gain; metabolic energy losses (kJ / kg ABW / day) = branchial and urinary N losses × 24.9 kJ/g of N; nutrient gain (mg / 100 g ABW / day) = [W_f × nutrient content in final whole body (%) – W_i × nutrient content in initial whole body (%)] / ABW / days × 1000; energy gain (kJ / kg ABW / day) = [W_f × energy content in final whole body – W_i × energy content in initial whole body] / ABW / days × 1000; nutrient or energy retention efficiency (% digestible intake) = nutrient (or energy) gain / digestible nutrient (or energy) intake × 100; metabolisable energy (kJ / kg ABW / day) = digestible energy intake – branchial and urinary energy losses; total heat production (kJ / kg ABW / day) = metabolisable energy – energy gain.

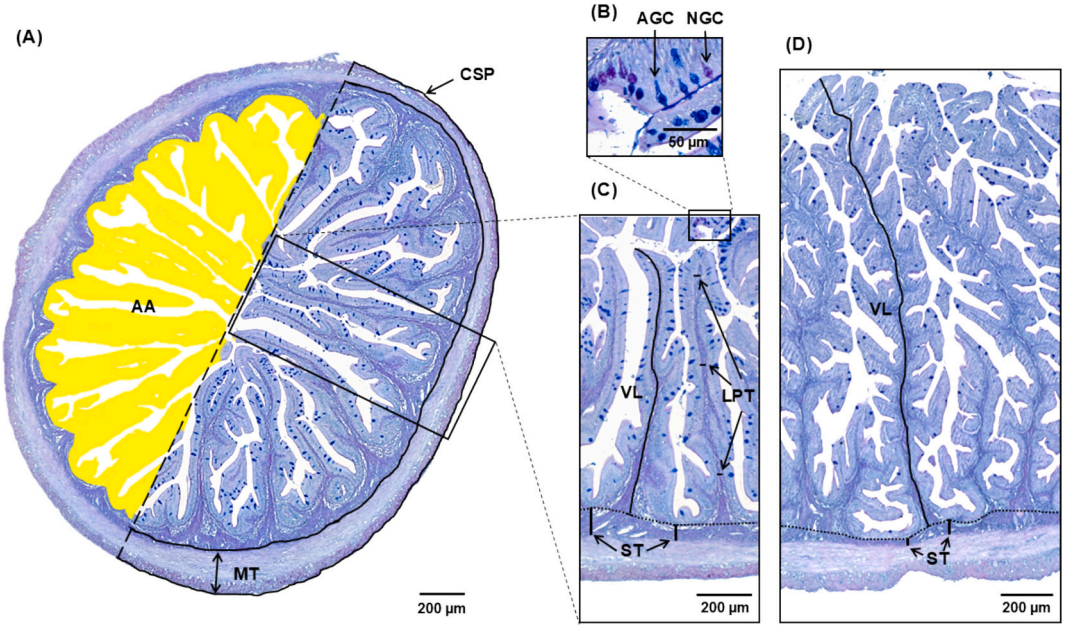

Fig. 1. Illustration of the histomorphometric parameters quantitatively analysed in the cross-sections of the anterior intestine of European seabass (staining with Alcian Blue/Periodic acid Schiff). (A) Absorption area (AA), cross-sectional perimeter (CSP), and muscularis thickness (MT) of fish fed with CTRL for 75 days; (B) Acid goblet cell (AGC) and neutral goblet cell (NGC) number and average area; (C) *villus* length (VL), *lamina propria* thickness (LPT), and submucosa thickness (ST); (D) VL and ST of fish fed with IM50 for 75 days. Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics, version 29.0 (IBM Corporation, U.S.A.), with significance established at 0.05. First, normality and homogeneity of variances were evaluated using the Shapiro-Wilk and Levene's tests, respectively. Then, a one-way analysis of variance (ANOVA) was conducted to identify statistically significant differences among the experimental groups. When this analysis showed significance, the Tukey HSD test was employed for individual mean comparison. Whenever normality or homogeneity of variances were not met, appropriate transformations were employed before the ANOVA. The Kruskal-Wallis test was applied if ANOVA assumptions were still not met.

3. Results

3.1. Chemical characterisation of the insect meals and experimental diets

Considering a K_p of 6.25, the crude protein (CP) levels determined for the BSFL and YM meals were 55.1% and 76.1% (on a DM basis), respectively. Using a K_p of 5.33, the estimated CP levels were 47.0% and 64.9% (on a DM basis), respectively (Table 1). The true protein values, obtained through the amino acid (AA) sum, corresponded to 48.5% and 63.3% of the DM, respectively (Table 2). Essential AAs (EAAs) constituted 45.8% of the total AAs in the BSFL meal, and 44.5% in the case of the YM meal. Among the EAAs, lysine (Lys), leucine, and valine were the most abundant in both IMs (Table 2). The crude fat (CF) content of the BSFL and YM meals corresponded to 9.0% and 6.2% of the DM, respectively (Table 1). Saturated fatty acids (SFAs) were the most abundant type of FAs in the BSFL meal, which contained 2.0% of lauric acid on a DM basis (Table 3). The most abundant FAs in the YM meal were mono- and polyunsaturated fatty acids (MUFAs and PUFAs), namely oleic acid (C18:1 n-9) and linoleic acid (C18:2 n-6), respectively (Table 3).

The diets used in the growth trial had a similar AA profile, with a ratio between EAAs and non-essential AAs (NEAAs) of 0.8 (Table 2). The diets also shared a similar FA profile. The sum of EPA and DHA represented 1.4% of the DM of all diets, and the ratio between n-6 PUFA and n-3 PUFA (Σn-6/Σn-3) was 1.0 (Table 3). The only FA whose content differed among the diets was lauric acid, with levels increasing as the dietary inclusion of IM mixture increased (Table 3). Likewise, chitin levels increased concomitantly with the dietary inclusion of the IM mixture (Table 1).

3.2. Growth performance, whole-body composition, and nutrient utilisation

All fish groups nearly quintupled their initial body weight after 75 days of feeding. The experimental diets were equally well-accepted by the seabass juveniles, resulting in a similar VFI among groups (1.8–1.9%/day). No significant differences were observed among the experimental groups concerning the DGI (2.0) and FCR (1.0–1.1) (Table 4). Likewise, no statistically significant differences were detected in the HSI and VSI, which ranged from 1.6% (IM50) to 1.8% (CTRL) and 7.5% (IM25) to 8.2% (CTRL), respectively (Table 4). The whole-body proximate composition was also similar across the experimental groups (Table 4). The apparent digestibility of protein (92–93%), fat (95–96%), P (63–72%) and energy (91–92%) were similar among diets ($p > 0.1$), resulting in a similar digestible protein/digestible energy ratio (Table 1).

The nutrient and energy balances of the fish fed with the experimental diets are described in Table 5. The gain and retention efficiency of nitrogen (N), energy, and phosphorus (P) did not differ among the experimental groups. Fish fed with IM50 exhibited significantly lower faecal lipid losses than the remaining experimental groups; IM50 fish also had the lowest faecal N losses, but only differed from IM25-fed fish. Furthermore, the digestible P intake was higher in fish fed IM3 and IM25 than in those fed the control. Fish fed with the diets containing IM had 20–27% lower faecal P losses than the control group.

3.3. Histological analysis of the liver and anterior intestine

Following the quantification of lipid droplets in the liver samples of fish fed with the experimental diets for 75 days, an average score of 3 was attributed to each group, and no significant differences were observed among groups (Fig. 2). Concerning the anterior intestine, the histomorphological analysis revealed that submucosa thickness was lower in fish fed with the IM diets than in the control (Table 6). Moreover, IM50-fed fish had 38 % longer *villi* than the control group (Table 6, Fig. 1). Despite a similar body weight among fish, trends towards significance were observed regarding the impact of the diets on the cross-sectional perimeter ($p = 0.063$) and absorption area ($p = 0.070$), with IM50-fed fish presenting the highest value for both parameters and CTRL-fed fish the lowest values (Table 6).

Table 4
Growth performance, feed utilisation, somatic indices, and whole-body composition (WBC) of European seabass juveniles fed with the experimental diets for 75 days.

	CTRL	IM3	IM25	IM50	*p*-value
Growth performance					
Initial body weight (g)	12.9 ± 0.02	12.9 ± 0.02	12.9 ± 0.02	12.9 ± 0.01	0.206
Final body weight (g)	57.7 ± 2.0	57.5 ± 1.1	57.7 ± 1.1	58.2 ± 2.3	0.959
Weight gain (g)	44.8 ± 2.0	44.6 ± 1.1	44.8 ± 1.1	45.3 ± 2.3	0.962
Final condition factor	1.18 ± 0.03	1.17 ± 0.02	1.17 ± 0.01	1.17 ± 0.03	0.906
VFI (%/day)	1.81 ± 0.05	1.85 ± 0.13	1.83 ± 0.12	1.75 ± 0.05	0.603
DGI	2.02 ± 0.06	2.02 ± 0.03	2.02 ± 0.03	2.04 ± 0.07	0.970
FCR	1.07 ± 0.04	1.10 ± 0.09	1.08 ± 0.07	1.03 ± 0.04	0.650
Somatic indices					
HSI (%)	1.79 ± 0.08	1.73 ± 0.15	1.66 ± 0.06	1.63 ± 0.08	0.264
VSI (%)	8.22 ± 0.71	7.75 ± 0.36	7.54 ± 1.04	7.62 ± 0.39	0.647
WBC (% wet weight)					
Dry matter	36.3 ± 1.2	36.5 ± 0.5	35.2 ± 0.6	36.1 ± 0.2	0.233
Crude protein	17.2 ± 0.2	17.4 ± 0.4	17.4 ± 0.6	17.5 ± 0.02	0.889
Crude fat	14.9 ± 1.1	15.3 ± 0.7	14.0 ± 0.4	14.6 ± 0.8	0.302
Gross energy (kJ/g)	9.52 ± 0.59	9.59 ± 0.26	9.30 ± 0.30	9.47 ± 0.31	0.819
Ash	3.93 ± 0.03	3.84 ± 0.08	3.72 ± 0.21	3.76 ± 0.53	0.807
Phosphorus	0.55 ± 0.02	0.54 ± 0.01	0.54 ± 0.03	0.53 ± 0.1	0.855

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. DGI – daily growth index; FCR – feed conversion ratio; HSI – hepatosomatic index; VFI – voluntary feed intake; VSI – viscerosomatic index. Initial WBC: Dry matter (%) – 30.9; Protein (% wet weight) – 17.0; Lipids (% wet weight) – 9.5; Energy (kJ/g) – 7.4; Phosphorus (% wet weight) – 0.57. Values are presented as the mean ± standard deviation; $n = 3$ for growth performance parameters; $n = 15$ for somatic indices; $n = 18$ for whole-body composition.

Table 5
Nitrogen, lipid, energy, and phosphorus balances of European seabass juveniles fed with the experimental diets.

	CTRL	IM3	IM25	IM50	*p*-value
Nitrogen (N) balance					
(mg/100 g ABW/day)					
Faecal N losses	10.1 ± 0.3^{ab}	10.0 ± 0.7^{ab}	10.3 ± 0.7^{a}	8.7 ± 0.2^{b}	0.023
Digestible N intake (DN)	122.0 ± 3.1	126.3 ± 9.1	123.6 ± 7.9	121.1 ± 3.2	0.772
Branchial and urinary N losses	75.1 ± 3.3	79.0 ± 10.8	76.2 ± 6.1	73.3 ± 3.6	0.770
N gain	46.9 ± 0.7	47.3 ± 1.7	47.5 ± 1.9	47.8 ± 0.9	0.869
N retention efficiency, %DN	38.4 ± 1.2	37.6 ± 3.9	38.4 ± 1.0	39.5 ± 1.4	0.795
Lipid (L) balance					
(mg/100 g ABW/day)					
Faecal L losses	17.9 ± 0.5^{a}	18.2 ± 1.3^{a}	18.1 ± 1.2^{a}	14.7 ± 0.4^{b}	0.004
Digestible L intake (DL)	319.9 ± 8.3	329.4 ± 23.8	321.8 ± 20.6	312.1 ± 8.4	0.667
Metabolic L losses	41.4 ± 29.6	42.9 ± 22.1	62.5 ± 19.6	38.9 ± 25.4	0.641
L gain	278.4 ± 26.0	286.5 ± 13.9	259.3 ± 6.8	273.1 ± 20.9	0.380
L retention efficiency, %DL	87.1 ± 9.0	87.2 ± 5.9	80.8 ± 4.8	87.6 ± 7.8	0.606
Energy (E) balance					
(kJ/kg ABW/day)					
Faecal E losses	34.5 ± 0.9	33.6 ± 2.4	35.6 ± 2.3	34.7 ± 0.9	0.619
Digestible E intake (DE)	372.2 ± 9.6	378.5 ± 27.3	377.9 ± 24.2	363.5 ± 9.7	0.768
Metabolic E losses	18.7 ± 0.8	19.7 ± 2.7	19.0 ± 1.5	18.3 ± 0.9	0.769
E gain	171.2 ± 15.0	172.4 ± 5.5	166.4 ± 5.2	70.5 ± 9.4	0.878
E retention efficiency, %DE	46.1 ± 4.7	45.7 ± 3.6	44.1 ± 2.5	46.9 ± 2.9	0.800
Metabolisable E	353.5 ± 8.8	358.8 ± 24.6	358.9 ± 22.7	345.2 ± 8.9	0.763
Total heat production	182.2 ± 20.6	186.4 ± 25.6	192.5 ± 21.2	174.7 ± 13.3	0.761
Phosphorus (P) balance					
(mg/100 g ABW/day)					
Faecal P losses	6.26 ± 0.16^{a}	5.13 ± 0.37^{b}	4.90 ± 0.31^{b}	4.57 ± 0.12^{b}	< 0.001
Digestible P intake (DP)	10.6 ± 0.3^{b}	12.3 ± 0.9^{a}	12.4 ± 0.8^{a}	12.0 ± 0.3^{ab}	0.024
Branchial and urinary P losses	1.30 ± 0.84	3.33 ± 0.95	3.41 ± 1.36	3.22 ± 1.28	0.140
P gain	9.26 ± 0.58	8.96 ± 0.24	9.00 ± 0.70	8.73 ± 1.47	0.832
P retention efficiency, %DP	87.9 ± 7.8	73.1 ± 5.6	72.9 ± 9.3	72.9 ± 10.9	0.164

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. Values are presented as the mean ± standard deviation ($n = 3$). Statistically significant differences among experimental groups are denoted using a different group of superscript letters ($p < 0.05$).

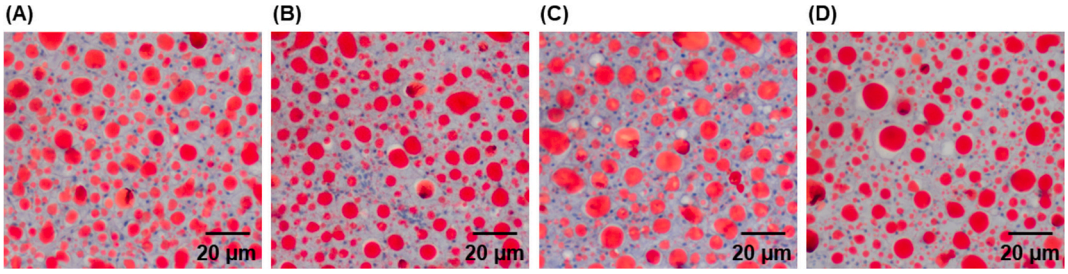

Fig. 2. Optical microscopy images of the liver of European seabass, showing no differences in lipid droplet quantity after feeding with the (A) CTRL, (B) IM3, (C) IM25 or (D) IM50 diets for 75 days (staining with Oil Red O). Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Quantitative histomorphometric analysis of the anterior intestine of European seabass juveniles fed with the experimental diets for 75 days.

	CTRL	IM3	IM25	IM50	*p*-value
Parameter					
Cross-sectional perimeter (mm)	11.9 ± 2.6	12.7 ± 2.0	12.4 ± 1.7	14.5 ± 1.9	0.063
Absorption area (mm^2)	4.09 ± 1.63	5.15 ± 1.48	4.38 ± 1.73	5.97 ± 1.41	0.070
Muscularis thickness (µm)	107.0 ± 32.7	75.7 ± 18.0	89.7 ± 16.9	86.3 ± 10.7	0.089
Submucosa thickness (µm)	64.7 ± 13.3^{a}	45.6 ± 11.1^{b}	44.5 ± 14.9^{b}	44.0 ± 10.3^{b}	0.003
Lamina propria thickness (µm)	48.2 ± 11.5	47.7 ± 2.2	42.5 ± 8.8	44.5 ± 7.0	0.495
Villi length (mm)	1.13 ± 0.22^{b}	1.41 ± 0.44^{ab}	1.19 ± 0.28^{ab}	1.55 ± 0.22^{a}	0.022
Goblet cells (GCs)	1711 ± 538	1845 ± 684	1669 ± 311	2081 ± 406	0.320
Acid GCs	1493 ± 457	1597 ± 611	1490 ± 260	1829 ± 365	0.340
Neutral GCs	218 ± 133	248 ± 128	179 ± 64	252 ± 63	0.342
GC average area (µm^2)	56.3 ± 13.9	46.9 ± 6.7	48.3 ± 12.5	47.7 ± 6.1	0.222

Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. Values are presented as the mean ± standard deviation ($n = 9$). Statistically significant differences among experimental groups are denoted using a different group of superscript letters ($p < 0.05$).

Table 7
Total lipid content (g/100 g of muscle) and fatty acid (FA) profile [% total FAs or mg/100 g of muscle] of the muscle of European seabass juveniles fed with the experimental diets for 75 days.

	CTRL	IM3	IM25	IM50	*p*-value
Total lipids	3.3 ± 0.3	3.5 ± 0.3	3.0 ± 0.8	2.8 ± 0.3	0.379
Saturated FA (SFA) (% FA)					
C12:0	0.02 ± 0.002^{c}	0.02 ± 0.002^{c}	0.07 ± 0.013^{b}	0.11 ± 0.006^{a}	< 0.001
C16:0	16.4 ± 0.3	16.6 ± 0.1	16.5 ± 0.2	16.5 ± 0.1	0.550
C18:0	3.94 ± 0.13	3.92 ± 0.06	4.06 ± 0.11	3.97 ± 0.17	0.573
ΣSFA[1]	23.1 ± 0.4	23.3 ± 0.1	23.3 ± 0.1	23.2 ± 0.2	0.610
Monounsaturated FA (MUFA) (% FA)					
C16:1 n-7	3.75 ± 0.04	3.80 ± 0.02	3.69 ± 0.20	3.70 ± 0.11	0.627
C18:1 n-9	34.1 ± 0.2	34.3 ± 0.3	33.4 ± 1.1	33.1 ± 0.9	0.262
C18:1 n-7	2.77 ± 0.05	2.79 ± 0.03	2.74 ± 0.03	2.71 ± 0.02	0.130
ΣMUFA[2]	44.1 ± 0.2	44.3 ± 0.4	43.4 ± 1.4	43.2 ± 1.0	0.409
Polyunsaturated FA (PUFA) (% FA)					
C18:2 n-6	10.9 ± 0.4	10.7 ± 0.1	10.8 ± 0.1	10.9 ± 0.1	0.760
C18:3 n-3	2.47 ± 0.09	2.47 ± 0.02	2.42 ± 0.02	2.42 ± 0.1	0.525
C20:5 n-3 (EPA)	6.02 ± 0.12	6.07 ± 0.13	6.19 ± 0.44	6.23 ± 0.24	0.735
C22:6 n-3 (DHA)	6.13 ± 0.20	5.92 ± 0.24	6.47 ± 0.82	6.49 ± 0.55	0.505
ΣPUFA[3]	29.7 ± 0.6	29.3 ± 0.4	30.1 ± 1.4	30.4 ± 0.8	0.475
Σn-3	16.8 ± 0.3	16.6 ± 0.3	17.3 ± 1.3	17.4 ± 0.8	0.560
Σn-6	12.5 ± 0.4	12.3 ± 0.1	12.5 ± 0.2	12.6 ± 0.1	0.477
Σn-6/Σn-3	0.77 ± 0.03	0.77 ± 0.02	0.75 ± 0.05	0.75 ± 0.04	0.729
EPA + DHA	12.1 ± 0.3	12.0 ± 0.4	12.7 ± 1.3	12.7 ± 0.8	0.597
DHA/EPA	1.00 ± 0.01	0.96 ± 0.02	1.03 ± 0.06	1.02 ± 0.05	0.281
Non-identified FA (% FA)	3.12 ± 0.04	3.13 ± 0.12	3.20 ± 0.08	3.19 ± 0.15	0.724
FA (mg/100 g muscle)					
C12:0	0.78 ± 0.09^{c}	0.92 ± 0.11^{c}	2.41 ± 0.74^{b}	3.52 ± 0.53^{a}	< 0.001
EPA	202 ± 26	207 ± 15	187 ± 31	181 ± 21	0.519
DHA	202 ± 24	197 ± 12	190 ± 19	184 ± 12	0.606
EPA + DHA	405 ± 50	405 ± 26	377 ± 49	364 ± 33	0.555

Diets: CTRL; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. Values are presented as the mean ± standard deviation (n = 3). Statistically significant differences among experimental groups are denoted using a different group of superscript letters ($p < 0.05$).

[1] SFAs sum (C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0).

[2] MUFAs sum (C14:1, C16:1 n-7, C17:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9, C22:1 n-9, C22:1 n-11, C24:1 n-9).

[3] PUFAs sum (C16:2 n-4, C16:3 n-4, C16:4 n-1, C18:2 n-6, C18:3 n-6, C18:3 n-3, C18:4 n-3, C20:2 n-6, C20:3 n-6, C20:3 n-3, C20:4 n-6, C20:4 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

3.4. Muscle total lipids and fatty acid composition

The muscle total lipid content and FA profile did not vary significantly among the experimental groups (Table 7). The only exception was the increasing lauric acid content with increasing dietary inclusion of the IM mixture (Table 7). Thus, the IM50-fed group presented the highest levels of lauric acid in the muscle (3.52 mg/100 g), followed by IM25-fed fish (2.41 mg/100 g), both being significantly higher than those determined for IM3- and CTRL-fed fish. SFAs, MUFAs, and PUFAs represented 23%, 43–44%, and 29–30% of the FAs in the muscle, respectively, and the EPA and DHA sum varied between 364 and 405 mg/100 g of wet muscle (Table 7).

3.5. Muscle antioxidant capacity and lipid peroxidation

No statistically significant differences were found among the experimental groups concerning the muscle oxygen radical absorbance capacity (ORAC) (Fig. 3A) and lipid peroxidation (LPO) levels (Fig. 3B). Nonetheless, a trend towards significance was observed regarding the impact of the diets on the muscle ORAC ($p = 0.055$), with IM50-fed fish presenting the highest value (118.1 μmol Trolox equivalents/g of muscle).

4. Discussion

Black soldier fly larvae (BSFL) and yellow mealworms (YMs) have been increasingly explored as novel ingredients for aquafeeds, including diets for European seabass juveniles. This study demonstrated the feasibility of incorporating a mixture of two IMs – BSFL meal and YM meal – to partially replace fishmeal (FM) in practical diets for this species. The experimental diets, containing low to moderate FM levels (7.5–14.6%), supported comparable growth performance, nutrient utilisation, and flesh quality to the control diet, which contained 15% FM. Overall, this work focuses on the potential of combining BSFL and YM meals in aquafeeds, which could increase the flexibility for feed formulation.

The first step towards evaluating an ingredient for incorporation in aquafeeds is its chemical characterisation. The protein content of feed ingredients is conventionally estimated from the total N content, often multiplied by a nitrogen-to-protein conversion factor (K_p) of 6.25, assuming that proteins are the only nitrogen (N) source in the matrix and contain approximately 16% N (Alhotan et al., 2024; Mariotti et al., 2008). Since IMs contain non-protein N (e.g., from chitin), researchers have made efforts to determine specific K_p values for more reliable crude protein (CP) estimation (Belghit et al., 2019; Janssen et al., 2017). In this work, the CP content of the BSFL and YM meals was estimated using both a K_p of 6.25 and 5.33, suggested by Boulos et al. (2020) for insect larvae. The former resulted in CP values that were 8% and 20% above the measured amino acid (AA) content of the BSFL and YM meals, respectively. In its turn, the K_p of 5.33 resulted in CP values that were closer to the AA sum (3% below for BSFL and 3% above for YM). Although the AA sum excluded tryptophan, this highlights the importance of selecting adequate K_p values to estimate the CP of emerging ingredients, ensuring the formulation of nutritionally balanced feeds. Additionally, it suggests that AA quantification remains the most reliable method for protein estimation in complex matrices like IMs.

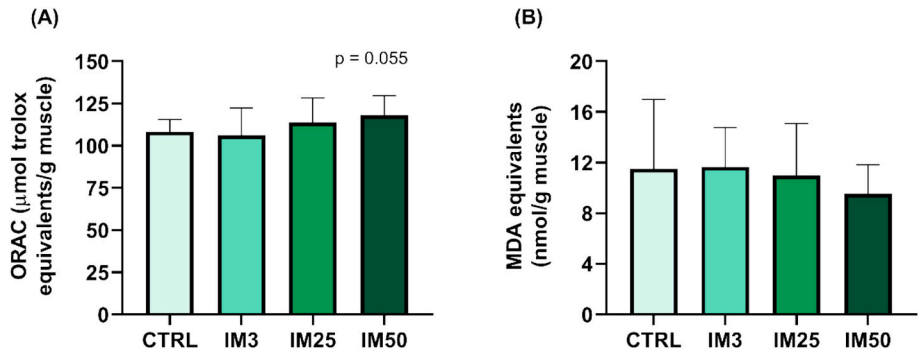

Fig. 3. (A) Oxygen radical absorbance capacity (ORAC, μmol of Trolox equivalents/g of muscle) and (B) levels of malondialdehyde (MDA) equivalents (nmol/g of muscle) in the muscle of European seabass juveniles fed with the experimental diets for 75 days. Values are presented as the mean ± standard deviation ($n = 15$). Diets: CTRL – control; IM3, IM25 and IM50 – diets in which 3%, 25% and 50% of the fishmeal protein in CTRL were replaced with a 50:50 mixture of black soldier fly larva meal and yellow mealworm meal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Concerning the AA profile, lysine (Lys), leucine and valine were among the most abundant EAAs in both IMs, in agreement with previous reports on defatted meals of BSFL and YMs (Adámková et al., 2020; Muñoz-Seijas et al., 2024; Smets et al., 2020) and their defatted meals (Basto et al., 2020; Matin et al., 2021). BSFL and YM meals have been reported to have a similar EAA profile to FM, but they typically contain less Lys and methionine (Met) (Belghit et al., 2019; Mastoraki et al., 2020). In this study, dietary supplementation with crystalline AAs was not necessary to meet the requirements of European seabass. Moreover, the AA profile of the control and IM-containing diets was highly similar, suggesting that, under the tested conditions, the IM mixture was an adequate FM substitute in terms of AA profile. Contrarily, supplementation with Lys or Met was deemed necessary to meet the requirements of seabass juveniles in other studies. In a study by Mastoraki et al. (2020), the dietary inclusion of 20% BSFL or YM meals to replace 30% FM in a control diet with 65% FM required Lys supplementation. In a study by Basto et al. (2021), the dietary inclusion of 18–36% YM meal to replace 40–80% FM in a control diet with 60% FM (or 60% YM meal as the only protein source) required Met supplementation. This likely resulted from the dietary inclusion of higher IM levels than those tested in the present study and the replacement of a higher FM quantity. Hence, it shows how the adequacy of using IMs as protein sources without AA supplementation depends on their inclusion level and the amount of FM they replace.

After 75 days of feeding with the control and IM-containing diets, no significant differences were observed among groups concerning voluntary feed intake (VFI), final body weight, daily growth index (DGI), or feed conversion ratio (FCR). Hence, the diets containing the IM mixture ensured a comparable growth performance and feed efficiency to the control. Similar findings have been reported in studies evaluating the inclusion of 1.2–20% BSFL to substitute 3–50% FM in control diets for European seabass juveniles containing 21–65% FM (Abdel-Tawwab et al., 2020; Magalhães et al., 2017; Mastoraki et al., 2020; Zarantoniello et al., 2023). Likewise, the dietary inclusion of 18–36% YM meal to replace 30–80% FM in control diets containing 45–65% FM, or the reliance on 60% YM as the only protein source, has been shown to adequately support the growth performance of seabass juveniles (Basto et al., 2021; Mastoraki et al., 2020). Concerning the FCR, contradictory results have been observed. Basto et al. (2021) reported that the dietary replacement of 40–80% FM with 18% or 36% YM meal resulted in a similar FCR to the control diet. Contrarily, Mastoraki et al. (2020) reported that the dietary inclusion of 19.5% YM meal to replace 30% FM increased the FCR. A negative impact of the dietary substitution of FM with IM on FCR has commonly been associated with the presence of chitin (Liland et al., 2021; Tran et al., 2022b) – a structural polysaccharide found in the exoskeleton of insects, suggested to reduce feed protein digestibility (Marono et al., 2015). Still, a meta-analysis by Tran et al. (2022b) suggests that IM utilisation at levels that ensure a dietary chitin content below 2.2% does not compromise the FCR for marine fish, which supports the observations of the present study. Like growth performance and feed efficiency, the viscero- and hepatosomatic indices (VSI and HSI) were also similar among the experimental groups, suggesting that the IM diets did not impact fat deposition in the viscera and liver. The latter was further confirmed by histological analysis.

When exploring alternative protein sources for aquafeeds, evaluating nutrient digestibility and utilisation by the fish is of the utmost importance, as they influence fish growth and waste production. In particular, N (grossly derived from protein metabolism) and phosphorus (P) are considered key contributors to the eutrophication of aquatic systems (Amirkolaie, 2011). When N and P are not retained in the fish, they are released into the water as faecal or non-faecal losses (Kokou and Fountoulaki, 2018), which should be minimised for lower environmental impact (Amirkolaie, 2011). In the present work, the gain and retention efficiency of N, energy and P were similar among groups. Hence, comparable levels were found in the whole body, independently of the diet. Still, faecal P losses were 20–27% lower in fish fed with the IM diets. This is likely related to the increased digestible P intake observed for these fish compared to the control (significant for IM3 and IM25 groups) and suggests that the IM diets contribute to lower solid P waste. Although the diets containing IM were supplemented with extra monocalcium phosphate (MCP), it is unlikely that the lower faecal P losses resulted from higher MCP digestibility, as lower faecal P losses have also been reported in other studies in which the diets were not supplemented with inorganic P. For instance, Basto et al. (2021) reported that including 18% defatted YM meal to replace 40% FM in a diet for seabass juveniles resulted in increased P digestibility and 43% lower faecal P losses. In a review article, Tran et al. (2022a) reported that, overall, the incorporation of BSFL or YM meals in aquafeeds decreases solid P waste production, which negatively correlates with the level of FM replacement by IM. Hence, P seems to be more bioavailable in insects than in FM, which contains more inorganic (tricalcium phosphate and hydroxyapatite) than organic P (Hua et al., 2005; NRC, 2011). In agreement, in a trial performed with European seabass juveniles (50 g), Pimentel-Rodrigues and Oliva-Teles (2007) reported that P digestibility in various FMs varied between 49 and 63%, whereas Basto et al. (2020) described P digestibility values of 73% and 90% for defatted BSFL and YM meals, respectively, in a trial with the same species (33 g).

Another factor that should be considered when exploring novel aquafeed ingredients is the impact on intestinal morphology and overall health. In the present study, the IM diets promoted similar morphological integrity of the anterior intestine to CTRL. However, fish fed with IM50 had 38% longer *villi* than the control, and there was a tendency towards higher cross-sectional perimeter and absorption area in the same fish. Although this could suggest that macronutrient digestibility in IM50 would be higher, this was not observed under the tested conditions. Feeding with the IM diets also reduced the submucosa thickness

by approximately 30%. The submucosa is a layer of connective tissue located between the mucosa and *muscularis*, providing support to a vascular network involved in nutrient absorption and inflammatory and immune responses (Ferreira et al., 2023; Li et al., 2020). Hence, the thickness of this layer may vary due to multiple factors, including cellularity (Basto-Silva et al., 2022). Randazzo et al. (2023) observed that the dietary replacement of 12% of a plant protein mixture (containing 39% soybean meal and 20% soy protein concentrate) with 8% BSFL meal reduced submucosal infiltrates in the distal intestine of European seabass. Similarly, Kumar et al. (2021) reported that dietary inclusion of BSFL meal ameliorated soybean meal-induced intestinal enteritis in rainbow trout. The chitin and lauric acid of IMs have been claimed to have anti-inflammatory potential (Henry et al., 2018; Randazzo et al., 2023), which could potentially explain the reduced submucosa thickness observed in fish fed with the IM diets. The histomorphological results obtained fall within the range previously reported for seabass juveniles (Pascon et al., 2021; Resende et al., 2022; Torrecillas et al., 2017). Despite relatively high coefficients of variation, especially for "absorption area" and "number of neutral/acid goblet cells", this variability is consistent with those studies, suggesting substantial biological variability among individuals. Increasing the number of biological replicates could address this in future studies, but these analyses are labour-intensive. In this connection, machine learning models have shown potential for standardising and accelerating histomorphological assessments (Oliveira et al., 2024), which may facilitate the analysis of larger sample sizes in future studies.

The dietary substitution of FM with BSFL or YM meals, which typically lack eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been shown to decrease the dietary n-3 long-chain polyunsaturated fatty acid content (Basto et al., 2021; Renna et al., 2017). In the present study, IM25 and IM50 were supplemented with additional salmon oil to meet the EPA and DHA requirements of European seabass (Houston, 2018). Hence, EPA and DHA levels were similar among the diets, and resulted in comparable levels in the muscle across experimental groups. Overall, the muscle total lipid and fatty acid (FA) content of IM-fed fish highly resembled those of the control group. Still, higher replacement of FM with the IM mixture resulted in higher lauric acid deposition: the muscle of IM25- and IM50-fed fish contained 3–4.5 times more lauric acid than the control. Similar observations have been reported with diets containing either BSFL meal or BSFL oil, increasing the deposition of this FA in Atlantic salmon (*Salmo salar*) (Radhakrishnan et al., 2024), gilthead seabream (Moutinho et al., 2024), Nile tilapia (*Oreochromis niloticus*) (Goda et al., 2024), rainbow trout (Renna et al., 2017), and Jian carp (*Cyprinus carpio*) muscle (Li et al., 2016). Lauric acid is known to have antimicrobial properties (Nitbani et al., 2022), which have also been observed in the lipidic fraction of BSFL (Franco et al., 2024). Moreover, its addition to pre-cooked minced fish products has been shown to reduce bacterial growth over storage (Pastoriza et al., 2002). Therefore, future studies ought to explore how increasing lauric acid levels in the muscle impact the microbial load of fish fillets over storage.

BSFL and YM meals have also been increasingly explored for their antioxidant capacity. Studies indicate that BSFL and YM meals and/or oils contain antioxidants, such as phenolic compounds, carotenoids and/or tocopherols, whose profile may vary with the rearing substrate (del Hierro et al., 2020; Martínez-Pineda et al., 2024; Morand-Laffargue et al., 2023). In the present study, these substrates included olive pomace, rich in hydroxytyrosol (Nunes et al., 2018), and wheat bran, rich in tocopherols (Zhou et al., 2004). For insight into whether potential antioxidants of the IM mixture could impact the antioxidant capacity of seabass muscle, the oxygen radical absorbance capacity (ORAC) assay (focused on the ability of antioxidants to scavenge peroxyl radicals – the main free radicals causing lipid oxidation in foods) was employed. A trend towards significance ($p = 0.055$) was observed for dietary impact, with IM25- and IM50-fed fish presenting the highest ORAC values. This may support the hypothesis that antioxidant compounds of the IM mixture are retained in the muscle, potentially enhancing shelf life and/or benefiting consumers' health (Mei et al., 2019; Radhakrishnan et al., 2024).

Despite the ORAC results, by the end of the trial, muscle lipid peroxidation (LPO) levels were similar among groups. Although there are no studies concerning the impact of YM meals on fish muscle antioxidant capacity or muscle LPO levels, Zarantoniello et al. (2023) showed that dietary inclusion of 1.2–8% BSFL meal to replace 3–20% of FM in a control diet for seabass juveniles with 40% FM did not affect muscle LPO levels. Contrarily, the inclusion of higher levels of BSFL meal – 19.5%, to replace 45% FM in a control diet with 32.4% FM – was shown to reduce LPO levels in the muscle of seabass juveniles (Moutinho et al., 2021). Moutinho et al. (2021) also reported that the diet with BSFL meal reduced the LPO rate over 3-day storage (2–4 °C). Although it is not clear whether this observation resulted from higher muscle antioxidant capacity, Radhakrishnan et al. (2024) reported that feeding Atlantic salmon with diets containing BSFL meal slightly increased β-tocopherol levels in the muscle. Hence, future work should focus on the identification of the antioxidants available in the IMs, the impact of the rearing substrate on their profile, and how this affects the muscle antioxidant capacity. Although the LPO values obtained in this study are consistent with those previously reported for European seabass (Filipa-Silva et al., 2023; Martins et al., 2024), the coefficients of variation were relatively high. This was likely due to biological variability, particularly given the low lipid content of seabass muscle (~ 3%, Table 7), which amplifies relative variation. Similar variability has been reported in European seabass (Albendea et al., 2023; Filipa-Silva et al., 2023) and other lean species, such as meagre (*Argyrosomus regius*) and flounder (*Paralichthys olivaceus*) (Gao et al., 2014; Monteiro et al., 2021), supporting this interpretation.

Summarily, this study evaluated the inclusion of a *H. illucens* and *T. molitor* larvae meal mixture in practical diets for European seabass juveniles. Previous studies had demonstrated that 50% FM replacement with BSFL meal, or full FM replacement with YM meal, can sustain seabass growth, although AA supplementation may be required depending on the replacement levels (Basto et al., 2021; Magalhães et al., 2017; Mastoraki et al., 2020). In our study, the diets were formulated to meet the EAA and FA requirements of the species – the latter achieved by adjusting fish oil levels in the IM diets. As a result, growth performance and muscle FA profiles were comparable across the experimental groups. Nonetheless, the inclusion of the IM mixture was associated with distinct physiological effects, likely attributable to specific components of the IMs. These included reduced faecal P losses in all IM-fed groups, increased lauric acid deposition in the muscle of fish fed IM25 and IM50, and a trend towards higher muscle antioxidant capacity in the IM50 group. These findings suggest that, beyond serving as a protein source, the IM mixture may provide functional benefits to diets for European seabass.

5. Conclusion

This study demonstrates the potential of a mixture of black soldier fly larva (BSFL) and yellow mealworm (YM) meals to partially substitute fishmeal (FM) in diets for European seabass. While the YM meal contributed with a higher protein and essential amino acid content to the feed, the BSFL meal provided lauric acid, which is increasingly recognised for its bioactive properties. The IM-containing diets ensured similar fish growth and feed efficiency to a commercial-like control diet with 15% FM. Additionally, faecal phosphorus (P) losses were reduced up to 27%, suggesting the production of lower levels of solid P waste. Fish intestinal morphology was also modulated, as submucosa thickness was reduced (potentially due to reduced inflammation), and, in the case of IM50-fed fish, *villi* were longer. Muscle nutritional quality was maintained, and the observed trend towards increased antioxidant capacity in the muscle of IM50-fed fish suggests the potential for longer fillet shelf life. The observed higher deposition of lauric acid could

potentially contribute to this, too, due to its antimicrobial properties.

CRediT authorship contribution statement

Rafaela S. Costa: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Ana Basto:** Writing – review & editing, Validation, Methodology. **Marta Monteiro:** Writing – review & editing, Validation, Methodology. **Bia Pinho:** Writing – review & editing, Methodology, Investigation. **Tiago Sá:** Writing – review & editing, Methodology, Investigation. **Marisa V. Santos:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Daniel Murta:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Johan W. Schrama:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Luisa M.P. Valente:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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