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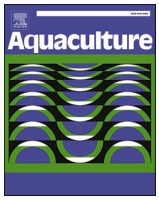

Towards sustainable aquaculture: Assessing polychaete meal (*Alitta virens*) as an effective fishmeal alternative in European seabass (*Dicentrarchus labrax*) diets

M. Monteiro [a], R.S. Costa [a], V. Sousa [a,b], A. Marques [a], T. Sá [a], L. Thoresen [c], S.A. Aldaghi [d], M. Costamagna [d], M. Perucca [d], K. Kousoulaki [c], L.M.P. Valente [a,b,*]

[a] *CIIMAR/CIMAR-LA, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal*

[b] *ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal*

[c] *Nofima, Nutrition and Feed Technology department, Kjerreidviken 16, 5141 Fyllingsdalen, Norway*

[d] *Project HUB-360, 10051 Avigliana, TO, Italy*

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ABSTRACT

In recent years, the pursuit of sustainable aquaculture practices has focused on alternatives to fisheries-derived ingredients, to avoid economic and environmental unsustainability. Low-trophic organisms, such as polychaetes, could be a suitable alternative to fishmeal due to their high protein content. This study aimed to investigate the feasibility of replacing fishmeal with the polychaete meal (*Alitta virens*; PM) in diets for European seabass (*Dicentrarchus labrax;* Wi, ~14.5 g). Four isoproteic (51% dry matter, DM) and isolipidic (17% DM) diets were fed to seabass juveniles: a fishmeal-based diet (FM) and three experimental diets with PM at inclusion levels of 2.5% (PM2.5), 5% (PM5), and 10% (PM10), replacing 10%, 20% and 40% of fishmeal, respectively. After 93 days of feeding, the effects of the PM on growth, digestibility, nutrient utilization, plasma metabolites, liver lipogenic activity, and anterior intestine histomorphology were analyzed. The environmental impact of all diets was also assessed. All diets were well-accepted by the fish, and promoted equal growth performance, ensuring high feed efficiency. The muscle EPA + DHA content in all dietary groups exceeded the recommended levels for human consumption. Hepatic lipogenesis and plasma triglyceride and non-esterified fatty acids (NEFA) levels were increased in fish fed PM2.5, suggesting that the PM promoted alterations in lipid metabolism, especially at low inclusion levels. PM2.5 also seemed to enhance the absorption capacity of the anterior intestine by increasing villus length and goblet cells number/area, albeit without impacting nutrient digestibility and growth performance. Furthermore, copper digestibility increased with higher levels of PM inclusion. The assessment of environmental sustainability indicated that the experimental diets incorporating PM present a more sustainable option compared to fishmeal. Overall, this study demonstrated that PM can serve as a sustainable alternative to fishmeal, replacing it by up to 40%, without compromising European seabass growth and nutrient utilization, while guaranteeing a high nutritional quality fillet for human consumption. The best environmental performance could be achieved with a diet comprising 5% to 10% (*w*/w) PM content. However, further research is needed to understand the underlying mechanisms of PM's effects on intermediary metabolism and its potential as a functional ingredient.

1. Introduction

Population growth and the increasing demand for high-quality fish products pose a current challenge to the aquaculture sector, urging the identification of sustainable practices to meet food demand. Fishmeal is considered a premium protein source in diets for aquaculture fish, and the high dependence on this feedstuff as the main protein source in aquafeeds has led to the overexploitation of marine resources and has

* Corresponding author at: CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal.
E-mail address: lvalente@icbas.up.pt (L.M.P. Valente).

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posed unbearable fluctuations in feed production costs (Naylor et al., 2021). Envisioning a more sustainable aquaculture development, recent efforts have been put forward to decrease the volume of unsustainable catches targeted for fishmeal production (from 30 Mt. to 16 Mt; FAO, 2022), which, in turn, has resulted in a lower supply of marine-harvested ingredients for aquafeed production. Coupled with the fast growth of the aquaculture industry, the limited supply of fishmeal has forced producers to use this commodity more strategically in new, balanced commercial diets that promote optimal fish growth and health. Thus, the feed industry was forced to explore multiple alternative protein sources, with plant-based feedstuffs being among the most used resources (Turchini et al., 2019).

For years, plant feedstuffs, such as soybean meal, have been the preferred alternative due to their high nutritional value, reasonable price and steady supply (Jia et al., 2022; Yang et al., 2011). However, these ingredients, especially when included at high levels, are associated with nutritional problems for carnivorous fish, such as low protein digestibility (Gaylord et al., 2008; Tacon and Metian, 2008), poor utilization of dietary nutrients (Adamidou et al., 2009), and an imbalance of amino acids (AAs) (Gaylord et al., 2010). Additionally, the armed conflict in Ukraine has had a significant impact on the supply of plant feedstuffs, resulting in major disruptions and substantial price increases (Psaltopoulos, 2022). Since the suitability and sustainability of vegetable ingredients in diets for carnivorous fish have been increasingly questioned (Colombo et al., 2022), other resources ought to be explored, including ingredients that do not compete directly with the human food supply. Therefore, promising candidates for inclusion in aquafeeds must fulfill several criteria. First, they need to meet fish nutritional requirements (Pelletier et al., 2018). Secondly, they must allow scaling up production to meet the increasing demand. Finally, they should be produced in environmentally, economically, and socially sustainable conditions to be competitive (Pelletier et al., 2018). In this sense, understanding nutrient digestibility is crucial for assessing new ingredients' potential environmental impact. By ensuring efficient nutrient absorption by fish and minimising waste release, particularly nitrogen and phosphorus into the aquatic environment, the overall sustainability and environmental impact of aquafeeds can be better managed (Chojnacka et al., 2021).

Low trophic marine species, like polychaetes, have emerged as potential ingredients for aquafeeds. These species have a well-balanced nutritional profile, characterized by high protein content and high levels of ω3 long-chain polyunsaturated fatty acids (LC-PUFA) (Jerónimo et al., 2021; Pan et al., 2021; Wang et al., 2019). Despite polychaete worms being commonly used as a supplement in maturation diets for shrimp (Chimsung, 2014) their potential as a novel protein source in aquafeeds is still relatively underexplored. Since there are a limited number of locations where marine annelid worms can be collected from and exploited in an economically and environmentally sustainable manner, large quantities of this biomass are very difficult to obtain, limiting their study as a protein source in fish. Only a few studies have examined the use of this ingredient, specifically looking at how it affects the growth performance of fish (Ahmad et al., 2021; Kals et al., 2017) Importantly, these studies have exclusively used biomass obtained from wild-caught specimens. However, the selective cultivation or co-cultivation of these organisms, either as the primary cultivated species or within integrated systems involving other species will allow to attain high biomass and reduce the heavy dependence on harvesting wild worms from their natural habitats (Wang et al., 2020). Recent research has focused on polychaete species such as *Nereis diversicolor* and *Alitta virens*, which can grow on aquaculture waste, efficiently handling sludge from land-based aquaculture and gaining valuable biomass while improving their nutritional value (Albrektsen et al., 2022; Wang et al., 2019). This advancement could pave the way for mass production of a valuable and novel feedstuff in aquafeeds, while also promoting a more aquaculture development.

The main goal of this study was to investigate the feasibility of utilizing *Alitta virens* as a substitute for high-quality fishmeal in diets for juvenile European seabass (*Dicentrarchus labrax*). The study focused on evaluating the effects of including polychaeta meal (PM) on growth performance, nutrient utilization, plasma biochemistry, lipid metabolism, muscle fatty acid composition, and anterior intestine morphology. Additionally, a life cycle assessment was conducted to evaluate the environmental implications of replacing fishmeal with increasing levels of PM.

2. Materials and methods

2.1. Ethical statement

This study was coordinated by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations), and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

2.2. Ingredient and experimental diets

A feeding trial and a digestibility trial were carried out with European seabass (*Dicentrarchus labrax*) juveniles. Four isoproteic (51% of dry matter, DM), isolipidic (17% DM), and isoenergetic (22 kJ g^{-1} DM) diets were formulated and extruded by Nofima (Norway), with a pellet size of 2.0 mm. A control diet (FM) was formulated to contain 25% of fishmeal. The remaining three diets incorporated 2.5% (PM2.5), 5% (PM5), and 10% (PM10) of spray-dried polychaete meal (PM – *Alitta virens*, from Topsy Baits, The Netherlands) replacing 10%, 20%, and 40% of fishmeal, respectively. The diets containing PM were supplemented with increasing levels of fish oil to achieve comparable levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Table 1). Yttrium oxide (Y) was added to the experimental diets (0.01% DM) to assess macronutrient, energy, and mineral digestibility. Proximate composition and mineral composition of the ingredient and diets are shown in Table 1. PM and diets fatty acid profile and amino acid profile are shown in Table 2.

2.3. Experimental conditions and feeding trial

Juvenile European seabass were transported from a commercial fish farm (Aquicultura Balear SAU, Spain) to the Fish Culture Experimental Unit of CIIMAR (Matosinhos, Portugal) and acclimated to the experimental conditions for 2 weeks, while feeding on a commercial diet (Aquasoja, Sorgal S.A.; 50% crude protein, 20% crude fat, as DM basis). Immediately before the trial, fish were lightly anesthetized with 2-phenoxyethanol (Sigma-Aldrich, MO, USA) at 60 μL L^{-1} for individual weighing (14.5 ± 1.0 g) and length determination (11.4 ± 0.3 cm). Then, 12 homogeneous groups of 40 fish were distributed among 160-L fiberglass tanks (3.6 kg m^{-3}) within a saltwater re-circulation system (22.0 ± 0.5 °C, 35‰ salinity, 10.6 L/ min flow rate and 12 L/12D photoperiod). Each diet was randomly assigned to triplicate groups of fish, which were fed until apparent satiation three times daily (9h, 12h30, and 16h30) for 13 weeks, using automatic feeders (Campos et al., 2019).

The feeding protocol was adjusted daily for each tank based on the presence or absence of uneaten feed after each meal. If all the feed dispensed by the automatic feeders was rapidly consumed, the daily total was increased by 5% the following day. Conversely, if some pellets were left uneaten, the daily quantity was reduced by 5% until no feed losses occurred. To calculate daily feed intake accurately, any remaining, uningested pellets were collected after each meal and weighed. Throughout the trial, nitrogenous compounds, salinity, temperature, dissolved oxygen, and pH were monitored and kept at levels recommended for this species (Blancheton, 2000).

Table 1
Experimental diets and proximate composition of the polychaete meal (*Alitta virens*) and diets.

Ingredient (%)	PM	FM	PM2.5	PM5	PM10
Fish meal LT [a]	–	25.0	22.5	20.0	15.0
Polychaete meal [b]	–	0.0	2.5	5.0	10.0
Wheat gluten [c]	–	5.04	5.19	5.44	6.04
Wheat [d]	–	6.0	5.9	5.7	5.2
Fish Oil [e]	–	9.2	9.4	9.6	10.0
Rapeseed oil [f]	–	2.8	2.4	2.0	1.3
SPC [g]	–	20.8	20.8	20.8	20.8
Horse beans [h]	–	14.0	14.0	14.0	14.0
Corn gluten [i]	–	7.50	7.50	7.50	7.50
Soybean meal [j]	–	5.0	5.0	5.0	5.0
Raps lecithin [k]	–	0.5	0.5	0.5	0.5
Choline chloride	–	0.5	0.5	0.5	0.5
Stay-C	–	0.05	0.05	0.05	0.05
Vitamin mix [l]	–	0.5	0.5	0.5	0.5
Mineral mix [m]	–	0.5	0.5	0.5	0.5
NaH_2PO_4	–	1.5	1.5	1.5	1.5
Lys (99%)	–	0.50	0.50	0.55	0.55
Methionine (99%)	–	0.20	0.25	0.25	0.25
Yttrium oxide	–	0.01	0.01	0.01	0.01
H_2O	–	0.40	0.50	0.60	0.80
Proximate composition					
Dry matter (%)	95.1	91.1	89.3	90.1	89.8
Ash (% DM)	16.4	8.9	8.7	8.6	8.5
Crude Protein (% DM)	65.5	51.8	50.7	51.3	51.0
Total Lipids (% DM)	10.9	16.6	16.5	16.5	16.6
Energy (kJ g^{-1} DM)	20.1	21.9	22.2	22.4	22.5
Mineral composition (mg 100 g^{-1} DM)					
Phosphorus	–	1545.7	1459.0	1452.2	1346.1
Iron	–	28.7	28.1	29.0	29.2
Copper	–	1.8	1.9	1.9	1.9
Manganese	–	7.0	6.7	6.5	6.6
Zinc	–	18.8	20.2	19.0	20.2
Selenium	–	0.12	0.10	0.16	0.10

[a] Pelagia AS, Bergen, Norway.
[b] Raw material from Topsy baits, Wilhelminadorp, The Netherlands, Processed for the current trial by Nofima AS, Bergen, Norway.
[c] Tereos SYRAL Belgium N.V., Aalst, Belgium.
[d] Norgesmøllene AS, Bergen, Norge.
[e] Pelagia AS, Bergen, Norway.
[f] EMMELEV, Otterup, Denmark.
[g] CJ Selecta S.A., Araguari MG, Brazil.
[h] Soufflet, Grand Est, France.
[i] Roquette Frères, Lestrem, France.
[j] Fiskå Mølle, Etne, Norway.
[k] Berg + Schmidt, Hamburg, Germany.
[l] Vilomix Norway AS, Hønefoss, Norway.
[m] Sigma-Aldrich Norway, Oslo, Norway.

2.4. Sampling

Prior to the start of the feeding trial and after a 24-h fasting period, 20 fish from the initial stock were sacrificed by anesthetic overdose with 2-phenoxyethanol (500 μL L^{-1}), and stored at −80 °C for initial whole-body composition analysis. After 6 weeks, an intermediate sampling was conducted: fish were lightly anesthetized as previously described, and bulk-weighed to monitor weight gain and feed consumption. At the end of the trial (93 days) and after a 24-h fasting period, all fish were lightly anesthetized for individual weighing (g) and length determination (cm). Blood from six fish per tank ($n = 18$) was collected from the caudal vein using heparinized syringes, and centrifuged (10 min, 5000 ×*g*) for plasma collection. Plasma aliquots were stored at −80 °C for metabolite quantification. Posteriorly, the same fish were sacrificed by a sharp blow on the head; liver and viscera were then weighed to determine the viscerosomatic and hepatosomatic indexes (VSI and HSI, respectively), and liver samples were stored at −80 °C for analysis of total lipids and the activity of lipogenesis-related enzymes. Dorsal muscle tissue was also collected for protein content, lipid content, and fatty acid analyses. A small part of the anterior intestine (right after the pyloric ceca) of 4 fish per tank was fixed in phosphate-buffered 4% formalin (pH 7) for 24 h and kept in 70% ethanol until further processing for histologic evaluation. Lastly, five fish per tank were stored at −80 °C for final whole-body composition analysis.

Table 2
Amino acid (% dry matter) profile and molecular weight distribution of the peptides in the soluble protein fraction (%) of the polychaete meal (*Alitta virens*) and experimental diets.

Amino acid	PM	FM	PM2.5	PM5	PM10
Essential amino acid (EAA)					
Arginine	3.8	3.2	3.0	3.1	3.1
Histidine	1.2	1.2	1.1	1.1	1.1
Lysine	3.8	3.5	3.3	3.3	3.2
Threonine	2.6	2.0	2.0	2.0	2.0
Isoleucine	2.5	2.4	2.3	2.4	2.4
Leucine	3.6	3.9	3.9	3.8	3.8
Valine	2.6	2.6	2.5	2.5	2.5
Methionine	1.7	1.7	1.6	1.6	1.6
Phenylalanine	2.6	2.9	2.8	2.9	2.9
ΣEAA	24.4	23.4	22.5	22.7	22.6
Non-essential amino acid (NEAA)					
Tyrosine	2.2	2.4	2.4	2.4	2.4
Alanine	4.5	2.5	2.5	2.5	2.5
Glycine	3.8	2.4	2.3	2.3	2.3
Proline	2.4	2.9	2.9	2.9	2.9
Serine	2.5	2.4	2.4	2.4	2.3
Cystine	0.80	0.86	0.86	0.86	0.87
Aspartate/asparagine	4.9	4.4	4.3	4.2	4.2
Glutamate/glutamine	7.3	8.6	8.8	8.6	8.7
Taurine	1.05	0.29	0.30	0.31	0.33
ΣNEAA	29.5	26.8	26.8	26.5	26.5
Σamino acids	53.7	50.1	49.2	49.2	49.1
Peptide (by molecular weight, Da)					
< 200	–	43.7	42.5	41.1	39.1
200–500	–	6.7	7.3	7.7	8.4
500–1000	–	2.4	2.6	2.8	3.2
1000–2000	–	2.8	3.2	3.6	4.3
2000–4000	–	4.3	4.8	5.3	6.3
4000–6000	–	5.7	5.8	6.0	6.3
6000–8000	–	6.8	6.7	6.6	6.6
8000–10,000	–	6.4	6.3	6.0	5.6
10,000–15,000	–	19.8	19.3	18.9	18.0
> 15,000	–	1.5	1.7	1.9	2.2

2.5. Digestibility trial

The digestibility trial was conducted immediately after the feeding trial, in a recirculating water system equipped with a battery of 12 fiberglass tanks of 50 L capacity, which were designed with a settling column connected to the outlet of each tank for faeces collection, according to Cho et al. (1982). Nine of the remaining fish from each tank (mean body weight 77.0 ± 0.5 g; initial density of 14 kg m^{-3}) were allocated to a new tank, but continued to be fed the same experimental diets as they were during the growth trial. Rearing conditions (temperature, salinity, dissolved oxygen, nitrogenous compound levels, and pH) were the same as described for the feeding trial. Prior to the start of faeces collection, a 15-day acclimation period was used for fish to adapt to the new system. To ensure consistent feed intake across all tanks, a predetermined quantity of feed was provided to each group of fish, 3 times daily, using automatic feeders. This approach aimed to maintain uniformity in the amount of feed consumed by the fish in every tank. At the end of the day, after the last meal, the tanks and settling columns were carefully cleaned to avoid contamination with feed leftovers. Faeces, in the settling column, were collected once daily (before the morning meal) during 21 days. Immediately after each daily collection, the faeces from each tank were centrifuged for 10 min at 7000 rpm (4 °C), and stored at −20 °C. *prior* to analysis, pooled faeces from each tank were freeze-dried, ground, and sifted.

2.6. Chemical analyses

2.6.1. Proximate composition of the ingredient, experimental diets, and faeces

Proximate composition analyses of PM, diets, whole fish (one pool per tank; $n = 3$) and faeces were performed according to AOAC (2006) methods, in duplicate. DM was quantified after drying in an oven at 105 °C for 24 h; ash was determined by combustion in a muffle furnace at 550 °C for 5 h; crude protein (CP; N × 6.25) was determined using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA); crude fat (CF) was quantified by petroleum ether extraction at 40–60 °C (Soxtec™ 2055, Foss, Höganäs, Sweden), and gross energy was measured in an adiabatic bomb calorimeter (IKA C2000-IKA-Werke GMBH & CO.KG, Staufen, Germany).

2.6.2. Mineral composition of the experimental diets

The content of macro- and microminerals (P, Zn, Fe, Se, Mn, Cu) in the diets and faeces was evaluated as described by Aspevik et al. (2021). Briefly, approximately 10 mg of sample were placed in a Teflon reactor vessel. Afterwards, 1 mL of HNO3 and 250 μL of H2O2 were added. The acid mineralization was conducted using high-pressure microwave digester irradiation (Ethos Easy, Milestone, Sorisole, Italy) at 800 W and 180 °C for 15 min. After treatment, the samples were left to cool at room temperature and filtered. Samples final volume was made up of 10 and 5 mL with distilled water, respectively. All solutions were diluted 1/100 before analysis.

The identification and quantification of minerals was performed using an inductively coupled plasma spectrometer mass detector (ICP-MS, Agilent model 7900). The operating conditions were as follows: carrier gas (1.0 L/min), Ar gas flow (15.0 L/min), RF power (1550 W), nebulizer pump speed (0.30 rps), and RF matching (1.80 V). Each mineral was quantified using a unique standard calibration curve and limits of detection (LOD) were calculated according to the following equation: LOD = 3sB/a, where 3sB is 3 times the standard deviation at zero concentration and a is the slope of the calibration curve. Distilled water was used as a blank and dried olive leaves (GSC-FOL/2018) were provided by the Spectroscopy Service of the University of Valencia and considered as a control sample for mineral content. It was analyzed in parallel to diet and faeces samples and blank to attest the accuracy of the method.

2.6.3. Amino acid composition of the ingredient and experimental diets

Amino acid analysis was performed according to Teodósio et al. (2022). Firstly, PM and the experimental diets were hydrolyzed with HCl 6 M for 72 h (116 °C), in nitrogen-flushed glass vials. Then, the samples were pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxy-succinimidyl carbamate), using the AccQ Tag method (Waters, USA). Ultra-high-performance liquid chromatography (UPLC) was performed using a Waters Reversed-Phase Amino Acid Analysis System, and norvaline as internal standard. Peaks were evaluated with EMPOWER software (Waters, USA). Tryptophan was not quantified, due to its partial loss during acid hydrolysis. Amino acid levels were expressed as % DM.

2.6.4. Total lipids and fatty acid profile

Total lipids were isolated and quantified as described by Folch et al. (1957), using chloroform in replacement of dichloromethane.

Fatty acid levels were quantified by gas chromatography. For this, direct acid transmethylation was performed as described by Parrish et al. (2015), with modifications. Briefly, PM, feed, and freeze-dried muscle samples (1 pool per tank) were milled and transferred (250–300 mg) to glass centrifuge tubes, to which 1 mL of internal standard solution (1 mg of tricosanoic acid/mL of toluene) and 3 mL of methylating solution were added. Then, the tubes were vortexed and heated at 100 °C for 1 h. After cooling, 1.8 mL of extraction solution with BHT (5 mg mL^{-1}) were added; the tubes were vortexed and centrifuged for collection of the fatty acid methyl esters (FAME – in the top, organic layer). This step was repeated twice (without the addition of BHT), and the organic phase was dried using anhydrous sodium sulfate. Lastly, the solvent was evaporated at 37 °C under a nitrogen stream, and FAME were transferred to vials with 1.5 mL of n-hexane. Samples were stored at −80 °C until analysis.

For FAME quantification, a Shimadzu Nexis GC-2030 gas chromatograph (GC, Kyoto, Japan) equipped with a flame-ionization detector (FID) and a Shimadzu AOC-20i auto-injector was used. Separation was carried out on a OmegaWax 250 capillary column (30 m × 0.25 mm I.D., film thickness of 0.25 μm), and operating conditions were as follows: split mode, with a split ratio of 1:50 and an injection volume of 1 μL. The injector and detector temperatures were kept at 250 °C and 280 °C, respectively. A flow rate of 25 mL/ min of helium as a carrier gas, 40 mL/ min of hydrogen, and 400 mL/ min of air was provided. The column thermal gradient was as follows: initial temperature of 50 °C for 2 min, which was increased at 50 °C min^{-1} to 174 °C, hold for 14 min, then increased 2 °C min^{-1} to 210 °C, hold for 50 min. FAME were identified by comparison with a known standard mixture (Sigma 47,885-U Supelco 37 Component FAME Mix, USA) and quantified using the software GCsolution for GC systems (Shimadzu, Kyoto, Japan). PM, feed, and muscle fatty acid levels were calculated using the internal standard as reference, and expressed as % DM or g 100 g^{-1} muscle wet weight.

2.7. Activity of lipogenesis-related hepatic enzymes

The activity of lipogenesis-related enzymes was determined as described by Monteiro et al. (2018). After liver homogenization in ice-cold buffer [100 mM Tris-HCl, 0.1 M EDTA, 0.1% (*V*/V) Triton X-100, 50 mM PMSF], samples were centrifuged for 20 min at 30000 ×*g* (4 °C), and the supernatant was stored at −80 °C until analysis. Prior to enzymatic activity assessment, the soluble protein content of the supernatant was determined according to (Bradford, 1976), using bovine ɣ-globulin as standard. Glucose-6-phosphate dehydrogenase (G6PD) activity was quantified using a reaction mixture of 1 M Tris-HCl (pH 7.8), 200 mM MgCl2, and 10 mM NADP; 20 mM glucose-6-phosphate was used as substrate solution. Malic enzyme (ME) activity was determined using the reaction mixture of 0.5 M glycylglycine (pH 7.4), 50 mM $MnCl_2$ and 10 mM NADP; 15 mM L-malate was used as substrate solution. Fatty acid synthase (FAS) activity was assessed with a reaction mixture of 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM NADPH, and 25 μM acetyl-CoA was used; 600 μM malonyl-CoA was used as substrate solution. Enzymatic activity was determined at 37 °C and absorbance was monitored over time in a microplate reader (BioTek Synergy™ HTX, Vermont, USA). Enzymatic activity values were expressed as milliunits of enzyme per mg of soluble hepatic protein, where one unit (U) is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per minute, at assay temperature.

2.8. Plasma metabolites

Plasma total protein, triglycerides, cholesterol, and non-esterified fatty acid (NEFA) levels were determined spectrophotometrically, using the following commercial kits: 1001291, 1,001,312, 1,001,090 (Spinreact, Spain) and 434–91,795 NEFA-HR(2) (Wako Chemicals, Germany), respectively. Absorbance was measured according to the instructions of the manufacturer, with adaptations for microplate readings (BioTek Synergy™ HTX, Vermont, USA).

2.9. Anterior intestine histology

After fixation, anterior intestine samples of four fish per tank ($n = 12$) were further processed according to standard histological procedures, embedded in paraffin and cut into 3-μm sections using a semi-automated rotary microtome (Leica RM 2245, Leica Biosystems, Nussloch, Germany). Samples were stained with specific Alcian blue/

PAS (pH 2.5) and observed under a light microscope (Olympus BX51; Olympus, Tokyo, Japan) with a camera (Olympus DP50; Olympus) for the measurement of the cross-sectional perimeter (mm), muscularis thickness (μm), submucosa width (μm), lamina propria width (μm), absorption area (mm^2), and villus length (μm) using an imaging software (Olympus® cellSens Standard 2.2) following procedures previously described in detail by Ferreira et al. (2023). Briefly, villus length was determined in the longest eight villi, following the villus curves from the tip to the base. Additionally, three measurements of the lamina propria width were made along each of these villi (one at the base, one in the middle and one at the villus apex) to determine the mean lamina propria width. Muscularis thickness and submucosa widths were determined as follows: muscularis thickness = radius of the section – radius of the section without muscularis; submucosa width = radius of the section – radius of the section without muscularis and submucosa. Quantification of neutral (magenta) and acid (blue) goblet cells was performed automatically by the imaging software, based on the color of the cells.

2.10. Life cycle assessment

2.10.1. LCA software and database

LCA analysis of products, processes, or services over their entire life cycle is a methodology for quantifying their environmental performance. In this study, a free and open-source tool for LCA modelling, OpenLCA software, has been utilized. The Ecoinvent v3.7 database, which offers a comprehensive compilation of life cycle inventory data for various materials and processes, served as the primary LCA database for this study.

2.10.2. Goal and scope definition

Based on the ISO 14040-44 standard for LCA studies, the LCA analysis in this study was carried out using the recommended framework (ISO 14040, 2006; ISO 14044, 2006). The system boundary for the analysis included all the stages of the formulated diet production, according to the "from cradle to gate" approach for producing 100 kg of each diet as the Technical Unit (TU). According to this approach all the mass-energy upstream flows have been collected and used in modelling.

2.10.3. Inventory analysis

Utilizing the ecoinvent database version 3.7, the inventory analysis in this study involved identifying and quantifying the materials and energy inputs and outputs associated with each step of diet production. The composition of ingredients used in the experimental diets (Table 1), and their respective percentages were utilized in the LCA analysis conducted in this study.

2.10.4. Impact assessment

The CML 2001 method was chosen to convert the data into environmental impact categories. This method allows for the assessment of a comprehensive range of impact potentials or impact categories (Guinee et al., 2002), for assessing the environmental burden associated to different effects. The output results of this analysis were based on the evaluation of producing 100 kg of diet formulation, which was defined as Technical Unit. This approach enabled the identification of the environmental burden associated with the production of a unit mass of fishmeal based diet (FM) formulation for the four compared diets. Nevertheless, in accordance with the Goal and scope definition of the LCA framework, a suitable Functional Unit of the system was defined. The effective nutritional value of the diets was chosen as the representative functionality of the different diet formulation. This concept incorporates the feeding process efficiency, which accounts for the diet attractiveness, feed intake, digestibility and bioconversion. In line with these considerations, the Nitrogen retention rate (%DN) was selected as a quantitative indicator, measured directly to represent the effective nutritional efficiency.

In line with the definition of the Functional unit the environmental impact of each diet formulation was referred to the associated "N retention efficiency, % DN". Furthermore, to assess the overall environmental impact and make a direct comparison of the four diet formulations the environmental relative performance indicator of the impact categories' average value (ERPI<ICs>) was calculated. First of all, the highest value of each impact category among all diet formulations was found and set as the reference maximum impact in the selected category (100%). In the same category all diet formulations impacts were compared to the reference one, obtaining a relative (dimensionless) performance indicator. At the end, the final ERPI<ICs> is calculated by averaging the relative impact values of all the categories in order to have a representative integral indicator of each diet environmental performance. In order to discriminate the technical performance from the functional performance of the considered diet formulations, this indicator was determined both for the results obtained for the TU and FU. Based on the uncertainty of the diets' key performance indicator values, the uncertainty propagation for the ERPI<ICs> associated to the FU was calculated and represented in Fig. 1.

2.11. Calculations

Growth performance and feed efficiency parameters were calculated as follows, where Wi and Wf are the fish initial and final weights (g), respectively, and ABW is the average body weight [(Wi + Wf) / 2, g]: Final condition factor (K) = Wf / (final length)^3 × 100; Daily Growth Index (DGI, g day^{-1}) = ($Wf^{1/3}$ − $Wi^{1/3}$) / days × 100; Voluntary Feed Intake (VFI, % day^{-1}) = feed intake / ABW / days × 100; Feed Conversion Ratio (FCR) = dry feed intake / (Wf − Wi); Protein Efficiency Ratio (PER) = (Wf − Wi) / protein intake per fish; nutrient or energy intake (g or kJ kg ABW^{-1} day^{-1}) = DM intake × dry nutrient (or energy) content in diet; nutrient or energy gain (g or kJ kg ABW^{-1} day^{-1}) = [Wf × final nutrient (or energy) composition of carcass (% wet weight) – Wi × initial nutrient (or energy) composition of carcass (% wet weight)] / ABW / days; nutrient or energy retention (% feed intake) = [Wf × final nutrient (or energy) composition of carcass (% wet weight) – Wi × initial nutrient (or energy) composition of carcass (% wet weight)] / dry matter intake × dry nutrient (or energy) content in diet. Somatic indices were also calculated, to assess animal condition at the end of the feeding trial: Hepatosomatic Index (HSI, %) = liver weight / Wf × 100; Viscerosomatic Index (VSI, %) = viscera weight / Wf × 100.

The apparent digestibility coefficients (ADC) of the experimental diets were calculated as: ADC of DM (%) = (1 – dietary Y level / faeces Y level) × 100; nutrient, energy or mineral ADC (%) = [1 – (dietary Y level / faeces Y level) × (faeces nutrient level / dietary nutrient level)] × 100. Calculations for nutrient and energy balance were performed as: digestible nutrient intake (g kg ABW^{-1} d^{-1}) = crude nutrient intake (g kg ABW^{-1} d^{-1}) × nutrient ADC; digestible energy intake (kJ kg ABW^{-1} d^{-1}) = crude energy intake (kJ kg ABW^{-1} d^{-1}) – fecal energy losses (kJ kg ABW^{-1} d^{-1}); nutrient or energy retention efficiency (% digestible intake) = nutrient (or energy) gain / digestible nutrient (or energy) intake × 100; total nutrient or energy losses (g or kJ kg ABW^{-1} d^{-1}) = nutrient (or energy) intake – nutrient (or energy) gain; fecal nutrient losses (g kg ABW^{-1} d^{-1}) = crude nutrient intake (g kg ABW^{-1} d^{-1}) × (1 – nutrient ADC/100); fecal energy losses (kJ kg ABW^{-1} d^{-1}) = crude energy intake (kJ kg ABW^{-1} d^{-1}) × (1 – energy ADC/100); metabolic nutrient losses (g kg ABW^{-1} d^{-1}) = digestible nutrient intake – nutrient gain; metabolizable energy (kJ kg ABW^{-1} d^{-1}) = digestible energy intake – metabolic energy loss; branchial and urinary energy losses (kJ kg ABW^{-1} d^{-1}) = metabolizable nitrogen losses × 24.9 kJ / digestible nitrogen; total heat production (kJ kg ABW^{-1} d^{-1}) = metabolizable energy – energy intake.

2.12. Statistical analysis

All data were tested for normality and homogeneity of variances

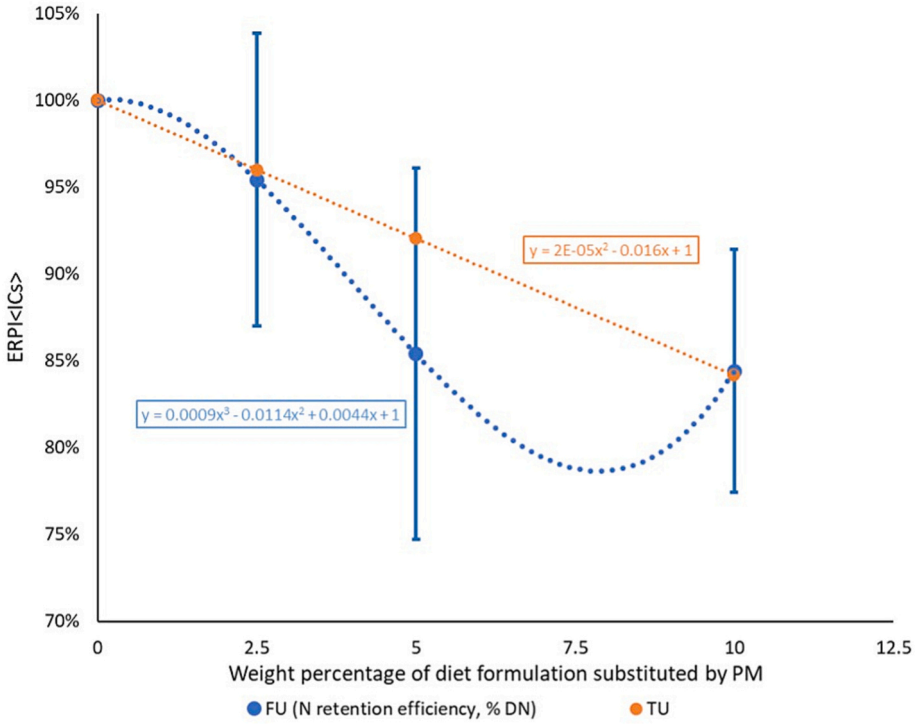

Fig. 1. Environmental relative performance indicator of the impact categories' average value (ERPI<ICs>) of the diets represent in percentage (%) based on the result of the 93-days growth trial result.

using Kolmogorov-Smirnov and Levene's test, respectively, and, if necessary, appropriately transformed. In addition, one-way analysis of variance (ANOVA) was performed to determine statistically significant differences among dietary treatments. When this test showed significance, individual means were compared using the Tukey HSD test. When ANOVA criteria were not fulfilled, data were submitted to the nonparametric test Kruskal-Wallis. Significant differences were considered when $P < 0.05$. All statistical analyses were performed using IBM SPSS, 28.0 (IBM Corp, 2021).

3. Results

3.1. Polychaete meal and experimental diet characterization

The PM had a crude protein content of 65.5% on a dry matter basis (DM), and its crude fat content was 10.9% DM. Energy content consisted of 20.1 kJ g^{-1} (Table 1) while ash content represented 16.4% DM. Total amino acids accounted for 53.7% of DM. Among them, 45.3% were essential amino acids (EAA), with arginine and lysine being the most abundant, followed by leucine. Glutamate/glutamine and aspartate/asparagine were the most abundant non-essential amino acids (NEAA). The most prevalent saturated fatty acid (SFA) was C16:0 (palmitic acid), while C18:1 n-9 (oleic acid, OA) was the most abundant monounsaturated fatty acid (MUFA). The most abundant polyunsaturated fatty acids (PUFA) were C18:2 n-6 (linoleic acid, LA) and C20:5 n-3 (EPA). EPA and DHA comprised 0.7% and 0.1% of DM, respectively. Additionally, fatty acids typically found in marine invertebrates, such as 18:1 n-13 and non-methylene-interrupted (NMI) C20 and C22 were detected in the diets at low levels (<1%), although the latter were only detected with ≥5% PM inclusion. The inclusion of PM in the diets resulted in increasing content of peptides with molecular weights (MW) ranging from 200 to 6000 Da. Conversely, the FM diet showed a higher abundance of peptides with MW ranging from 6000 to 15,000 Da. However, peptides exceeding 15,000 Da were predominantly found in the PM10 diet, while those below 200 Da were more abundant in the FM diet. The amino acid profile was very similar across diets, with EAA comprising 45.8–46.7% of the total amino acids. Regarding the lipid profile, the FM diet exhibited higher levels of neutral lipids, particularly triglycerides, indicating a greater concentration of non-polar lipids. Additionally, PM5 diet showed the highest levels of free fatty acids, while the PM10 diet exhibited the highest cholesterol levels. Fatty acid composition was very similar among all diets, with MUFA representing the largest fraction of total fatty acids (47.5–49.5%), while SFA and PUFA represented 18.9–19.9% and 27.8–28.2% of total fatty acids, respectively. EPA + DHA levels remained similar among the diets (10% total fatty acids, representing 1.3% DM). The amino acid and peptide profiles of the experimental diets are shown in Table 2, while the lipid and fatty acid profiles are shown in Table 3.

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

All experimental diets were well-accepted by the European seabass juveniles and mortality throughout the feeding trial was very low (< 1%) and similar among treatments. After 93 days, all groups of fish had quintupled their initial body weight, reaching an average of 77.9 g (Table 4). Growth performance and feed utilization parameters, including FCR, DGI, VFI, and PER, were not significantly affected by the dietary inclusion of PM (Table 4). Likewise, HSI and VSI were similar among groups, although fish fed PM5 exhibited the highest numerical HSI (1.89%) and VSI (6.64%) (Table 4).

Nutrient retention and gain were not significantly affected by the dietary treatments, but PM5-fed fish showed the highest lipid and protein retention, resulting in the highest lipid, protein and energy gain (142.6 g kg ABW^{-1} day^{-1}) (data not shown). As a result of similar nutrient utilization, whole-body composition was similar across the groups (Table 4). Albeit not statistically significant, protein, lipid and energy content of fish fed PM5 were the highest among the experimental groups.

Table 3
Lipid and fatty acid profile of the experimental diets (g^{-1} 100 g lipids) and LC-PUFA (%DM).

	PM	FM	PM 2.5	PM 5	PM 10
Neutral lipids	–	95.0	89.0	91.0	92.0
Polar lipids	–	2.4	1.8	2.0	2.3
Triglycerides	–	93.0	86.0	88.0	89.0
Free fatty acids	–	1.6	1.5	1.8	1.1
Cholesterol	–	1.1	1.1	1.2	1.4
Phosphatidylcholine	–	2.4	1.8	2.0	2.3
Fatty acids					
C14:0	0.6	4.5	4.7	4.7	4.8
C16:0	12.7	12.0	12.3	12.2	12.6
C18:0	3.6	1.6	1.6	1.6	1.6
ΣSFA	18.0	19.6	19.9	19.6	18.9
C16:1 n-7	2.6	3.4	3.5	3.5	3.6
C18:1 n-9 (OA)	13.2	22.0	21.0	20.3	18.9
C18:1 n-7	4.2	1.9	1.9	1.9	1.8
C18:1n-13	4.8	–	0.1	0.2	0.3
C20:1 n-9	2.8	8.4	8.5	8.6	8.7
ΣMUFA	26.2	49.5	49.0	48.7	47.5
C18:2 n-6 (LA)	8.7	11.6	11.4	11.1	11.2
C18:3 n-3 (ALA)	1.7	2.4	2.3	2.2	2.1
C18:4 n-3	0.05	1.7	1.8	1.8	1.8
C20 NMI	1.8	–	–	–	0.1
C20:4 n-3	0.2	0.4	0.4	0.4	0.4
C20:5 n-3 (EPA)	9.0	4.7	4.8	5.0	5.2
C22:5 n-3	1.5	0.4	0.4	0.5	0.5
C22:6 n-3 (DHA)	1.1	5.4	5.4	5.2	5.2
C22 NMI	5.4	–	–	0.2	0.4
ΣPUFA	33.6	27.9	27.9	27.8	28.2
Σ n-3	14.3	15.2	15.2	15.2	15.3
Σ n-6	18.3	12.1	12.1	11.9	12.2
Σ n-3/Σ n-6	0.8	1.2	1.3	1.3	1.3
LC-PUFA (%DM)					
EPA	0.7	0.6	0.6	0.7	0.6
DHA	0.1	0.7	0.7	0.7	0.6
EPA + DHA	0.8	1.3	1.3	1.3	1.3

∑SFA is the sum of saturated fatty acids, which includes C14:0, C16:0, C18:0, C20:0, and C22:0; ∑MUFA is the sum of monounsaturated fatty acids: C16:1n-7, C18:1n-9, C18:1n-7, C18:1n-5, C20:1n-9, C20:1n-7, C22:1n-11, C22:1n-9, C22:1n-7, and C24:1n-9; ∑PUFA is the sum of polyunsaturated fatty acids: C16:2n-4, C16:3n-4; C18:2n-6, C18:3n-3, C18:3n-6, C18:4n-3, C20:2n-6, C20:3n-3, C20:3n-6, C20:4n-3, C20:4n-6, C20:5n-3, C21:5n-3, C22:4n-6, C22:5n-3, and C22:6n-3; ∑n-3 is the sum of n-3 PUFA; ∑n-6 is the sum of n-6 PUFA.

3.3. Apparent digestibility of the experimental diets and fish nutrient balance

As shown in Table 5, the ADC of the macronutrients and energy in the experimental diets was very similar. This resulted in similar nitrogen and energy balances among all fish groups. It was, however, noted that fish fed PM5 exhibited the highest efficiency in retaining N and E (Table 5). Nevertheless, mineral digestibility showed significant differences among diets, specifically concerning copper that had a significantly higher ADC value in fish fed PM10 (79.6%) compared to those fed the FM (73.4%) diet.

3.4. Muscle proximate composition and fatty acid profile

Muscle protein content was not significantly affected by dietary inclusion of PM, representing, on average, 20% of the fish wet weight (Table 6). Likewise, there were no significant differences regarding muscle total lipid content. Still, the highest muscle lipid content was observed for fish fed PM5 (4.5% of the wet weight), and the lowest for fish fed PM10 (3.5%) (Table 6).

Overall, muscle fatty acid composition remained similar among dietary groups (Table 6), reflecting the dietary fatty acid profile. However, there was a significant decrease in OA in fish fed PM10 compared to those fed the FM diet. Moreover, a concurrent decrease in alpha-linolenic acid (ALA) was observed in all fish groups fed diets with increasing PM. Still, EPA and DHA levels in the muscle remained similar, reaching an average of 160 mg and 240 mg per 100 g of muscle, respectively. Fatty acids such as 5-octadecenoic (C18:1 n-13) were detected in the muscle of fish fed PM5 and PM10, increasing significantly in a dose-response manner. In contrast, NMI 22C was only detected in fish fed PM10.

3.5. Hepatic lipogenesis and liver lipid content

The activity levels of ME, G6PD, and FAS in the liver of the European seabass fed the experimental diets are shown in Fig. 2. Although no significant differences could be perceived in ME activity, the lowest activity was registered in fish fed PM5 (9.46 mU mg $protein^{-1}$). G6PD activity was significantly lower in fish fed PM5 (65.32 mU mg $protein^{-1}$) compared to those fed FM (81.60 mU mg $protein^{-1}$). Furthermore, FAS activity was significantly lower in fish fed FM (8.42 mU mg $protein^{-1}$)

Table 4
Growth performance, feed utilization, and somatic indexes of European seabass fed the experimental diets for 93 days. Values are the mean ± SD.

Growth Performance	FM	PM2.5	PM5	PM10	*p*-value
Initial body weight (g)	14.47 ± 0.03	14.52 ± 0.01	14.49 ± 0.02	14.52 ± 0.01	1.000
Final body weight (g)	76.67 ± 0.23	78.07 ± 1.60	79.10 ± 3.80	77.77 ± 3.01	0.374
Weight gain (g)	62.19 ± 0.26	63.55 ± 1.61	64.61 ± 3.79	63.25 ± 3.02	0.359
Final K	1.17 ± 0.03	1.21 ± 0.01	1.19 ± 0.04	1.19 ± 0.01	0.558
DGI (g day^{-1})	1.95 ± 0.01	1.97 ± 0.03	1.99 ± 0.07	1.97 ± 0.06	0.357
VFI (% day^{-1})	1.54 ± 0.07	1.54 ± 0.03	1.55 ± 0.03	1.52 ± 0.07	0.333
FCR	1.05 ± 0.04	1.05 ± 0.02	1.04 ± 0.01	1.03 ± 0.03	0.327
PER	1.84 ± 0.07	1.89 ± 0.03	1.87 ± 0.02	1.90 ± 0.06	0.617
Somatic Indexes					
HSI (%)	1.69 ± 0.16	1.79 ± 0.10	1.89 ± 0.11	1.63 ± 0.10	0.188
VSI (%)	6.43 ± 0.27	6.44 ± 0.58	6.64 ± 0.20	6.58 ± 0.96	0.913
WBC (% wet weight)					
Dry matter	33.4 ± 0.6	33.3 ± 1.0	35.1 ± 0.4	33.8 ± 1.7	0.224
Protein	17.1 ± 1.4	16.9 ± 0.7	17.9 ± 1.3	16.8 ± 0.3	0.533
Lipids	12.8 ± 0.9	13.3 ± 1.3	14.7 ± 0.2	13.4 ± 2.0	0.337
Energy (kJ g^{-1})	8.6 ± 0.1	8.6 ± 0.5	9.1 ± 0.2	8.9 ± 0.4	0.268
Ash	3.2 ± 0.1	3.0 ± 0.2	3.0 ± 0.3	2.9 ± 0.2	0.426

K – Condition factor; DGI – Daily Growth Index; VFI – Voluntary Feed Intake; FCR – Feed Conversion Ratio; PER – Protein Efficiency Ratio; HSI – Hepatosomatic Index; VSI – Viscerosomatic Index.
Initial WBC: Dry matter (%) – 28.3; Protein (% wet weight) – 16.0; Lipids (% wet weight) – 9.26; Energy (kJ g^{-1}) – 7.02.

Table 5
Apparent digestibility coefficients (ADC, %) of the macronutrients, energy, and minerals in the experimental diets, and nutrient balances of European seabass fed the experimental diets for 93 days. Values are the mean ± SD ($n = 3$).

ADC (%)	FM	PM2.5	PM5	PM10	p-value
Dry matter	80.7 ± 1.2	81.8 ± 1.4	80.2 ± 1.9	84.2 ± 3.6	0.208
Crude protein	95.7 ± 0.2	95.9 ± 0.1	95.8 ± 0.1	96.6 ± 0.8	0.083
Crude fat	98.7 ± 0.2	98.7 ± 0.2	98.6 ± 0.1	98.7 ± 0.2	0.864
Gross energy	91.2 ± 0.8	90.7 ± 0.6	90.5 ± 1.4	91.3 ± 0.1	0.631
Phosphorous	61.5 ± 6.0	59.1 ± 1.0	57.8 ± 3.8	60.0 ± 5.7	0.779
Iron	21.6 ± 1.2	22.3 ± 4.4	14.4 ± 5.0	28.3 ± 12.2	0.320
Copper	73.4 ± 2.1^{b}	78.1 ± 2.4^{ab}	77.1 ± 2.5^{ab}	79.6 ± 1.3^{a}	0.035
Manganese	45.0 ± 5.3	45.4 ± 6.3	40.3 ± 5.3	52.1 ± 6.3	0.182
Nitrogen (N) balance (g kg ABW^{-1} day^{-1})					
Digestible N intake (DN)	1.22 ± 0.05	1.20 ± 0.02	1.21 ± 0.03	1.20 ± 0.05	0.887
N gain	0.41 ± 0.04	0.40 ± 0.02	0.44 ± 0.04	0.40 ± 0.01	0.492
N retention efficiency, % DN	33.4 ± 2.0	33.6 ± 2.0	36.0 ± 3.2	33.3 ± 1.2	0.461
Fecal N losses	0.055 ± 0.005	0.052 ± 0.000	0.054 ± 0.002	0.042 ± 0.012	0.104
Metabolic N losses	0.81 ± 0.01	0.80 ± 0.03	0.78 ± 0.03	0.80 ± 0.04	0.611
Total N losses	0.87 ± 0.02	0.85 ± 0.03	0.83 ± 0.04	0.84 ± 0.05	0.803
Energy (E) balance (kJ kg ABW^{-1} day^{-1})					
Digestible E intake (DE)	306.5 ± 12.3	310.0 ± 5.0	312.7 ± 10.7	312.5 ± 14.3	0.890
E retention efficiency, % DE	42.9 ± 2.1	42.8 ± 3.6	45.6 ± 0.9	43.8 ± 2.1	0.482
Metabolizable E	286.3 ± 12.1	290.2 ± 4.8	293.4 ± 10.9	292.6 ± 13.3	0.851
Fecal E losses	29.8 ± 3.3	31.8 ± 2.2	32.7 ± 4.1	29.8 ± 1.4	0.544
Branchial + urinary E losses	20.2 ± 0.2	19.8 ± 0.7	19.3 ± 0.9	19.9 ± 1.0	0.611
Total E losses	204.8 ± 15.1	209.2 ± 14.5	202.9 ± 3.7	205.6 ± 11.6	0.931
Total heat production	154.9 ± 13.2	157.6 ± 12.1	150.8 ± 8.2	155.9 ± 9.9	0.890

Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).

than in fish fed PM2.5 (11.32 mU mg protein^{-1}). No significant differences were found between these groups and fish fed PM5 or PM10. Even though significant alterations in lipogenic activity were observed among the dietary groups, the overall levels of liver total lipid remained unaffected, accounting for on average of 31.7% of the liver weight (Fig. 2).

3.6. Plasma metabolites

Plasma cholesterol, triglycerides, NEFA, and protein levels of European seabass fed the experimental diets are depicted in Fig. 3. Cholesterol levels showed no significant variation among the fish groups, ranging between 366.9 and 384.6 mg dL^{-1} for fish fed FM, PM2.5 and

Table 6
Protein, lipids (g 100 g^{-1} wet weight), fatty acid (g 100 g^{-1} lipids) and LC- PUFA (%WW) composition of the dorsal muscle of European seabass fed the experimental diets for 93 days Values are the mean ± SD (n = 3).

	FM	PM2.5	PM5	PM10	p-value
Protein	20.7 ± 0.9	20.1 ± 0.5	19.7 ± 0.2	20.6 ± 1.0	0.239
Lipids	4.0 ± 0.2	3.8 ± 0.3	4.5 ± 0.8	3.5 ± 0.7	0.353
Fatty acids					
C14:0	3.3 ± 0.05	3.3 ± 0.05	3.4 ± 0.2	3.4 ± 0.1	0.363
C16:0	16.9 ± 0.2	17.2 ± 0.3	17.3 ± 0.2	17.6 ± 0.5	0.147
C18:0	3.1 ± 0.1	3.0 ± 0.04	3.1 ± 0.1	3.1 ± 0.2	0.528
ΣSFA	24.0 ± 0.2	24.2 ± 0.3	24.5 ± 0.4	24.8 ± 0.6	0.153
C16:1 n-7	3.7 ± 0.1	3.8 ± 0.2	3.8 ± 0.1	3.8 ± 0.2	0.792
C18:1 n-9 (OA)	25.7 ± 0.2 ^{a}	25.0 ± 0.9 ^{ab}	24.7 ± 0.1 ^{ab}	22.9 ± 1.3 ^{b}	0.025
C18:1 n-7	2.0 ± 0.01	2.0 ± 0.01	2.01 ± 0.01	1.99 ± 0.04	0.230
C18:1 n-13	–	–	0.11 ± 0.001^{b}	0.22 ± 0.003^{a}	<0.001
C20:1 n-9	5.9 ± 0.07	6.1 ± 0.05	6.0 ± 0.07	6.1 ± 0.20	0.152
ΣMUFA	45.0 ± 0.3	44.8 ± 1.1	44.1 ± 1.0	42.7 ± 1.9	0.187
C18:2 n-6 (LA)	9.0 ± 0.04	8.7 ± 0.18	8.8 ± 0.12	8.6 ± 0.41	0.291
C18:3 n-3 (ALA)	1.8 ± 0.02 ^{a}	1.7 ± 0.02 ^{b}	1.7 ± 0.01 ^{b}	1.5 ± 0.07 ^{c}	<0.001
C18:4 n-3	1.1 ± 0.01	1.1 ± 0.02	1.12 ± 0.04	1.13 ± 0.03	0.451
C20:5 n-3 (EPA)	5.0 ± 0.03	5.0 ± 0.2	5.2 ± 0.2	5.6 ± 0.4	0.128
C22:5 n-3	0.6 ± 0.01	0.6 ± 0.04	0.6 ± 0.05	0.6 ± 0.07	0.240
C22:6 n-3 (DHA)	8.2 ± 0.1	8.0 ± 0.8	8.2 ± 0.8	8.6 ± 1.2	0.887
C22 NMI	–	–	–	0.2 ± 0.004	n/a
ΣPUFA	27.4 ± 0.2	27.2 ± 1.4	27.8 ± 1.3	28.5 ± 2.2	0.726
Σn-3	16.9 ± 0.2	16.9 ± 1.2	17.3 ± 1.1	17.9 ± 1.8	0.699
Σn-6	10.1 ± 0.005	9.9 ± 0.2	10.1 ± 0.2	10.2 ± 0.5	0.778
Σn-3/ Σn-6	1.7 ± 0.02	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	0.626
LC-PUFA (%WW)					
EPA	0.15 ± 0.01	0.16 ± 0.02	0.16 ± 0.03	0.16 ± 0.03	0.964
DHA	0.25 ± 0.02	0.24 ± 0.03	0.24 ± 0.04	0.24 ± 0.03	0.99
EPA + DHA	0.40 ± 0.03	0.40 ± 0.04	0.40 ± 0.07	0.41 ± 0.06	0.996

∑SFA is the sum of saturated fatty acids, which includes C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0; ∑MUFA is the sum of monounsaturated fatty acids: C14:1, C16:1n-7, C17:1n-7, C18:1n-9, C18:1n-7, C20:1n-9, C22:1n-9, C22:1n-11, C24:1n-9; ∑PUFA is the sum of polyunsaturated fatty acids: C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-6, C18:3n-3, C18:3n-6, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-3, C20:4n-6, C20:5n-3, C22:5n-6, C22:5n-3, C22:6n-3; ∑n-3 is the sum of n-3 PUFA; ∑n-6 is the sum of n-6 PUFA.

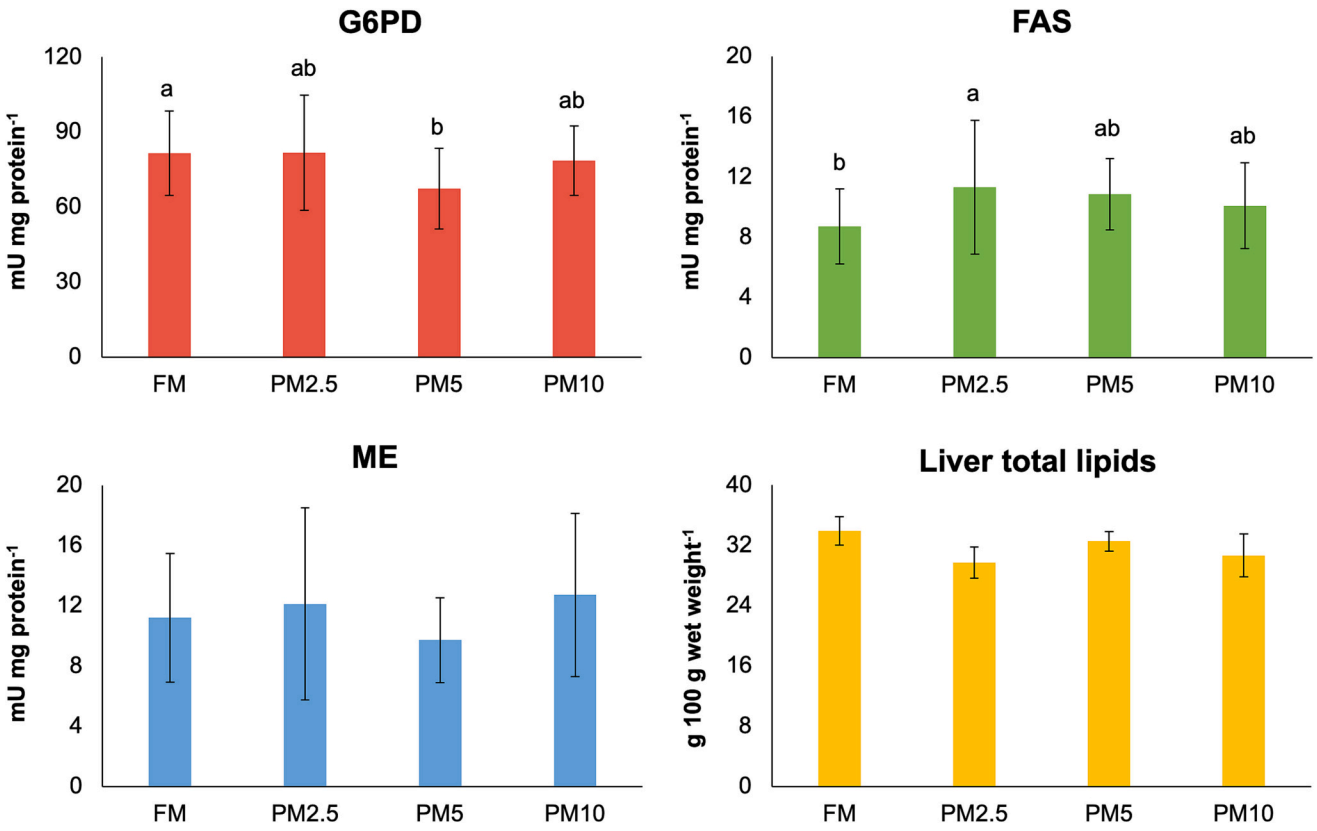

Fig. 2. Hepatic lipogenic capacity of European seabass measured by G6PH, ME and FAS enzymatic activities, along with liver total lipids. Results are expressed as mean ± SE ($n = 18$). Different letters indicate significant differences ($p < 0.05$).

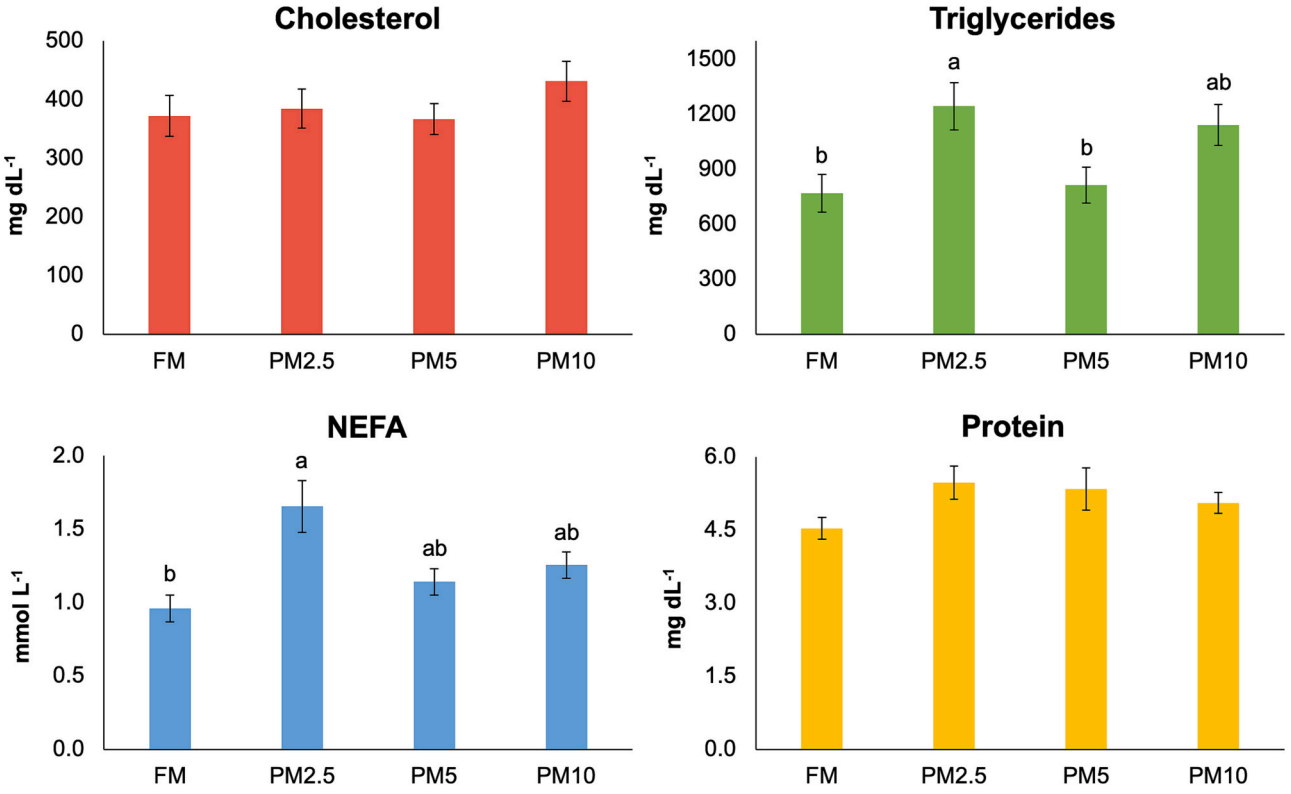

Fig. 3. European seabass plasma biochemistry. Results expressed as mean ± SE (n = 18). Different letters indicate significant differences ($p < 0.05$).

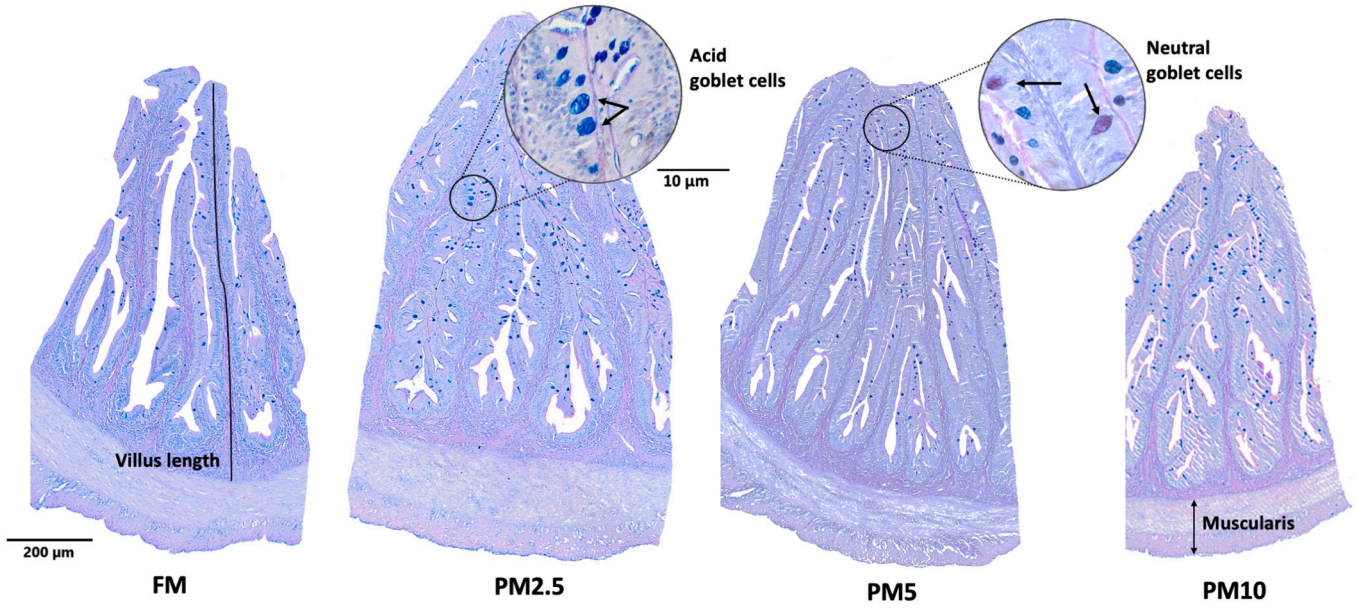

Fig. 4. Transversal cross-section of the anterior intestine of European seabass fed the dietary treatments for 93 days. Magnification: 100×. Scale bars: 200 µm.

Table 7
Histomorphology of the anterior intestine of European seabass juveniles fed the experimental diets for 93 days. Values are the mean ± SD ($n = 12$).

Parameter	FM	PM2.5	PM5	PM10	*p*-value
Weight (g)[1]	74.7 ± 8.0	76.6 ± 6.2	81.2 ± 7.8	80.6 ± 7.3	0.101
Length (cm)[1]	18.7 ± 0.6	18.7 ± 0.6	19.0 ± 0.6	19.1 ± 0.6	0.319
Cross-sectional perimeter (mm)	6.9 ± 0.9	8.7 ± 3.5	7.4 ± 2.9	6.2 ± 1.3	0.087
Absorption area (mm^2)	1.7 ± 0.5	3.2 ± 2.7	2.4 ± 2.5	1.5 ± 0.6	0.183
Muscularis thickness (µm)	67.0 ± 15.6^{ab}	89.3 ± 41.1^{a}	75.1 ± 24.5^{ab}	58.6 ± 19.3^{b}	0.038
Submucosa width (µm)	24.6 ± 4.8	30.1 ± 13.8	27.5 ± 9.7	22.2 ± 4.7	0.234
Lamina propria width (µm)	23.8 ± 5.1	30.5 ± 14.5	24.9 ± 9.3	24.2 ± 6.0	0.491
Villus length (µm)	705.9 ± 75.8^{ab}	1034.7 ± 469.2^{a}	814.9 ± 298.8^{ab}	675.4 ± 123.7^{b}	0.024
Goblet cells (GC)	1266.7 ± 451.2	1237.3 ± 658.5	1405.4 ± 477.0	1138.9 ± 512.3	0.673
Acid GC	1245.8 ± 456.2	1190.3 ± 670.7	1337.3 ± 457.7	1082.4 ± 525.8	0.700
Neutral GC	20.9 ± 21.7	47.0 ± 40.6	68.2 ± 95.8	56.6 ± 46.2	0.596
Average area GC (μm^2)	11.7 ± 1.0^{b}	26.2 ± 19.6^{a}	15.8 ± 11.5^{ab}	12.7 ± 1.0^{ab}	0.021

[1] Average weight and length of the fish selected for histologic analysis. Different superscript letters indicate significant differences among dietary treatments.

PM5, while reaching the highest value for fish fed PM10 (431.3 mg dL^{-1}). Likewise, plasma total protein remained unaffected by the dietary treatments, with the lowest value observed in fish fed FM (4.5 mg mL^{-1}) and the highest in fish fed PM2.5 (5.5 mg mL^{-1}). Plasma triglyceride levels were significantly higher in fish fed PM2.5 compared to those fed FM or PM5 (768.2 and 814.4 mg dL^{-1}, respectively). Fish fed PM2.5 also exhibited significantly higher levels of NEFA (1.7 mmol L^{-1}) compared to fish fed FM (1.0 mmol L^{-1}). However, the plasma NEFA content of fish fed PM5 and PM10 did not differ significantly from the former two groups.

3.7. Anterior intestine histomorphology

The anterior intestine structure of all fish used for histologic analysis was well-preserved (Fig. 4). No statistically significant differences were found among the dietary groups in terms of cross-sectional perimeter, absorption area, submucosa width, and lamina propria width (Table 8). However, the *muscularis* of fish fed PM5 was significantly thicker (89.3 µm) compared to those fed the PM10 diet (58.6 µm). On the other hand, the mean villus length of fish fed PM2.5 (1034.7 µm) was significantly higher than that of fish fed PM10 (675.4 µm), but it did not differ significantly from fish fed FM (705.9 µm) or PM5 (814.9 µm) (Table 7). The number of acid and neutral goblet cells, as well as the total goblet cell count, were not significantly affected by the dietary treatments. However, the goblet cell area was significantly higher in fish fed PM2.5 (26.2 μm^2) in relation to fish fed FM (11.7 μm^2) (Table 7).

3.8. Environmental impact assessment

The environmental impacts of the four diets based on the technical unit (TU), that is, the production of 100 kg of each diet, are reported in Table 9. It is evident that all impact categories present a consistent trend in the diet formulations. Specifically, as the percentages of substitution of reference fishmeal with PM increase, there is a progressive decrease in environmental impacts across all categories. The orange trend line labelled "TU" in Fig. 1 illustrates how the technical unit environmental relative performance indicator (TU-ERPI<ICs>), expressed in percentage, changes with the increasing incorporation of PM (2.5%, 5% and 10% WW) to replace fishmeal at increasingly levels (10%, 20% and 40%). The TU-ERPI<ICs> accounts for the impacts associated with the production of the different diets, their ingredients, and the relative upstream flows within the studied production system. Fig. 1 also reports the functional unit ERPI<ICs>, which incorporates the relative impacts of the diets production while considering the diets' functionality level. From the TU-ERPI<ICs> results obtained based on the technical unit analysis, it can be observed that incorporating higher levels of PM in the diet formulation results in a progressive reduction of environmental impacts. To better interpret the normalized environmental impacts (FU-ERPI<ICs>) in comparison with TU-ERPI<ICs> values, a *t*-test has been conducted, assuming that the ERPI<ICs> values exhibit a normal

Table 8
Environmental Impacts of the four diet formulations based on their Technical Unit.

Impact Category	Unit	FM	PM2.5	PM5	PM10
Abiotic depletion	kg Sb eq	9.22E-03	8.85E-03	8.47E-03	7.72E-03
Abiotic depletion (fossil fuels)	MJ	1.74E+03	1.67E+03	1.61E+03	1.46E+03
Acidification	kg SO_2 eq	1.48E+00	1.40E+00	1.33E+00	1.19E+00
Eutrophication	kg PO_4^{3-} eq	6.04E-01	5.89E-01	5.74E-01	5.46E-01
Fresh water aquatic ecotox.	kg 1,4-dB eq	9.09E+01	8.72E+01	8.37E+01	7.67E+01
Global warming (GWP100a)	kg CO_2 eq	2.30E+02	2.23E+02	2.17E+02	2.03E+02
Human toxicity	kg 1,4-dB eq	9.40E+01	9.00E+01	8.61E+01	7.81E+01
Marine aquatic ecotoxicity	kg 1,4-dB eq	2.11E+05	1.98E+05	1.86E+05	1.61E+05
Ozone layer depletion (ODP)	kg CFC-11 eq	1.50E-05	1.44E-05	1.38E-05	1.26E-05
Photochemical oxidation	kg C_2H_4 eq	7.05E-02	6.84E-02	6.64E-02	6.23E-02
Terrestrial ecotoxicity	kg 1,4-dB eq	9.72E+00	9.28E+00	8.83E+00	8.06E+00

distribution. The test results, with a confidence interval of 95%, suggest that the ERPI<ICs> values for PM2.5 and PM10 on both FU-ERPI<ICs> and TU-ERPI<ICs> are similar, confirming the decreasing trend of environmental impact with increasing levels of PM in the diet formulation. Nevertheless, when viewed from a different perspective, the statistics test reveals that the TU-ERPI<ICs> and FU-ERPI<ICs> values corresponding to PM5 are discriminated with a 95% confidence level. This indicates a positive influence of PM inclusion in the diet formulation on its nutritional performance for this specific diet. Interpolation of the average values with a third order polynomial indicates that the FU-ERPI<ICs> may reach a minimum, suggesting that the best environmental performance could be achieved with a diet comprising 5% to 10% (*w*/w) PM content.

4. Discussion

In recent years, driven by higher prices and limited availability of marine ingredients, market dynamics dictated a marked shift towards the use of, predominantly, plant-based alternatives (Naylor et al., 2021; Tacon et al., 2021). However, researchers' concerns about several challenges associated with plant feedstuffs (Colombo, 2020; Colombo et al., 2022) have prompted the exploration of more suitable alternative feedstuffs, such as low-trophic organisms. This study successfully demonstrated that commercially available PM can effectively replace up to 40% of high-quality fishmeal in European seabass diets without compromising growth, feed utilization and flesh nutritional value, while also improving environmental performance.

The partial replacement of FM with PM did not significantly impact the growth performance or nutrient utilization of *D. labrax*. Only a few reports have explored the use of PM in fish feeds. For example, a recent study demonstrated that PM obtained from *A. virens* can serve as the main protein source in diets for rainbow trout, resulting in similar growth performances between fish fed a PM-based diet and those fed a commercial diet (Thum et al., 2022). Additionally, the inclusion of PM from *Nereis* sp., comprising 40% of the dietary protein, has been reported to promote growth and survival in tilapia larvae, further supporting this ingredient as a valuable alternative protein source in aquafeeds (Ahmad et al., 2021). Although no specific data on the impact of PM inclusion in fish whole body composition is available, studies involving other low-trophic organisms (e.g. insects) have reported alterations, particularly concerning whole fish lipid content. Some studies reported an increase in whole European seabass lipid levels (Basto et al., 2021), while others observed a decrease (Mastoraki et al., 2020). However, the present work indicates that PM inclusion did not have a significant effect on the overall fish composition of the fish. This suggests that the influence of PM on lipid metabolism may be milder compared to other low-trophic organisms commonly used in aquafeed formulation.

To the best of the authors' understanding, this study represents the first investigation into the influence of PM on nutrient digestibility. The findings unveiled that substituting fishmeal with up to 40% PM did not yield any significant effects on macronutrient apparent digestibility coefficient (ADC). However, the inclusion of PM did significantly affect the digestibility of minerals. Mineral digestibility is not often evaluated in marine species, due to the possible interference of waterborne minerals in the analysis (Storebakken et al., 2000). This is particular critical when faeces collection rely on decantation systems. In fact, in the present study, Zinc ADC was not possible to calculate due to the high levels of this mineral present in the local coastal area (Couto and Ribeiro, 2022; Ribeiro et al., 2022), resulting in a negative ADC (data not shown). Nonetheless, as the water conditions were equal among the whole RAS system, it is expected that they affected ADCs in a similar way, meaning that differences among diets should still be valid and due to the diet itself. Regarding mineral ADC, the values observed are within the range reported by Resende et al. (2022) for European seabass. In the present work, Copper (Cu) digestibility increased concomitantly with the increase in PM levels. Cu plays crucial roles in metabolic processes and is involved in various enzymatic complexes, including in the regulation of lipid metabolism (Chen et al., 2015; NRC, 2011; Tseng et al., 2023). While limited research has focused on the effects of dietary copper on fish lipid metabolism, some studies have demonstrated that higher levels of Cu significantly decrease the overall lipid content in Atlantic salmon (*Salmo salar*) after six weeks of feeding (Berntssen et al., 1999). Conversely, another study indicated that a diet containing up to 2 mg of Cu per 100 g caused changes in gene expression related to lipid metabolism in large yellow croaker, *Larimichthys croceus* (Meng et al., 2016). In the present study, although alterations in lipid metabolism were observed, these changes were primarily associated with fish fed PM2.5 (~1.9 mg Cu per 100 g diet) and could not be attributed solely to increasing levels of Cu resulting from higher inclusion levels of PM.

Fishmeal replacement with PM did not impact significantly most of the parameters evaluated in the anterior intestine which are within the range of values described in the literature for this species (NRC, 2011). However, PM2.5 led to the increase of the *villus* length and *muscularis thickness,* and increased goblet cell (GC) area, especially when compared to fish fed PM10. It has been described that an increase in these parameters can be linked to increased nutrient uptake, leading to an increase in nutrient digestibility and fish growth (Messina et al., 2019). However, in the present work, the distinct impacts of PM2.5 on the intestinal morphology were not sufficient to significantly affect the nutrient digestibility and overall fish performance.

In the present study, plasma metabolites levels were found consistent with those reported for European seabass (Campos et al., 2019; Messina et al., 2019; Parma et al., 2023). However, a significant increase in NEFA and triglycerides concentration was observed in fish fed PM2.5. Higher plasmatic NEFA could be attributed to the mobilization of lipids from adipose tissue into the circulation, which may be influenced by the combination of fasting and diet factors (Lu et al., 2019). Additionally, the increased activity of hepatic FAS induced by PM2.5 could have augmented the availability of fatty acids for the synthesis of complex lipids, specifically triglycerides. Typically, an increased flux of triglycerides, would be associated with a dysregulated hepatic metabolism

resulting in lipid accumulation, particularly in cases of overnutrition (Alves-Bezerra and Cohen, 2017). However, under normal circumstances, high fluxes through these pathways do not compromise liver function, and the liver stores only small amounts of fatty acids as triglycerides (Ye et al., 2019; Zhang et al., 2019). This would be consistent with the hepatic lipid levels observed in the present study, which were not increased in fish fed PM2.5. Moreover, in this study, the hepatic lipogenic enzyme activities in seabass were consistent with previous research, in this species, with higher G6PD activity than ME activity (Campos et al., 2019; Monteiro et al., 2018). While G6PD was significantly reduced in fish fed PM5, ME activity did not reveal any clear trend. On the other hand, the increased FAS activity observed in fish fed PM2.5 aligns with increased plasmatic levels of NEFA and triglycerides. The effects of fishmeal replacement in lipogenic enzymes' activity seem to be quite variable, but it seems to be often connected with possible dietary amino acid imbalances. For instances, a deficiency of the exclusively ketogenic amino acids, such as lysine, can lead to increased catabolism of other amino acids, leaving thus more carbon chains and glucogenic substrates available for lipogenesis (Teodósio et al., 2022). However, in the present work, dietary amino acid profiles were very similar, even at 40% replacement of fishmeal (PM10), and thus unlikely to be behind the metabolic alterations observed. Moreover, differences in whole-body lipid, HSI, or lipid and energy balances among dietary treatments were not observed, suggesting observed metabolic alterations are mild. Although more research is needed to understand how low inclusion levels of PM (2.5%) precisely affect fish lipid metabolism, the present findings indicate that PM holds promise as a functional ingredient. By mobilizing fish NEFA and triglycerides, the use of PM2.5 could help mitigate the detrimental effects of prolonged starvation (Hsieh and Shiau, 2000) or exposure to low temperatures (Deng et al., 2020; Wang et al., 2022), which are known to have a significant impact on energy homeostasis and fish metabolic function. However, these alterations were not observed with higher inclusion levels and were not sufficient to affect the lipid deposition, as no significant differences in the whole body, liver or muscle lipid content.

Fish consumption, in general, is associated with health benefits due to its high levels of n − 3 PUFA. These FAs have been proven to prevent cardiovascular and neurological diseases (Calon and Cole, 2007; Lavie et al., 2009) and are a quality trait searched by consumers. At the end of the trial, EPA + DHA content of fish fillet (0.4 g per 100 g WW) was well above the European Food Safety Authority (EFSA) recommended daily intake of EPA + DHA, regardless of the dietary treatment (0.25 g per day for healthy human individuals)(EFSA, 2016). Nevertheless, in the present work, fishmeal replacement with *A. virens* (wild-harvested) necessitated a slight increase (<1%) in fish oil supplementation to maintain the recommended dietary EPA + DHA levels for European sea bass. However, polychaetes have been found to possess active PUFA biosynthesizing systems (Kabeya et al., 2020), and their nutritional composition can be enhanced through adjustments in dietary and abiotic factors (Aguado-Giménez et al., 2023; Malzahn et al., 2023; Wang et al., 2019). This makes polychaetes promising candidates for fish meal replacement, eliminating the need for dietary fish oil supplementation. Moreover, these marine invertebrates possess a number of distinctive fatty acids, that can be biosynthesized endogenously, including NMI fatty acids (Barnathan, 2009; Monroig et al., 2022). The presence of competitive interaction between NMI fatty acid and PUFAs in marine organisms has been suggested (Zhukova, 2019), but the specific biological roles and functions of these fatty acids remain unclear. Nevertheless, these fatty acids have been reported to possess a multitude of advantages for human well-being, including anti-inflammatory properties that could have the potential to positively influence the health of fish as well (Zhukova, 2023).

Besides ensuring high-quality fish for consumption, a pressing concern in modern aquaculture is to minimize the environmental footprint of fish production (Mitra, 2021). In this study, we conducted an environmental impact assessment to evaluate the substitution of fishmeal with PM. The results revealed that increasing percentages of inclusion of PM resulted in progressively decreasing environmental impacts. This trend is due to the lower specific environmental impact associated to PM in comparison with other ingredients incorporated into the reference formulation, in particular, the fishmeal component. Therefore, even though the compared amount (in mass) of the FM and the other three experimental diets is the same in all formulations, the one with increasing PM content shows a better environmental performance. To date, LCA studies on intensive aquaculture have covered a wide range of aquatic species, including European seabass (Zoli et al., 2023). Despite the diversity of LCAs addressing the environmental characterization of intensive aquaculture practices, a common conclusion can be drawn: the leading role played by feed (Maiolo et al., 2020). As in the present work, Goyal et al. (2021) also showed the potential of novel ingredients based on low-trophic organisms, such as *Hermetia ilucens*, to improve the sustainability of aquafeeds, as alternative protein sources. Nevertheless, when considering the results based on the functional unit analysis (FU-ERPI<ICs>), it becomes apparent that increasing the PM content by more than a certain amount (8% of the total diet ingredients) does compromise performance of the diet from a nutritional perspective. In fact, growth performance remained similar despite the levels of PM included in the diet. Nevertheless, further assessments are needed to prove a limiting value of PM to maximize both functional and environmental performance.

5. Conclusion

Overall, the results of this study clearly show that fishmeal replacement by PM, up 40% does not compromise growth or nutrient utilization, while also representing a more environmentally sustainable protein source in aquafeeds. Additionally, all experimental diets ensured high flesh quality, providing high levels of EPA + DHA, surpassing the 250 mg recommended by EFSA for human daily consumption. Nevertheless, the influence of PM2.5 on the intestinal histomorphology of European seabass appears to be beneficial, while also affecting the regulation of lipid metabolism. These findings present intriguing prospects for further investigation on the functional potential of PM.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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