The use of fishery and aquaculture by-products with *Nannochloropsis* sp. allows total dietary replacement of wild-caught fishmeal, fish oil and soy protein in European sea bass juveniles

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ABSTRACT

Five experimental diets (CTRL, 50FMFO, 50FMFO-50MIC, 0FMFO-50MIC, 0FMFO-100MIC) were formulated to replace wild-caught fishmeal (FM), wild-caught fish oil (FO) and soy protein using fisheries, aquaculture by-products (BP) and microalgae (MIC). Fifty European sea bass juveniles were distributed in 15 tanks (initial body weight 46.66 ± 0.04 g) and reared in a recirculating aquaculture system for 88 days. Temperature, salinity, oxygen and photoperiod were kept constant throughout the experiment (22 ± 0.5 °C, 25 g L^{-1} and 8.0 ± 1.0 mg L^{-1}, 12:12 light/dark, respectively). Growth, feed intake (FI), proximal composition, nutritional index, apparent digestibility, somatometric indexes, blood plasma biochemistry and digestive enzyme activity were evaluated. Also, gut microbiota composition was assessed through next-generation sequencing. Results showed that growth performance and feed digestibility were not affected by FM, FO and soy replacement using BP and MIC. Dietary replacement of 100% FM and FO with circular substitutes and 50% replacement of soymeal with microalgae increased the activity of alkaline phosphatase and chymotrypsin. Moreover, the inclusion of BP and MIC had positive effects on the gut microbiota richness and abundance. In conclusion, the utilization of BP and MIC represents a valuable alternative to FM and FO as well as soy protein in feed for European sea bass juveniles.

1. Introduction

Fish by-products are playing a major role in reducing food losses and waste, enhancing food security and nutrition, promoting environmental sustainability and climate change mitigation across various food systems (FAO, 2019; Olesen et al., 2023). Recently, in the aquaculture sector, there has been a growing interest in utilizing fisheries and aquaculture by-products as valuable alternative raw materials to fish meal (FM) and fish oil (FO) derived from wild stocks. This interest comes from the aim to reduce the carbon footprint of finfish production and to preserve wild fish stocks (Newton et al., 2023). In this regard, fish processing procedures can generate a huge amount of discharged material (up to 70%), consisting of head, skin, bones and viscera (Kandyliari et al., 2020). However, the discharge material is still rich in micro and macronutrients such as proteins, lipids and essential fatty acids, minerals and vitamins (Mutalipassi et al., 2021). This material can be processed at an industrial scale with advanced technologies to extract highly nutritious fish meal and oil, ensuring that no part of the fish goes to waste (Coppola et al., 2021). Thus, fish by-products have been already integrated into 30% and 50% of the world FM and FO production, respectively and represent an extremely promising ingredient to be used by aquaculture feed industries (IFFO, 2022).

Microalgae are renowned in fish nutrition for their high content of EPA and DHA, essential fatty acids crucial for various life stages, from larvae to adults. Consequently, microalgae are frequently incorporated into feed formulations (Ansari et al., 2021). They also provide varying protein percentages, ranging from 18% to 46%, with some species reaching up to 69%, making them a significant source of essential amino acids (Nagarajan et al., 2021). Due to their nutritional profile and the growing demand for alternative aquafeed ingredients, the global market for microalgae is expected to surge from $32.6 billion annually in 2017 to $53.43 billion by 2026 (Nagarajan et al., 2021). Moreover, microalgae are highly sustainable, capable of thriving on diverse substrates and in various conditions, including waste materials such as wastewater (Gamboa-Delgado and Márquez-Reyes, 2018). Positive results with the addition of microalgae as protein sources in aquafeed have been reported in salmon (Gong et al., 2019) and many Mediterranean species, such as meagre (Estevez et al., 2022), gilthead sea bream (Carvalho et al., 2021) and European sea bass (Pascon et al., 2021). In this latest species, different microalgae species such as *Tetraselmis* sp. (Tulli et al., 2012), *Isochrysis* sp. (Tibaldi et al., 2015) and *Pavlova viridis* (Haas et al., 2016) have been tested as a replacement of fish meal and fish oil, with the percentage ranging from 20% to 100% of microalgae biomass. Recently, *Nannochloropsis* sp. showed promising results on growth performances (Ayala et al., 2023) and intestinal health in gilthead sea bream (Saez et al., 2022) and European sea bass (Haas et al., 2016; Castro et al., 2016).

Considering these various factors, it is important to note that despite numerous studies examining the effects of a diet based solely on fish oil and fish meal trimmings or microalgae on European sea bass, there is a scarcity of literature addressing the combined impact of these ingredients on both growth and health. Further research is needed to understand the potential distinct effects of incorporating both these components in the diet of European sea bass, providing valuable insights into optimizing nutrition and enhancing the well-being of this species. In this scenario, we postulated that blending FM and FO obtained from trimming with microalgae in the European sea bass diet could serve as valuable alternatives to wild-caught fish and soy meal. This combination holds the promise of enhancing growth performance and gut health, leveraging the nutritional attributes of these ingredients while advancing aquaculture sustainability. Following a methodology akin to Marchi et al. (2023a) for European sea bass, this study focused on earlier juvenile specimens. This choice was prompted by the acknowledgment of potentially heightened nutritional requirements, particularly concerning protein and fatty acid composition. Although this approach may entail increased feed costs, the potential to enhance animal quality justifies such investment.

Table 1
Ingredients and proximate composition of the experimental diets.

Composition (%)	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC
Ingredients, % of the diet					
[1]Fish meal Prime	30.0	15.0	15.0	–	–
[2]Fish meal trimming	–	15.0	15.0	30.0	30.0
[3]Fish oil EXTRA	11.5	5.75	5.75	–	–
[4]Fish oil trimming	–	5.75	5.75	11.5	11.5
[5]Soy Protein Concentrate	15.0	15.0	7.50	7.50	–
[6]Microalgae	–	–	7.50	7.50	15.0
[7]Wheat meal	9.66	9.30	7.77	6.77	5.00
[8]Wheat gluten	15.0	15.0	16.5	16.5	16.5
[9]Pea Protein	12.0	12.0	12.5	12.0	12.5
[10]Rapeseed oil	6.00	5.75	5.30	5.00	5.00
Vit C 35%	0.05	0.05	0.05	0.05	0.05
Vit E 50%	0.02	0.02	0.02	0.02	0.02
Dl-Methionine	0.06	0.075	0.055	0.35	0.38
L-Lysine 50%	–	0.60	0.60	0.80	0.67
L-Valine	–			0.40	0.89
L-Threonine				0.70	0.87
L-Tryptophan	–			0.20	0.92
[11]Vitamins-Premix	0.70	0.70	0.70	0.70	0.70
Yttrium oxide	0.01	0.01	0.01	0.01	0.01
Proximate composition, % on a wet weight basis					
Moisture	6.88	5.80	6.85	6.95	6.57
Protein	50.2	50.8	50.3	48.7	48.2
Lipid	20.9	21.4	21.6	23.0	23.5
Ash	6.79	7.72	8.95	9.80	11.2
Gross energy (MJ kg^{-1} DM)	21.6	21.5	21.4	21.3	21.1

[1] Origin: Chile; Composition: protein 67%, lipid 10%, ash 14%. Antioxidant: butylated hydroxyanisole, BHA, 40 ppm; Butylated hydroxytoluene BHT, 200 ppm.

[2] Origin: Morocco; Obtained from Atlantic mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*). Composition: protein 63%, lipid 10%, ash 20%. BHA, 80 ppm; BHT, 80 ppm.

[3] Origin: Chile. Antioxidant: BHA 90 ppm, BHT 80 ppm, propylgallate, PG 40 ppm.

[4] Origin: EU. Obtained from Atlantic salmon (*Salmo salar*). Antioxidant: BHA 70 ppm, BHT 145 ppm, PG 40 ppm.

[5] Origin: Serbia. Composition: protein, 61%, lipid, 0.3%.

[6] Origin: Spain; Obtained from processed Nannocloropsis (Almeria, Spain).

[7] Origin: Spain; Composition: 12% protein.

[8] Origin: Spain; Composition: 78% crude protein.

[9] Origin: Spain; Obtain from Pisane, Emilio Peña; Composition: 85% crude protein, 1.5% crude lipid.

[10] Origin: Spain; Obtain from Roviroli SL.

[11] Lifebioencapsulation SL (Almería, Spain). Vitamins (mg kg^{-1}): vitamin A (retinyl acetate), 2000,000 UI; vitamin D3 (DL-cholecalciferol), 200,000 UI; vitamin K3 (menadione sodium bisulphite), 2500 mg; vitamin B1 (thiamine hydrochloride), 3000 mg; vitamin B2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine (Betafin S1), 50,000 mg. Minerals (mg kg^{-1}): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 18.6%; (186,000 mg); KCl, 2.41%; (24,100 mg); NaCl, 4.0% (40,000 mg).

2. Materials and methods

2.1. Experimental diets

Five experimental diets were developed to sequentially substitute wild-caught FM and FO with fisheries and aquaculture by-products, alongside the replacement of soy protein concentrate with microalgae (MIC). Specifically, wild-caught FM (FM Prime) and FO (FO Extra) were first half and then totally replaced by FM and FO trimming (50% FM, 50% FO), (50FMFO), while soy protein concentrate was partially replaced by microalgae (50% FM, 50% FO, 50% MIC), (50FMFO-50MIC). Then wild-caught FM and FO were totally replaced by FM and FO trimming with a concomitant partial (0%FM, 0%FO, 50%MIC), (0FMFO-50MIC) or total (0% FM, 0%FO, 100%MIC) (0FMFO-100MIC) replacement of soy protein concentrate by the microalgae *Nannochloropsis* sp. Diets were produced with a diameter of 3 mm by the University of Almeria, Spain. The formulation was designed by Agricola Italiana Alimentare S.p.a – AIA, Verona, Italy; in accordance with the University of Almeria and the University of Bologna. Briefly, all ingredients were mixed in a 120 L mixer, grounded with a hammer mill (UPZ 100, Hosokawa-Alpine, Augsburg, Germany) to 0.5 mm. The diets were extruded in a twin-screw extruder (Evolum 25, Clextral, Firminy, France), fitted with 3 mm die holes. The extruder barrel consisted of four sections and the temperature profile in each segment (from inlet to outlet) was 90, 95, 95, and 105 °C, respectively. The pellets were dried after extrusion at 27 °C using a drying chamber (Airfrio, Almería, Spain), and cooled at ambient temperature. Vacuum fat coating was done on the following day in a Pegasus PG-10VC LAB vacuum coater (Dinnissen, Sevenum, The Netherlands). Ingredients, proximate and fatty acids composition of the experimental diets are shown in Table 1 and Table 2.

2.2. Fish and rearing trial

The trial took place at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea bass juveniles were obtained from an Italian hatchery, located along the Adriatic coast. At the beginning of the trial, 50 fish per tank with an initial body weight of about 46.66 ± 0.04 g, were randomly distributed into 15 square tanks with a capacity of 800 L. Each diet was randomly assigned and administered to triplicate groups,

over 88 days. Tanks were provided with natural seawater and connected to a closed recirculation system (overall water volume: 20 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25 mJ/cm^2: 32m^3 h^{-1}, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). The overall water renewal amount in the system was 5% daily, while water exchange rate was 100% every hour. During the experiment, temperature was kept at 22 ± 0.5 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) thanks to the connection with a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Each day, ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were monitored by spectrophotometer (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany) and salinity (25 g L^{-1}) was measured by a refractometer (106 ATC, Giorgio Bormac S.r.l., Carpi, Italy). Sodium bicarbonate was added if needed to keep pH constant at 7.8–8.0 (Hanna Portable pH Meter HI991001, WJF Instrumentation Ltd. Alberta, Canada). In each tank animals were fed to satiation with automatic belt feeders (Scharfling, F.02.001, Scubla, Italy) twice a day, set to release gradually pellets for one hour. The uneaten pellets of each tank were collected, dried overnight at 105 °C, and weighed for feed intake (FI) calculation (Parma et al., 2019).

2.3. Sampling

At the outset and upon conclusion of the experiment, all animals within each tank were anesthetized with MS222 at a concentration of 100 mg L^{-1} and weighed. Specific growth rate (SGR) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined using a pooled sample of 10 fish initially and a pooled sample of 5 fish per tank at the end of the trial. For gut microbiota analysis, 12 h post-meal at the conclusion of the trial, digesta content from posterior intestine of five fish per tank was collected and immediately stored at -80 °C (Parma et al., 2020). At the same time 10 fish per tank were euthanized to collect feces to determine the apparent digestibility coefficient (ADC) of dry matter and protein using the indirect method with diets containing yttrium oxide (Busti et al., 2020). Blood was also collected from 5 fish per tank for the assessment of plasma biochemistry. Blood samples were centrifuged (3000 ×g, 10 min,

Table 2
Fatty acid profile (% of total fatty acid methyl esters, FAME) of the experimental diets.

Fatty acids	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC
14:0	2.20 ± 0.36	2.29 ± 0.37	2.33 ± 0.37	2.33 ± 0.37	2.24 ± 0.37
16:0	14.1 ± 1.70	14.6 ± 1.80	13.3 ± 1.60	13.9 ± 1.70	15.6 ± 1.90
16:1n7	2.73 ± 0.00	3.75 ± 0.00	3.72 ± 0.00	4.49 ± 0.00	2.82 ± 0.00
18:0	3.94 ± 0.56	3.86 ± 0.56	3.55 ± 0.51	3.54 ± 0.51	4.26 ± 0.65
18:1n9	28.5 ± 3.10	27.0 ± 3.00	28.1 ± 3.00	27.1 ± 2.90	26.8 ± 3.00
18:1n7	2.53 ± 0.39	2.44 ± 0.39	2.56 ± 0.39	2.46 ± 0.38	2.42 ± 0.38
18:2n6	12.2 ± 1.60	11.9 ± 1.60	11.5 ± 1.40	11.3 ± 1.40	11.9 ± 1.60
18:3n3	3.67 ± 0.53	3.44 ± 0.51	3.56 ± 0.51	3.42 ± 0.50	3.42 ± 0.50
18:4n3	0.64 ± 0.16	0.63 ± 0.15	0.79 ± 0.19	0.69 ± 0.17	0.52 ± 0.12
20:1n9	2.01 ± 0.33	1.96 ± 0.33	2.82 ± 0.42	2.48 ± 0.38	1.25 ± 0.26
20:4n6	0.93 ± 0.22	1.01 ± 0.24	0.92 ± 0.21	1.06 ± 0.22	1.22 ± 0.26
20:4n3	< 0.0050	< 0.0050	< 0.0050	< 0.0050	< 0.0050
20:5n3	4.38 ± 0.62	5.58 ± 0.80	5.35 ± 0.72	6.15 ± 0.86	4.60 ± 0.65
22:5n3	1.18 ± 0.25	1.16 ± 0.25	1.36 ± 0.25	1.26 ± 0.24	1.03 ± 0.24
22:6n3	12.2 ± 1.60	12.0 ± 1.50	10.5 ± 1.40	10.7 ± 1.40	14.1 ± 1.80
Σ SFA	23.1 ± 1.9	23.7 ± 2.00	21.7 ± 1.70	22.4 ± 1.80	25.3 ± 2.10
Σ MUFA	39.2 ± 4.00	38.6 ± 3.90	41.7 ± 3.80	40.5 ± 3.70	35.6 ± 3.90
Σ PUFA	37.5 ± 2.50	37.7 ± 2.40	36.4 ± 2.30	37.2 ± 2.30	39.1 ± 2.60
Σ n-3	22.6 ± 1.80	23.3 ± 1.80	22.2 ± 1.70	22.9 ± 1.80	24.1 ± 2.00
Σ n-6	15.0 ± 1.70	14.4 ± 1.70	14.2 ± 1.50	14.4 ± 1.50	15.1 ± 1.70
Σ n-9	31.6 ± 3.10	30.0 ± 3.00	32.1 ± 3.10	30.7 ± 2.90	28.9 ± 3.00
n-3/n-6	1.51 ± 0.21	1.62 ± 0.23	1.56 ± 0.21	1.59 ± 0.21	1.60 ± 0.23
n-3 PUFA	17.8	18.7	17.2	18.1	19.7
EPA/DHA	0.36	0.46	0.51	0.57	0.33

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

4 °C) and plasma aliquots were stored at −80 °C until analysis (Pelusio et al., 2021). To assess enzymatic activity, 4 fish per tank were euthanized and the entire intestine was collected from each fish. Samples were stored at −80 °C until analyses. The experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna (ID 1136/2019), in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.4. Calculations

Following, the employed formulae:

$$\text{Specific growth rate (SGR)} \ (\%\text{day}^{-1}) = 100^* (ln \ \text{FBW} - ln \ \text{IBW}) / \text{days}$$ (where FBW represent the final body weight and IBW and the initial body weights).

$$\text{Feed intake} \ \left(\text{FI}, \text{g kg} \ \Delta\text{BW}^{-1} \ \text{day}^{-1}\right) = ((100^* \text{total feed ingestion}) / (\Delta\text{BW})) / \text{days}.$$

Feed conversion ratio (FCR) = feed intake/weight gain.

Protein efficiency rate (PER) = (FBW–IBW)/protein intake.

Apparent Digestibility (ADC (%))
= 100–[100*(Yttrium in feed/Yttrium in feces)*
(nutrient in feces/nutrient in feed)].

Gross protein efficiency (GPE) (%)
= 100*[(%final body protein*FBW) − (%initial body protein*IBW)]
/total protein intake fish.

Lipid efficiency rate (LER) = (FBW − IBW)/lipid intake.

Gross lipid efficiency (GLE) (%)
= 100*[(%final body lipid*FBW) − (%initial body lipid*IBW)]
/total lipid intake fish.

2.5. Proximate composition analysis

To determine moisture content, samples were dried in an oven at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method, multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer (1959) extraction method. Samples were incinerated to a constant weight in a muffle oven at 450 °C to estimate ash content (AOAC, 2010). The concentrations of yttrium oxide in both diets and feces were measured through Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using equipment from Perkin Elmer, MA, United States, following the method described by Busti et al. (2020).

2.6. Metabolic parameters in plasma

To determine plasma parameters was used 500 μL of sample on an automated analyser (AU 480; Olympus/Beckman Coulter, Brea, CA, United States) according to the manufacturer's instructions (Parma et al., 2023). The levels of glucose (GLU), urea, creatine, uric acid, total bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), cholesterol (CHOL), triglycerides (TRIG), total protein (TP), albumin (ALB), calcium (Ca^{+2}), phosphorus (P), potassium (K$^+$) sodium (Na$^+$), iron (Fe), chloride (Cl), magnesium (Mg) were determined. The Albumin/Globulin (ALB/GLO) ratio, lactate (LAC) and Current Calcium (Cur.Ca) were calculated.

2.7. Determination of digestive enzyme activities

From 4 fish per tank, the entire digestive tract was extracted, and segments of the intestines were pooled. All assays were conducted in triplicate, respecting both proximal and distal regions of the intestine. To determine digestive enzyme activities, intestinal segments were manually homogenized in distilled water at 4 °C to achieve a final concentration of 0.5 g mL^{-1}. Subsequently, the homogenized material was centrifuged (16,000 $\times g$ for 12 min at 4 °C), and supernatants obtained after centrifugation were immediately stored at −20 °C until further analysis. Total soluble protein was quantified according to Bradford (1976) using bovine serum albumin as a standard. The activities of some of the most important digestive enzymes were measured spectrophotometrically, and specific enzymatic activity was expressed as unit per gram of tissue (Alarcon et al., 1998). Total alkaline protease (TAP) activity was assessed using 5 g L^{-1} casein in 50 mM Tris HCl (pH 9.0) as substrate, with one unit of TAP defined as the amount of enzyme that released 1 μg of tyrosine per minute. This calculation considered an extinction coefficient for tyrosine of 0.008 μg^{-1}mL^{-1}cm^{-1}. The activity of TAP was measured spectrophotometrically at 280 nm. To determine the activities of trypsin and chymotrypsin, 0.5 mM BAPNA (N-a-benzoyl-DL-arginine-4nitroanilide) (Erlanger et al., 1961) and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-Pnitroanilide) (DelMar et al., 1979) respectively, were prepared as substrate in 50 mM Tris-HCl, 10 mM CaCl$_2$ buffer (pH 8.5). Leucine aminopeptidase activity was assayed using 2 mM L-leucine-pnitroanilide (LpNa) in 100 mM Tris-HCl buffer, pH 8.8 while alkaline phosphatase was assessed with *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.5, containing 1 mM MgCl$_2$ as substrates (Vizcaíno et al., 2014). Trypsin, chymotrypsin, and leucine aminopeptidase activities were measured spectrophotometrically at 405 nm and one unit of activity (U) was defined as the amount of enzyme that releases 1 μmol of p-nitroanilide (pNA) per minute, considering the extinction coefficient 8800 M cm^{-1}. Moreover, one unit of alkaline phosphatase activity was defined as the amount of enzyme that 1 μg of nitrophenyl released per minute considering a coefficient molar extinction of p-nitrophenol, 17, 800 M cm^{-1}, measured at 405 nm (Galafat et al., 2022).

2.8. Gut bacterial community DNA extraction, sequencing and analysis

At the end of the feeding trial, total DNA was extracted from individual distal gut content (300 mg per fish) obtained from a total of 75 fish (15 fish per tank), as previously reported by Parma et al. (2016). Total DNA was then quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at −20 °C until further processing. The amplification of the V3-V4 hypervariable regions of the 16S rRNA bacterial gene was carried out using the 341F and 785R primers with overhang sequencing adapters attached and 2 x KAPA HiFi HotStart ReadyMix (KAPA Byosistems). As already described by Parma et al. (2020), the thermal cycle consists of 30 amplification cycles and at the end PCR products were purified and the indexed libraries were prepared following Illumina protocol "16S Metagenomic Sequencing

Library Preparation". Libraries were normalized to 4 nM and pooled together, the resulting pool was denatured with 0.2 N NaOH and diluted to 6 pM with 20% Phix control. Sequencing was performed on the Illumina MiSeq platform using a 2×250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed by combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; https://qiime2.org). High-quality reads, obtained after a filtering step for length (min/max = 350/550 bp) and quality step using USEARCH with a max error rate of 3% (Edgar, 2010), were cleaned and clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using a hybrid method combining VSEARCH and q2 classifier trained on the SILVA database release 138.1 (Bokulich et al., 2018). Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith's Phylogenetic Diversity (faith_pd) (Faith, 1992); Shannon_entropy index (Shannon, 1948), and number of observed ASVs (observed features). Unweighted UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

2.9. Statistical analysis

All data are presented as mean ± standard deviation (SD). As experimental unit single tank was used to evaluate growth performance and a pool of ten fish was considered the experimental unit for the analysis of carcass composition, nutritional indices and digestibility evaluation. Five individual fish per tank were used for analysing somatic indices, blood biochemistry and gut microbiota community profiles. Preceding ANOVA, normality and homogeneity of variance were checked using Shapiro-Wilk and Brown-Forsythe tests, respectively. Tukey's post hoc test was performed. All statistical analyses were performed using GraphPad 8.0.1. The differences among treatments were considered significant at $p \leq 0.05$. Microbiota analysis and respective plots were produced using R software (https://www.r-project.org/) with "vegan" (http://www.cran.r-project.org/package-vegan/), "Made4" (Culhane et al., 2005) and "stats" packages (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html). Data separation was tested by a permutation test with pseudo-F ratios (function "Adonis" in the "vegan" package). When required, Wilcoxon and Kruskal–Wallis tests were used to assess significant differences in alpha diversity and taxon relative abundance between groups. When necessary, p-values were corrected for multiple testing with Benjamini-Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered as statistically significant.

3. Results

3.1. Growth

Results of growth performance and FI are summarized in Table 3. No statistical differences were evaluated for all parameters considered (IBW, FBW, WG, FCR and SGR).

3.2. Proximate composition

Results of body composition, nutritional indices and somatic indices are summarized in Table 4. Values of moisture were significantly higher in CTRL diet compared to 50FMFO, 50FMFO-50MIC and 0FMFO-50MIC. Protein body content presented lower value in diet 50FMFO-50MIC than in 50FMFO and 0FMFO-100MIC diets. Lipid body content was significantly higher in 50FMFO, 50FMFO-50MIC and 0FMFO-50MIC compared to control diet. No statistical differences were detected for ash, apparent digestibility and all nutritional indexes considered. Table 5 presents results of fatty acid composition collected from the flesh of animals. No statistical differences were found.

3.3. Plasma results

Plasma parameter results are shown in Table 6. Creatinine value was higher in CTRL than other treatments. AL*P* values in 0FMFO-50MIC and 0FMFO-100MIC were higher compared to 50FMFO-50MIC. Values of CHOL were higher in CTRL compared to 50FMFO-50MIC and 0FMFO-100MIC diets. CTRL presented the highest values of HDL while 50FMFO was higher than 0FMFO-50MIC and 0FMFO-100MIC diets. TP values were statistically higher in CTRL, and lowest in 0FMFO-100MIC diet. Fe values were lower in 50FMFO compared to CTRL. Diet 0FMFO-100MIC presented higher values of Na than 50FMFO-50MIC and CTRL diets. Cl was lower in CTRL compared to 0FMFO-100MIC.

3.4. Digestive enzymes activities

The enzyme activity results assessed at the end of the trial were summarized in Table 7. LANP, TAP, and trypsin activity did not exhibit any significant differences among treatments, considering both the proximal and distal intestinal regions. However, AP and chymotrypsin showed significant differences among treatments and between segments. Alkaline phosphatase activity in proximal segment presented lower values in CTRL and 0FMFO-100MIC diets compared to 50FMFO and 50FMFO-50MIC diets. In the distal tract of the intestine, alkaline phosphatase activity was lower in 50FMFO and 0FMFO-100MIC groups compared to the 50FMFO-50MIC group. In the proximal region,

Table 3

Growth performance and feed intake of European sea bass juveniles fed the experimental diets over 88 days.

Experimental diets						
	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	*p value*
IBW	46.6 ± 0.18	46.6 ± 0.10	46.7 ± 0.17	46.7 ± 0.11	46.7 ± 0.62	0.9942
FBW	169 ± 2.37	164 ± 2.44	163 ± 2.72	159 ± 4.69	176 ± 26.5	0.5653
WG	119 ± 2.32	118 ± 2.38	117 ± 2.57	112 ± 4.63	129 ± 26.8	0.5704
SGR	1.45 ± 0.02	1.43 ± 0.02	1.43 ± 0.04	1.39 ± 0.03	1.50 ± 0.17	0.5818
FI	1.67 ± 0.01	1.73 ± 0.01	1.66 ± 0.01	1.73 ± 0.06	1.63 ± 0.16	0.4371
FCR	1.32 ± 0.03	1.36 ± 0.01	1.31 ± 0.01	1.42 ± 0.09	1.29 ± 0.23	0.6317
Survival	100 ± 0.00	100 ± 0.00	99.30 ± 1.20	100 ± 0.00	100 ± 0.00	0.4516

Data are given as the mean ($n = 3$) ± SD. In each line, different superscript letters indicate significant differences among treatments ($p \leq 0.05$).

IBW = Initial body weight (g).

FBW = Final body weight (g).

WG = Weight gain (g).

SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days.

Feed intake (FI, g kg ΔBW^{-1} day^{-1}) = ((1000*total feed ingestion)/(ΔBW))/days.

FCR = Feed conversion rate = feed intake, g / weight gain, g.

Survival = Survival (%).

Table 4
Body composition and nutritional indices measured in European sea bass juveniles.

	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	*p value*
Whole body composition, %						
Moisture	65.0 ± 1.80[b]	62.2 ± 0.4[a]	62 ± 1.3[a]	62.1 ± 1.6[a]	63.9 ± 1.2[ab]	**0.0023**
Protein	16.8 ± 0.4[ab]	17.3 ± 0.5[b]	16.0 ± 1.08[a]	17 ± 0.3[ab]	17.1 ± 0.1[b]	**0.0126**
Lipid	14.5 ± 1.9[a]	17.6 ± 0.7[b]	18.1 ± 0.5[b]	17.8 ± 1.6[b]	16 ± 1.1[ab]	**0.0003**
Ash	2.90 ± 0.36	2.57 ± 0.17	3.16 ± 0.92	2.57 ± 0.92	2.84 ± 0.10	0.1632
Apparent digestibility						
Protein	72.5 ± 4.4	76.8 ± 5.64	74.2 ± 6.58	76 ± 2.8	75.3 ± 3.88	0.6128
Dry Matter	95.3 ± 1.25	96.2 ± 0.78	95.4 ± 1.06	94.7 ± 0.69	93.9 ± 1.89	0.2078
Nutritional indices						
PER	2.49 ± 0.05	2.35 ± 0.05	2.31 ± 0.05	2.30 ± 0.02	2.66 ± 0.55	0.4022
GPE	42.3 ± 0.61	41.5 ± 1.36	36.4 ± 3.86	39.8 ± 1.01	46.0 ± 9.77	0.2445
LER	5.73 ± 0.11	5.64 ± 0.11	5.47 ± 0.12	5.03 ± 0.05	5.51 ± 1.14	0.5475
GLE	87.1 ± 16.9	107 ± 6.54	110 ± 5.78	99.9 ± 12.6	94.5 ± 14.9	0.2137

Data are given as the mean (n = 3 ± SD). In each line, different superscript letters indicate significant differences among treatments (*p* values ≤ 0.05 are indicated in bold).

PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).

GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.

LER = Lipid efficiency rate = (FBW - IBW)/lipid intake.

GLE = Gross lipid efficiency = 100*[(% final body lipid*FBW) - (% initial body lipid*IBW)]/total lipid intake fish.

Table 5
Fatty acid composition (% of total fatty acid methyl esters, FAME) of European sea bass.

	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	*p Value*
14:00	2.17 ± 0.01	2.15 ± 0.02	2.37 ± 0.42	1.82 ± 0.60	2.12 ± 0.07	0.4302
16:00	15.4 ± 0.29	15.5 ± 0.24	16.1 ± 1.11	15.6 ± 0.73	15.7 ± 0.50	0.6964
16:1n7	4.34 ± 0.08	4.22 ± 0.11	4.38 ± 0.41	4.19 ± 0.30	4.16 ± 0.13	0.7783
18:00	3.8 ± 0.10	3.83 ± 0.04	3.85 ± 0.23	3.94 ± 0.38	3.93 ± 0.15	0.9152
18:1n9	35.7 ± 0.22	34.8 ± 1.05	34.8 ± 1.37	36.0 ± 0.65	36.0 ± 0.8	0.3444
18:1n7	0.34 ± 0.01	0.29 ± 0.05	0.29 ± 0.02	0.31 ± 0.02	0.31 ± 0.00	0.2491
18:2n6	11.1 ± 0.02	11.1 ± 0.25	11.4 ± 0.38	11.1 ± 0.12	11.1 ± 0.10	0.4136
18:3n3	2.59 ± 0.01	2.62 ± 0.04	2.52 ± 0.12	2.6 ± 0.08	2.58 ± 0.06	0.5477
18:4n3	0.55 ± 0.03	0.52 ± 0.08	0.62 ± 0.07	0.58 ± 0.09	0.55 ± 0.12	0.7013
20:1n9	3.29 ± 0.26	3.04 ± 0.51	3.18 ± 0.35	3.16 ± 0.40	3.13 ± 0.35	0.9545
20:4n6	0.65 ± 0.01	0.73 ± 0.10	0.68 ± 0.15	0.67 ± 0.02	0.68 ± 0.04	0.8492
20:4n3	0.33 ± 0.02	0.31 ± 0.03	0.32 ± 0.03	0.78 ± 0.8	0.30 ± 0.04	0.4633
20:5n3	3.36 ± 0.05	3.4 ± 0.12	3.32 ± 0.04	2.99 ± 0.44	3.18 ± 0.30	0.3344
22:5n3	0.97 ± 0.19	1.04 ± 0.04	1.02 ± 0.02	1.02 ± 0.06	1.00 ± 0.09	0.9228
22:6n3	8.53 ± 0.18	9.55 ± 1.24	8.4 ± 1.44	8.59 ± 0.29	8.66 ± 0.32	0.5438
Σ SFA	21.4 ± 0.37	21.5 ± 0.20	22.4 ± 1.47	21.4 ± 1.22	21.7 ± 0.58	0.6737
Σ MUFA	43.7 ± 0.44	42.4 ± 1.73	42.7 ± 1.50	43.7 ± 0.28	43.4 ± 0.37	0.4526
Σ PUFA	28.1 ± 0.26	29.3 ± 1.58	28.3 ± 1.18	28.3 ± 0.23	28.1 ± 0.69	0.5534
Σ n-3	16.3 ± 0.27	17.5 ± 1.24	16.2 ± 1.34	16.6 ± 0.26	16.3 ± 0.78	0.4762
Σ n-6	11.7 ± 0.03	11.8 ± 0.36	12.1 ± 0.29	11.8 ± 0.13	11.8 ± 0.09	0.3834
Σ n-9	3.64 ± 0.25	3.34 ± 0.57	3.47 ± 0.36	3.48 ± 0.37	3.45 ± 0.35	0.9178
n-3/n-6	1.39 ± 0.02	1.47 ± 0.06	1.34 ± 0.13	1.40 ± 0.03	1.37 ± 0.07	0.3674
n-3 PUFA	13.2	14.3	13.1	13.4	13.2	0.4805
EPA/DHA	0.39	0.35	0.4	0.34	0.36	0.5764

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

chymotrypsin exhibited higher values in the 50FMFO and 50FMFO-50MIC diets compared to the 0FMFO-50MIC diet. Conversely, in the distal segment, the 50FMFO-50MIC diet displayed the highest value compared to other diets, with 50FMFO, 50FMFO-50MIC and 0FMFO-50MIC showing higher values compared to CTRL and 50FMFO-100MIC.

3.5. Gut microbiota

The 16S rRNA gene sequencing was performed on a total of 75 distal intestine content samples, yielding 306,385 high-quality reads (mean ± SD, 4085 ± 2618) and clustered into a total of 1155 ASVs. In order to assess the effects of replacement of FM, FO and SP, on the gut bacteria community during the growth process of sea bass, the gut microbiota (GM) was analysed for each dietary group at the end of the trial. Principal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances was used to evaluate the GM variations between samples (beta-diversity). Moreover, the gut microbial community diversity, within each dietary group, was represented with faith-PD, Shannon_entropy and observed features. According to our results (Fig. 1), in terms of overall GM composition, we observed a significant separation between groups. Specifically, CTRL and 50FMFO groups were segregated compared to the other groups (50FMFO-50MIC, 0FMFO-50MIC, 0FMFO-100MIC) (Adonis, *p* < 0.001). At the same time, focusing on the internal ecosystem diversity of the fish gut microbiota, we observed a significant increase of both faith_pd and Shannon indices of 0FMFO-50MIC and 0FMFO-100MIC groups compared to the control group (Wilcoxon rank-sum test, *p* < 0.05). On the other hand, within the treatment groups we observed a significant increase in the Shannon index of 50FMFO-50MIC, 0FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO group (*p* < 0.05), while regarding faith_pd index

Table 6
Plasma biochemistry in European sea bass fed with experimental diets.

	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	p Value
GLUC	91.0 ± 19.1	86.4 ± 18.5	76.2 ± 13.4	81.4 ± 17.6	81.6 ± 20.4	0.2229
Urea	10.2 ± 1.95	9.29 ± 2.66	9.17 ± 1.24	8.99 ± 2.11	8.96 ± 1.3	0.3233
CREA	0.43 ± 0.09[b]	0.26 ± 0.07[a]	0.26 ± 0.05[a]	0.20 ± 0.04[a]	0.21 ± 0.04[a]	**<0.0001**
Uric Ac	0.12 ± 0.1	0.13 ± 0.1	0.08 ± 0.05	0.10 ± 0.04	0.13 ± 0.08	0.3803
Tot Bil	0.08 ± 0.04	0.07 ± 0.03	0.07 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	0.7749
Ast	77.4 ± 40.2	78.0 ± 61.8	68.1 ± 60.3	84.4 ± 87.5	37.5 ± 21.3	0.2084
Alt	5.46 ± 2.23	4.20 ± 1.97	4.26 ± 2.91	5.14 ± 4.22	2.85 ± 1.35	0.1026
Alp	59.9 ± 11.8[ab]	62.1 ± 16[ab]	55.1 ± 6.21[a]	69.5 ± 12.84[b]	68.1 ± 12.9[b]	**0.0132**
Ck	6436 ± 4445	4081 ± 4551	3064 ± 2891	5078 ± 5856	2220 ± 1659	0.0677
LDH	77.2 ± 22.6	72.5 ± 35.7	72.9 ± 60.7	75.5 ± 60.1	55.4 ± 30.4	0.6806
Ca^{2+}	15.8 ± 1.26	15.2 ± 1.05	14.8 ± 1.05	15.1 ± 1.3	15.3 ± 1.33	0.2166
P	14.3 ± 1.53	13.2 ± 1.51	13.6 ± 0.84	13.2 ± 1.32	13.9 ± 1.30	0.1266
Mg	3.67 ± 0.50	3.45 ± 0.53	3.22 ± 0.63	3.48 ± 0.66	3.43 ± 0.66	0.3887
CHOL	280 ± 100[b]	234 ± 69.3[ab]	202 ± 65.4[a]	218 ± 51.2[ab]	189 ± 51.6[a]	**0.0084**
HDL	78.6 ± 25.4[c]	59.1 ± 10.2[b]	52.5 ± 11.9[ab]	42.1 ± 15.3[a]	42.2 ± 8.89[a]	**<0.0001**
TRIG	1758 ± 385	1717 ± 345	1668 ± 251	1778 ± 392	1682 ± 411	0.9075
TP	4.87 ± 0.45[b]	4.77 ± 0.5[ab]	4.48 ± 0.53[ab]	4.56 ± 0.58[ab]	4.32 ± 0.45[a]	**0.0277**
Alb	1.38 ± 0.16	1.33 ± 0.15	1.27 ± 0.14	1.34 ± 0.20	1.26 ± 0.12	0.2234
Alb/Glo	0.39 ± 0.02	0.38 ± 0.02	0.39 ± 0.03	0.41 ± 0.04	0.41 ± 0.03	0.0779
LAC	42.5 ± 9.7	44.3 ± 5.17	37.8 ± 7.42	42.1 ± 9.13	45.4 ± 9.96	0.1457
$CurCa^{2+}$	228 ± 37.8	202 ± 32.1	202 ± 22.1	200 ± 29.9	214 ± 34.1	0.0974
Na/K	94.7 ± 14.0	88.8 ± 14.8	89.4 ± 19.9	89.5 ± 28.5	102 ± 23.3	0.3101
Fe	137 ± 24.3[b]	110 ± 28.2[a]	116 ± 21.6[ab]	127 ± 22.5[ab]	130 ± 21.3[ab]	**0.0184**
Na^+	174 ± 5.35[a]	178 ± 3.39[ab]	176 ± 3.39[a]	177 ± 3.73[ab]	181 ± 5.68[b]	**0.0006**
K^+	1.88 ± 0.3	2.06 ± 0.39	2.06 ± 0.45	2.15 ± 0.59	1.86 ± 0.44	0.3155
Cl	138 ± 5.51[a]	143 ± 3.05[ab]	143 ± 3.50[ab]	142 ± 4.38[ab]	146 ± 5.64[b]	**0.0010**

Data are given as the mean ($n = 15$ diet^{-1}) ± SD. Different letters indicate significant difference (One-way ANOVA, p values ≤ 0.05 are indicated in bold) between treatments. GLU, glucose, (mg dL^{-1}); Urea, (mg dL^{-1}); CREA, creatinine, (mg dL^{-1}); Uric Ac, uric acid, (mg dL^{-1}); Tot Bil, total bilirubin, (mg dL^{-1}); Ast, aspartate aminotransferase, (U L^{-1}); Alt, alanine aminotransferase (U L^{-1}); Alp, alkaline phosphatase (U L^{-1}); Ck, creatine kinase, (U L^{-1}); LDH, lactate dehydrogenase, (U L^{-1}); Ca^{+2}, calcium, (mg dL^{-1}); P, inorganic phosphorus;, (mg dL^{-1}); Mg, magnesium, (mg dL^{-1}); CHOL, cholesterol, (mg dL^{-1}); HDL, high density lipoprotein; TRIG, triglycerides, (mg dL^{-1}); TP, total protein, (mg dL^{-1}); Alb, albumin, (g dL^{-1}); Alb/Glo, albumin/globulin; LAC, lactate (mmol L^{-1}); $CurCa^{2+}$, current calcium (mg dL^{-1}); Na/K, sodium/potassium; Fe, iron, (µg dL^{-1}); Na^+, sodium, (mEq L^{-1}); K^+, potassium, (mEq L^{-1}); Cl, chloride, (mEq L^{-1}); SD, standard deviation.

Table 7
Digestive enzymes activities (U g tissue^{-1}) measured in European sea bass.

	CTRL	50FMFO	50FMFO-50MIC	0FMFO-50MIC	0FMFO-100MIC	p Value
Proximal Intestine						
AP	5.94 ± 0.88[a]	8.58 ± 0.84[b]	9.24 ± 1.51[b]	13.3 ± 0.84[c]	4.82 ± 0.97[a]	**<0.0001**
LANP	0.76 ± 0.1	0.74 ± 0.08	0.71 ± 0.09	0.77 ± 0.1	0.63 ± 0.21	0.2761
Trypsin	0.15 ± 0.08	0.24 ± 0.12	0.23 ± 0.14	0.32 ± 0.16	0.39 ± 0.19	0.1546
CT	1.74 ± 1.84[a]	8.20 ± 1.94[c]	11.0 ± 1.97[c]	3.57 ± 1.61 [b]	1.46 ± 1.92[a]	**<0.0001**
TAP	1008 ± 668	830 ± 429	1431 ± 381	917 ± 325	1515 ± 684	0.0217
Distal Intestine						
AP	4.91 ± 2.57[ab]	4.58 ± 4.61[a]	5.88 ± 4.42[b]	8.19 ± 3.59[c]	4.07 ± 3.65[a]	**<0.0001**
LANP	0.78 ± 0.08	0.60 ± 0.1	0.76 ± 0.09	0.80 ± 0.1	0.67 ± 0.21	0.0611
Trypsin	0.54 ± 0.18	0.51 ± 0.26	0.58 ± 0.27	0.82 ± 0.46	0.34 ± 0.34	0.0742
CT	3.92 ± 1.87[a]	8.29 ± 1.29[b]	14.3 ± 1.68[c]	6.73 ± 0.98[b]	1.39 ± 0.62[a]	**<0.0001**
TAP	2096 ± 943	2607 ± 868	2449 ± 1694	2665 ± 756	3318 ± 1616	0.3435

Data are given as the mean ($n = 3 \pm$ SD). In each line, different superscript letters indicate significant differences among treatments (p values ≤ 0.05 are indicated in bold).
AP: Alkaline phosphatase.
LANP: Leucine aminopeptidase.
CT: Chymotrypsin.
TAP: Total alkaline protease.

only 0FMFO-100MIC group shown a significantly higher value compared to 50FMFO group ($p < 0.05$). While only 50FMFO group showed a significant reduction of Shannon index compared to the CTRL group ($p < 0.05$). The overall GM composition at different phylogenetic levels was investigated, as reported at phylum and family level in Fig. 2, and Supplementary Table 1, while specific genera significant variations were highlighted in Fig. 3. More specifically, at phylum level the most abundant taxa observed was Firmicutes (with an overall relative abundance mean of 97%). The most represented families were *Streptococcaceae* (r.ab. mean ± SEM 60.0 ± 3.5% CTRL; 28.4 ± 1.8% 50FMFO; 20.3 ± 1.6% 50FMFO-50MIC; 35.1 ± 4.0% 0FMFO-50MIC; 34.6 ± 3.2%

0FMFO-100MIC), and *Lactobacillaceae* (34.4 ± 3.7% CTRL; 68.5 ± 1.9% 50FMFO; 55.0 ± 3.8% 50FMFO-50MIC; 38.0 ± 4.6% 0FMFO-50MIC; 27.2 ± 6.2% 0FMFO-100MIC) all belonging to Firmicutes phylum. Focusing on the specific variation between each group at genera level, we observed a significant decrease in the relative abundance of *Lactococcus*, *Bifidobacterium*, *Granulicatella*, *Lacteicaseibacillus* and *Streptococcus* genera in treatment groups compared to the CTRL group (Wilcoxon rank-sum test, $p < 0.05$), as shown in Fig. 3. On the other hand, we observed a significant relative abundance increase of *Pediococcus*, *Clostridium* sensu stricto *1*, *Leuconostoc* and *Turicibacter* genera in treatment groups compared to the CTRL group (Wilcoxon rank-sum
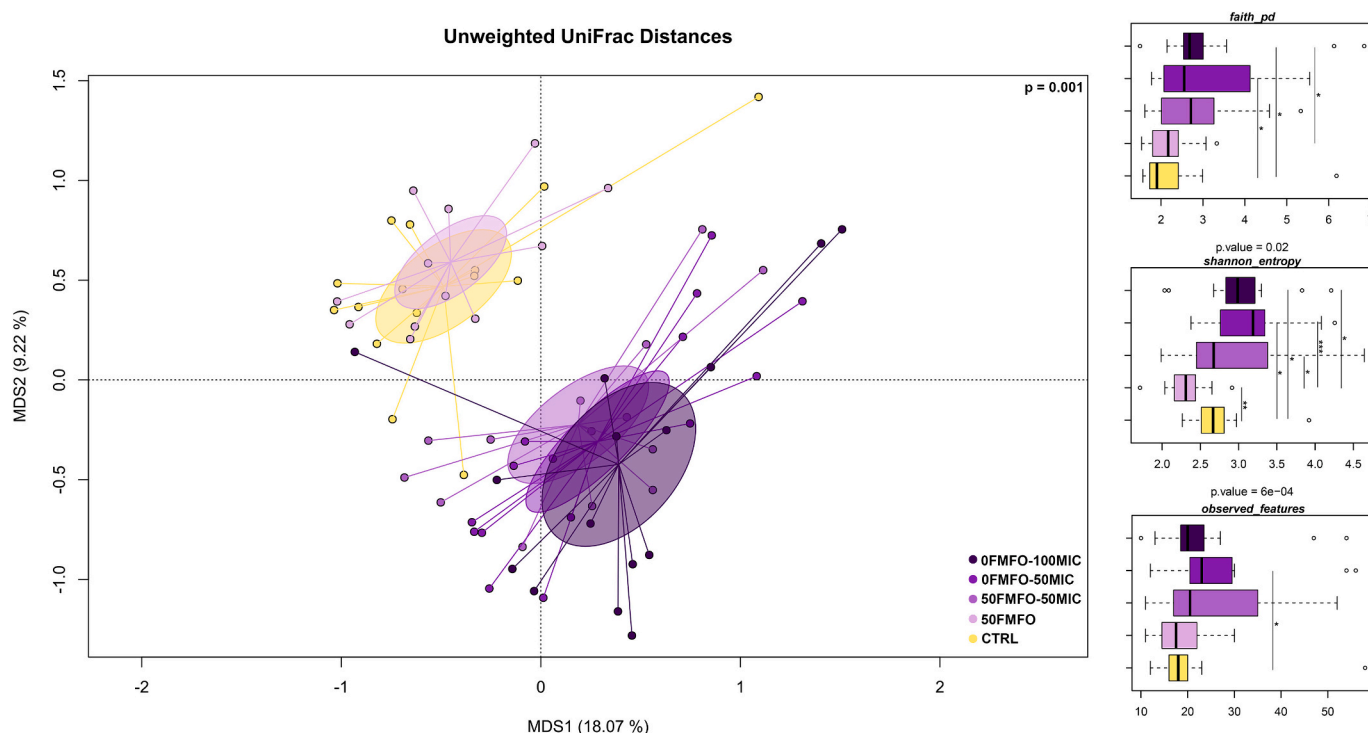
Fig. 1. Beta diversity and alpha diversity of gut microbiota of sea bass fed with experimental diets over 88 days. On the left, Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between gut microbiota composition of animals fed with experimental diets. Significant separations were highlighted (permutation test with pseudo-F ratios Adonis; $p = 0.001$). On the right, Boxplots of alpha diversity values with 3 metrics, faith_pd, shannon_entropy and observed_features (ASVs). Faith_pd and Shannon indices shown significant general variations (Kruskal-Wallis test $p < 0.05$) of alpha diversity among dietary groups, with specific significant variations between groups highlighted by a line and a different number of * based on the p value (Wilcoxon rank-sum test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
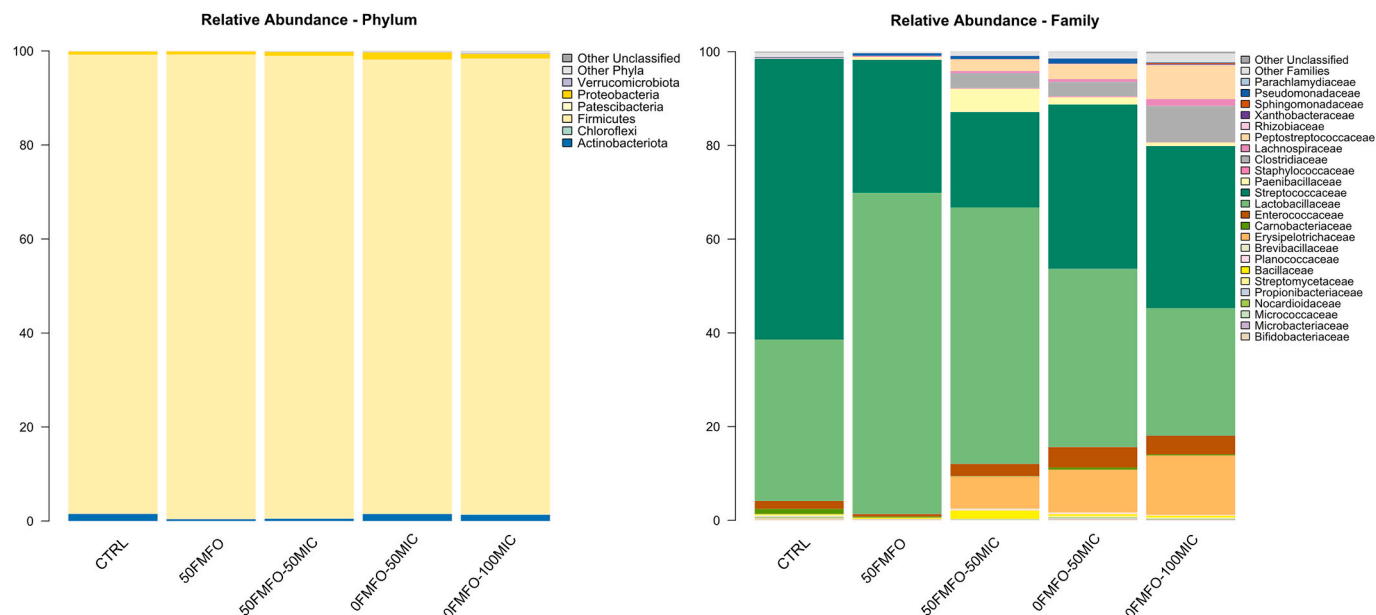
Fig. 2. Microbiota composition of distal gut content of sea bass fed experimental diets. Bar plot summarizing the microbiota composition at phylum (left) and family (right) of fish intestinal content. Only phyla and families with a relative abundance ≥0.5% in at least 2 samples are shown.

test, $p < 0.05$; Fig. 3). Focusing the attention on the pinpoint variations within the treatment groups, we observed a significant decrease of *Pediococcus* genus in 0FMFO-100MIC group compared to 50FMFO ($p < 0.05$), a significant decrease of *Bifidobacterium* genus in 50FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO group ($p < 0.05$) and a significant decrease in the relative abundance of *Leuconostoc* genus in

the 0FMFO-50MIC and 0FMFO-100MIC groups compared to 50FMFO ($p < 0.05$; Fig. 3). Furthermore, always compared to 50FMFO group, we observed a significant increase in the relative abundance of *Clostridium* sensu stricto *1* and *Turicibacter* genera in all the other treated groups ($p < 0.01$; Fig. 3).
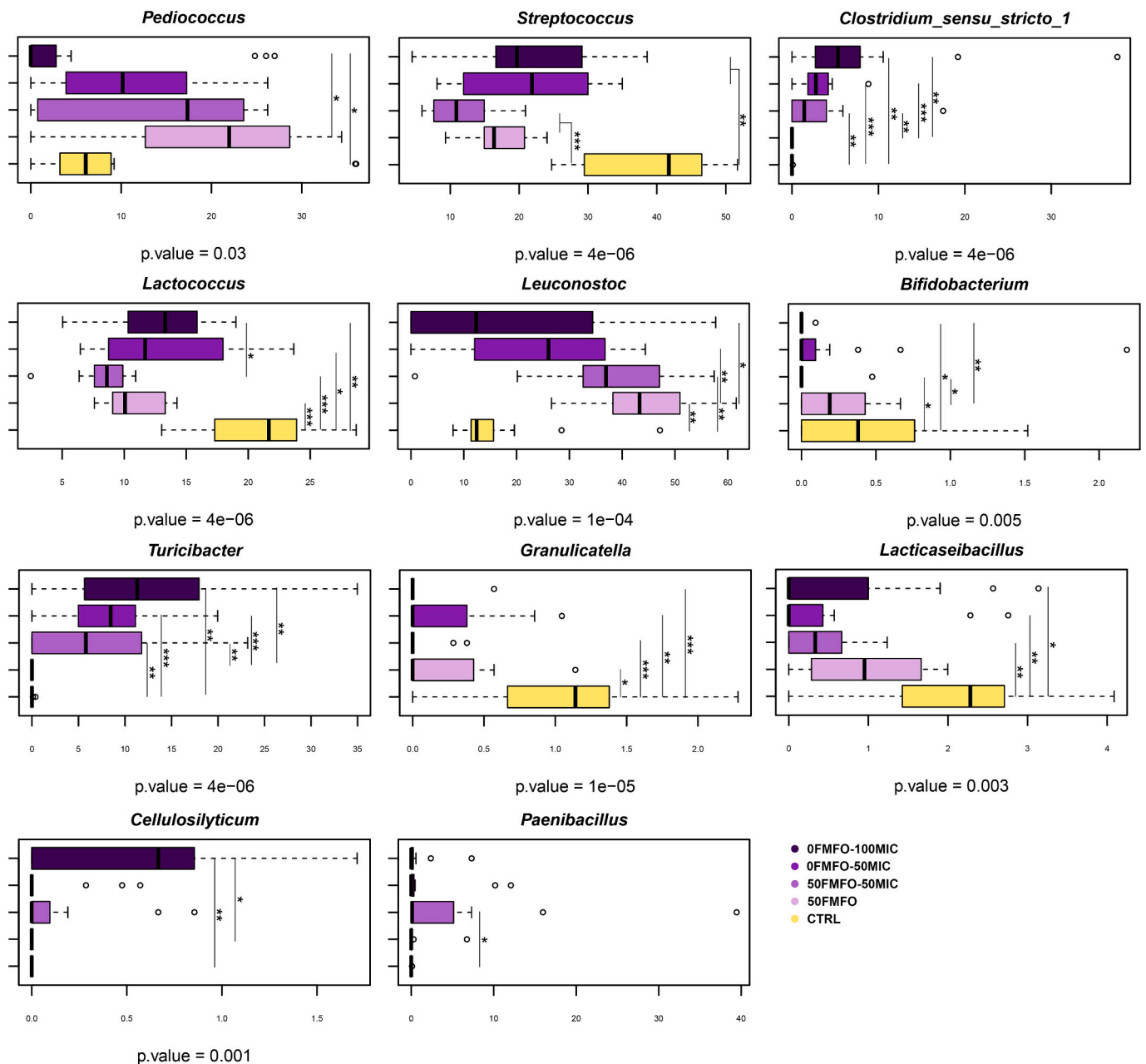
Fig. 3. Taxonomic composition of bacterial communities of distal gut content of sea bass fed experimental diets. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Only genera with a mean relative abundance $\geq 0.5\%$ in at least 2 samples were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

4. Discussion

In aquaculture, sustainable feed production and circularity are crucial for long-term viability and environmental conservation. Responsible ingredient sourcing and waste reduction support marine ecosystem health, while circular models prioritize resource efficiency and waste reduction, enhancing economic sustainability and minimizing environmental impact. The focus of the current study aligns perfectly with these principles as circular ingredients are tested for European sea bass aquaculture. Utilizing bycatch and trimmings as feed ingredients reduces waste and minimizes the environmental impact of fishing activities, contributing to more responsible and sustainable fishing practices. This approach maximizes the utilization of harvested fish and alleviates pressure on wild fish populations, promoting more efficient resource use within the aquaculture industry. Additionally, substituting soymeal with microalgae, such as *Nannochloropsis*, offers a sustainable alternative with potential nutritional and health benefits, including a blend of essential amino acids, healthy fatty acids as well as vitamins and pigments (Nagappan et al., 2021). Marchi et al. (2023a, 2023b) showed that the use of fishery and aquaculture by-products from mackerel and sardines is a valid strategy to totally replace wild-caught FM and FO in European sea bass at the on-growing stage. Similarly, the results of growth, FI and feed utilization reported in the present study confirm also for juveniles the possibility of totally replace wild-caught FM and FO using trimmings without any negative effect on the overall growth and feed efficiency indicators. In some cases, the substitution of wild-caught FM with by-product meal or with microalgae led to an improvement of growth and feed utilization as for the olive

flounder (*Paralichthys olivaceus*) fed with tuna by-product (Kim et al., 2014) or for Nile tilapia (*Oreochromis niloticus*) fed with a microalgae-blend (Sarker et al., 2020). For Pangasius catfish, the utilization of by-products from the filleting industry has been implemented in several farms (Paripatananont, 2002). However, the quality of FM and FO from aquatic by-product may vary from the wild-caught one where FM produced from by-products and trimmings contains a lower protein content (as low as 50–55%) and high ash levels (up to 20–30%). Similar to the findings of this study, multiple prior research efforts have demonstrated that integrating dietary microalgae into fish feed typically does not lead to significant deviations in growth performance compared to control diets. For example, *Nannochloropsis oceanica* in Atlantic salmon, *Isochrysis* sp. and *Tetraselmis suecica* in European sea bass, *Arthrospira maxima* in red tilapia and *Scenedesmus almeriensis* in gilthead seabream, have all been investigated (Gong et al., 2020; Tibaldi et al., 2015; Vizcaíno et al., 2014, Tulli et al., 2012). Specifically, recent studies on European sea bass have shown that incorporating dietary microalgae levels of up to 15–20% has resulted in growth and feed efficiency parameters similar to those observed in the present study. These parameters include specific growth rates (SGR) ranging from 1.00 to 1.70 and feed conversion ratios (FCR) from 1.15 to 1.64 (Pascon et al., 2021; Valente et al., 2019). Concerning body composition, protein content was similar in fish fed 50% FM and FO from by-products compared to control diet. However, when SBM was substituted by 50% of MIC and FM and FO were replaced by trimming derivates, body protein content was lower compared to 50FMFO. Moreover, lipid composition was higher when FM and FO were replaced with by-product but also with partial substitution with MIC compared to the control diet. This increment is in accordance with a previous study conducted on European sea bass (Mota et al., 2023) and gilthead sea bream (Valente et al., 2019) fed with *Nannochloropsis* enriched diet, caused by high proportion of LC-PUFA in microalgae.

In contrast with previous study by Randazzo et al. (2023), where a blend of two microalgae whole cell dry biomass was utilized, in the present study, values of digestibility and nutritional indices were not affected by microalgae inclusion. Additionally, fatty acid composition values are often used to evaluate the efficiency of microalgae-enriched diets to replace FO. In this study, the inclusion of microalgae allowed the maintenance of essential FA levels, such as DHA, EPA and ARA. This is in accordance with a previous study conducted on European sea bass juveniles fed with 5 enriched diets with two different species of microalgae (*Pavlova viridis* and *Nannochloropsis* sp.) at two percentages as replacement of FO. Here it was shown how the percentages of the microalgae inclusion can influence the fatty acid composition of the animal fillet (Haas et al., 2016).

Analyses of plasma biochemistry reveal that the experimental diets had minor effects on the health of the organism. However, only a few plasma parameters showed significant differences between treatment groups. Among these, creatinine (CREA), a metabolic waste product of creatine produced at the kidney level (He et al., 2020), emerged as notable. Creatine, a nitrogenous organic acid naturally found in metabolically active tissues of all vertebrates, primarily serves to assess muscle condition (Fazial et al., 2018). Elevated levels of creatinine were observed in the blood of fish fed wild-caught fishmeal-fish oil (FMFO), indicating heightened creatine metabolism in these fish. This is likely due to the spontaneous formation of creatinine during the conversion of creatine to phosphocreatine, as proposed by Marchi et al. (2023a, 2023b). Additionally, the experimental diet influenced plasma cholesterol levels, showing a decrease when microalgae were incorporated. This reduction occurred alongside a decrease in soy protein concentrate, which is recognized for its hypercholesterolemic effects in European sea bass (Bonvini et al., 2018). In contrast, earlier studies did not observe any change in plasma cholesterol levels in European sea bass and Nile tilapia fed with *Nannochloropsis oceanica* (Batista et al., 2020) and *Nannochloropsis oculata* (Zahran et al., 2023), respectively. Nevertheless, microalgae, including *Nannochloropsis* species, are acknowledged as rich

sources of phytosterols that could support lowering blood cholesterol levels (Randhir et al., 2020). Plasma proteins serve as reliable indicators of the well-being of well-nourished animals (Peres et al., 2014). Even if the results of TP decreased, they remained within the standard range. Plasma electrolytes are valuable indicators of cellular health. In this study, all values tended to increase, particularly with the higher level of 0FMFO-100MIC compared to the control diet, which exhibited lower values, with a difference of 4%. Therefore, the partial and total replacement of wild-caught fishmeal and fish oil with soy derivatives and microalgae did not adversely affect animal health. Enzymatic activity is often used as an indicator of digestibility and assimilation in fish feed enriched with microalgae. In this study, we observed no differences in trypsin activity between treatments excluding any potential anti-trypsin action of the vegetable ingredients employed (Biswas et al., 2022). Moreover, the activity of AP reached its peak in fish fed diet with total replacement of wild-caught FM and FO and with half replacement of soy derivates with microalgae (50FMFO-50MIC). Alkaline phosphatase is a key enzyme of the intestinal brush borders, serving as an indicator of the intestinal integrity and as a general marker of nutrient absorption. The increases of these activities may enhance the overall efficiency of digestive and absorptive processes (Silva et al., 2010; Vizcaíno et al., 2014).

A clear impact of diets has been shown also through the analyses of the gut microbiome. In particular, the inclusion of a different percentage of microalgae drove the shifting of the gut microbiome community, clearly segregating from the gut bacterial communities of fish fed diet without microalgae. In particular, Clostridiaceae, Planococcaceae, Peptostreptococcaceae, Enterococcaceae and Erysipelotrichaceae, were the most significant family responsible for driving the segregation between diets. These six families are found extremely abundant in carnivorous fish species and are well known as promoters of healthy intestinal epithelium (Egerton et al., 2018). At genera level, most promising results are represented by *Pediococcus, Leuconostoc* and *Turicibacter*, belonging to Firmicutes phylum, with a relative increasing in abundance from control diet to diet with higher replacement of FM, FO and SP with microalgae. *Pediococcus* is a genus of bacterium recognized and widely used as a probiotic in aquaculture for marine species such as Atlantic salmon (Jaramillo-Torres et al., 2019) as well as in European eel larval stages (Politis et al., 2023). Additionally, a positive effect of this probiotic on fish health, increasing growth, influencing body composition and promoting intestinal health has been shown also for European sea bass (Eissa et al., 2022). Moreover, probiotic *P. acidilactici* supplementation worked positively in combination with β-glucans or fructooligosaccharides (FOS), in a synergic effect to promote fish growth performance (Torrecillas et al., 2018). Few studies have investigated the use of microalgae as a potential prebiotic (Oviedo-Olvera et al., 2023), however recent studies aimed to show the positive effect of different microalgae strains on probiotics development and efficacy (Patel et al., 2021). *Turicibacter* is a genus of Firmicutes, largely present in European sea bass gut microbiota (Ofek et al., 2021) and other commercial species such as Tilapia. *Turicibacter* genus have a recognized role in the modulation of bacterial colonization, regulation of host energy metabolism, and host immunity (Bereded et al., 2022). Moreover, *Turicibacter* could produce short-chain fatty acids, and it was positively correlated with the content of butyric acid which is a functional fatty acid that inhibits enteritis and repairs the intestine (Hao et al., 2022). The positive effects of *Nannochloropsis* inclusion in feed on the intestinal microbiota of European has been recently demonstrate by Ferreira et al. (2022), marking the importance of integrating microalgae in fish feed, not only for their sustainability as aquafeed but also for their positive effect on the gut health.

5. Conclusion

This study highlighted the potential of totally replacing wild-caught FM and FO and soy protein using by-products from fisheries and aquaculture of mackerel and sardine as well as salmon trimming and the

microalgae *Nannochloropsis* sp., without affecting growth performance. Growth parameters considered in this study do not seem to be affected by the substitution of wild-caught FM and FO and soy protein with more sustainable alternatives. Moreover, the study showed that the experimental diets influenced the activity of the main digestive enzymes in the proximal and distal part of the intestine. Specifically, dietary replacement of 100% FM and FO with circular substitutes and 50% replacement of soymeal with microalgae increase the activity of alkaline phosphatase and chymotrypsin. Based on the data collected in this study, we emphasize the multiple advantages connected with the use of microalgae as a protein source not only having no negative effects on the growth and health of the animal but, on the contrary, promoting important functions such as the absorption of nutrients at intestinal level, avoiding antinutritional factors. Moreover, we observed that the diets with by-products from fishery and aquaculture and microalgae had a positive effect on the richness and abundance of the microbiota, favouring those strains with a demonstrated beneficial effect on the animal's health. Hence, the integration of trimmed fish meal, oil, and microalgae presents a promising alternative to the conventional ingredients extensively employed in aquafeed production. Embracing the utilization of these components on a broader scale is imperative, not solely due to their benign impact on the organism's health and physiology but also owing to their circularity and reduced environmental footprint. By promoting the adoption of such sustainable practices, the aquaculture industry can make significant steps towards promoting ecological balance and mitigating its ecological footprint. This shift not only reflects a commitment to responsible resource management but also paves the way for a more resilient and environmentally conscious approach to aquaculture production.

CRediT authorship contribution statement

A. Marchi: Writing – review & editing, Writing – original draft, Methodology, Investigation. **E. Benini:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **F. Dondi:** Writing – review & editing, Methodology, Investigation. **M.G. Ferrari:** Methodology, Investigation. **D. Scicchitano:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **G. Palladino:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **M. Candela:** Writing – review & editing, Methodology, Conceptualization. **R. Cerri:** Writing – review & editing, Methodology, Investigation. **A. Di Biase:** Writing – review & editing, Methodology, Investigation, Conceptualization. **A.J. Vizcaíno:** Writing – review & editing, Methodology, Investigation. **F.J. Alarcón-López:** Writing - review & editing, Methodology, Investigation. **F.G. Acién:** Writing – review & editing, Methodology, Investigation. **P.P. Gatta:** Funding acquisition, Conceptualization. **A. Bonaldo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **L. Parma:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741015.

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