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Dietary *Paecilomyces variotii* single-cell protein supports growth and intestinal health in gilthead sea bream

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ABSTRACT

The effects of dietary single-cell proteins (SCP) derived from the filamentous fungus *Paecilomyces variotii* (PV) on growth, plasma biochemistry, and gut health were assessed in gilthead sea bream. Fish, with an initial average weight of 120.3 ± 0.02 g, were fed four experimental diets for 104 days, incorporating different levels of PV SCP meal (0% CTRL, 5% SCP5, 7.5% SCP7.5, and 10% SCP10) as a partial replacement for fishmeal (FM) starting from a FM content of 22% in the control diet. At the end of the trial fish were subjected to a 2-h acute crowding stress (80 kg m^3 biomass). At the end of the growth trial, there were no significant differences in final body weight (FBW), specific growth rate (SGR), feed conversion rate (FCR), or feed intake (FI), indicating that growth and feed utilization were not adversely affected by the inclusion of SCP. Instead, the diet containing PV upregulated the expression of genes related to nutrient transport, such as *peptide transporter 1* (*pept1*), *fatty acid-binding protein* (*fabp2*), *sodium/glucose cotransporter 1* (*slc5a1*), and *aquaporin 8* (*aquap8*), suggesting an enhanced capacity for nutrient uptake. Varying levels of dietary SCP led to an increased abundance of potentially beneficial lactic acid bacteria, as well as the genus *Paenibacillus*, known for its chitinase activity, which may enhance nutrient availability through chitin degradation. Crowding stress (T2) significantly altered most plasma parameters, reflecting a negative impact on the fish's metabolic functions. Notably, fish fed SCP-based diets exhibited substantially lower plasma levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK), suggesting a mitigating effect on stress-induced tissue damage. This protective role is further supported by the upregulation of *glutathione reductase* (*gr*) expression, indicating enhanced antioxidant defenses capable of counteracting oxidative damage in muscle and other tissues. In conclusion, these findings confirm the potential of the filamentous fungus *Paecilomyces variotii* as a valuable protein source for gilthead sea bream, not only as a partial substitute for conventional proteins in aquafeeds but also as a functional ingredient with health-promoting properties.

1. Introduction

In recent decades, plant-based protein sources have been utilized as alternatives to fish meal (FM) in aquafeeds. However, these ingredients pose significant sustainability concerns, including a high carbon footprint and direct competition with crops intended for human consumption (Wilkinson and Young, 2020). This highlights the need for novel, sustainable, and nutritionally adequate feed ingredients. Among these, single-cell proteins (SCP), microbial biomass derived from yeast, bacteria, fungi, and microalgae, represent a promising alternative due to their efficient production through fermentation and reduced environmental impact (Sharif et al., 2021; Marchi et al., 2023). SCPs can be

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cultivated on organic-rich industrial and agricultural residues and require less land, water, and time than traditional plant-based sources (Sharif et al., 2021). In particular, SCP derived from filamentous fungi are gaining attention because of their ability to utilize diverse low-cost substrates, their consistent nutritional value (40–60% protein), and their content of essential amino acids, polyunsaturated fatty acids, vitamins, and minerals (Karimi et al., 2019; Hooft et al., 2024). Furthermore, fungal cell walls contain functional components such as mannan-oligosaccharides (MOS), β-glucan, chitin, and chitosan, which may provide health benefits in fish by enhancing growth performance (Refstie et al., 2010; Staykov et al., 2007), modulating gut structure (Schmidt et al., 2017), exhibiting antioxidant activity (Karimi et al., 2023), and improving disease resistance (Gokulakrishnan et al., 2023).

Recent studies have evaluated the nutritional value of the filamentous fungi *Paecilomyces variotii* (PV) in fish, mostly in salmonids. In Atlantic salmon (*Salmo salar*), PV successfully replaced up to 20% of the dietary crude protein derived from FM and vegetable ingredients, thereby improving feed conversion ratio (FCR) and nutrient utilization (Hooft et al., 2024). A study on rainbow trout (*Oncorhynchus mykiss*) demonstrated that the use of PV as an alternative protein source in aquafeeds has the potential to enhance sustainability without compromising feed efficiency. In fact, diets incorporating PV could maintain FCR comparable to those of conventional soy-based feeds while offering environmental advantages (Bergman et al., 2024). In addition, PV has shown immunomodulatory properties. In Atlantic salmon, exposure to PV in primary head kidney and spleen leukocyte cultures induced pro-inflammatory cytokines and antimicrobial effectors (Mensah et al., 2024). Similarly, dietary inclusion of 4% and 16% enhanced specific IgM production against *Vibrio anguillarum* and reduced proteins associated with inflammatory pathways, including Salmonella infection, apoptosis, and necroptosis, in the head kidney (Mensah et al., 2025). Other studies have explored the effects of dietary filamentous fungi in fish. In reba carp (*Cirrhinus reba*), SCP derived from the filamentous fungus *Aspergillus niger,* led to improvements in growth performance (Chakraborty et al., 2022). In rainbow trout (*Oncorhynchus mykiss*), dietary inclusion of *Neurospora intermedia* modulated the gut microbiota and increased the abundance of lactic acid bacteria (Singh et al., 2021). Similarly, in Gibel carp (*Carassius auratus* gibelio), dietary supplementation with *Geotrichum candidum* improved growth and feed utilization (Noor-Ul et al., 2020).

To date, no studies have evaluated the use of PV-derived SCP in diets for gilthead sea bream (*Sparus aurata*), a key species in Mediterranean aquaculture. The present study aims to address this gap by testing, for the first time in this species the effects of dietary PV-SCP inclusion. The trial employed practical diets based on currently used commercial formulations for the species, ensuring high relevance for aquaculture applications. A holistic approach was applied, integrating growth performance, plasma biochemistry and microbiota profiling. Furthermore, we assessed intestinal gene expression related to stress, immunity, nutrient absorption, and barrier integrity, and applied a crowding stress challenge to evaluate the functional potential of PV under farming-like conditions.

2. Materials and methods

2.1. Ethics statement

Experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, under European directive 2010/63/UE relating to the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethical Committee of the University of Bologna (Italy) (protocol N° 237,050).

2.2. Experimental diets

Four experimental diets (pellet size: 4.0 mm) were formulated and produced using extrusion technology by Sparos Lda (Olhão, Portugal). Each diet contained increasing levels of SCP from PV as a substitute for FM: a control diet (CTRL) with 0% SCP, a diet with 5% SCP (SCP5), a diet with 7.5% SCP (SCP7.5), and a diet with 10% SCP (SCP10). The composition and nutritional content of the diets are presented in Table 1. SCP inclusion partially replaced FM by up to 36.5%. The diets were formulated using FM and a combination of vegetable ingredients, following a previously established approach for this fish species (Busti et al., 2025). The SCP meal used in this study was produced by RISE Processum AB (Örnsköldsvik, Sweden). Fungal biomass was used as the SCP. *P. variotii* was grown on hydrolysate from forestry residues (thin tops and branches) in 10 m^3 fermenters following the procedure described by Alriksson et al. (2014) and summarized briefly as follow: One 50 L bioreactor and one 500 L bioreactor were used for the inoculum production, and one 10,000 L bioreactor was used for the final SCP production. All cultivations were performed at 30 °C, pH 6.0, and the stirring was increased with increasing culture broth viscosity to maintain a sufficient oxygen level. A 20 L inoculum was prepared in the 50 L bioreactor and added to the 500 L inoculum reactor, which had been filled to a volume of 180 L (hydrolysate, salts, and trace elements). The

Table 1
Ingredients and proximate composition of the four experimental diets provided to sea bream (*Sparus aurata*) over 104 days. Three diets contained different inclusion levels of SCP from Paecilomyces variotii in substitution of fish meal (FM).

Ingredients%	CTRL	SCP5	SCP7.5	SCP10
Eperimental diets				
Fish meal[1]	22.00	17.99	15.97	13.96
Processum[2]	0.00	5.00	7.50	10.00
Soybean meal	11.44	11.45	11.45	11.45
Wheat flour	9.82	8.06	7.23	6.41
Corn gluten meal	26.40	26.41	26.42	26.42
Wheat gluten meal	3.07	3.07	3.07	3.07
Soybean Protein Concentrate	13.20	13.80	14.11	14.42
Rapeseed oil	7.52	6.95	6.72	6.50
Fish oil	3.22	3.75	3.96	4.17
Hydrolyzed fish meal (appetizer)	1.03	1.03	1.03	1.03
Aminoacid Mix (Met, Lys and Tau)	0.75	0.87	0.93	1.00
Monoammonium Phosphate	0.79	0.86	0.84	0.82
Vitamin and Mineral Premix[3]	0.76	0.76	0.76	0.76
Proximate Composition (%)				
Moisture	7.67	7.58	7.84	7.07
[4]Crude protein	48.5	48.2	48.2	48.8
[5]Crude fat	14.8	14.6	14.1	14.7
[6]Ash	5.67	5.40	5.22	5.19
[7]Crude Energy In feed (cal/g)	5249.3	5235.0	5231.2	5227.3

[1] Protein content in FM: 66%.
[2] Origin: Processum (Sweden). Proximate composition (g/100 g): Moisture 5.25, Proteins 46.5, Fibres13.9, Lipids 8.13, Ash 5.98.
[3] Vitamins and mineral premix (mg/kg diet, in vivo NSA: Portugal): vit. D 0.05 mg, vit. A 2.38 mg, vit. E 324.7 mg, inositol 158.4 mg, niacin 182.2 mg, pantothenic acid 67.7 mg, vit. B2 27.4 mg, vit. B1 27.7 mg, vit. B6 24.3 mg, folic acid 6.52 mg, vit. K 5.39 mg, biotin 0.96 mg, vit. B12 0.05 mg, choline 1314.6 mg, vit. C 250.3 mg, calcium 0.87 mg, cobalt 0.38 mg, copper 48.62 mg, iron 494.7 mg, magnesium 21.3 mg, manganese 25.9 mg, molybdate 0.97 mg, nickel 0.80 mg, phosphorus 0.51 mg, potassium 0.83 mg, sodium 0.14 mg, selenium 0.83 mg, sulfur 0.35 mg, zinc 52.67 mg.
[4] Crude protein was detected as total nitrogen (N*6.25) using Kjeldahl's method in accordance with AOAC International (2010).
[5] Total lipids were obtained following the Bligh and Dyer's method (Bligh and Dyer, 1959).
[6] Ash content was estimated by incineration in a muffle oven at 450 °C overnight.
[7] Crude energy was measured by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, United States).

inoculum growth lasted 24 h, after which 200 L culture broth was used to inoculate the 10,000 L reactor. The cultivation at this scale followed a fed-batch process. Samples were withdrawn during the cultivation for analyses of biomass, monosaccharides, and aliphatic acids. The harvested biomass was separated from the liquid fraction using a filter press. The proximate composition and amino acid profile of SCP and experimental diets are detailed in Table 2. Fatty acids composition of experimental diets is reported in Supplementary Table 1.

2.3. Fish and feeding trial

The trial was carried out at the Laboratory of Aquaculture within the Department of Veterinary Medical Sciences of the University of Bologna, located in Cesenatico, Italy. Fish were provided by an Italian fish farm (Il Vigneto a.r.l., Grosseto, Italy) and originated from a single batch. They were acclimated to laboratory conditions for one week. At the beginning of the trial, 40 specimens (initial weight: 120.3 ± 0.02 g) per tank were randomly placed in 12 square tanks, each with a capacity of 450 L. Experimental diets were randomly assigned in a triplicate design and administered by hand to visual satiation twice a day, at 8:30 AM and 4:00 PM, six days a week, over 104 days. Visual satiation was defined as the point at which only a few pellets reached the bottom of the tank, indicating that the fish had stopped feeding actively. Feeding procedures were designed to avoid feed losses; however, in cases uneaten pellets were observed, they were collected, dried overnight at 105 °C, and weighed for the overall feed intake calculation (Marchi et al., 2023). Tanks were supplied with natural seawater (originating from the Adriatic Sea, off the Cesenatico coast) and connected to a closed recirculation system (RAS) with an overall water volume of 6 m^3. Oxygen levels were maintained at 8.0 ± 1.0 mg L^{-1}, the temperature at 24 ± 1.0 °C, and the salinity at 25 g L^{-1}.

2.4. Crowding challenge

At the end of the growth trial (104-day feeding period), the animals underwent a 36-h fasting period. Following this, a crowding stress challenge was initiated by reducing the water level in the tanks for 2 h, increasing the biomass density from ~14 kg m^{-3} to 80 kg m^{-3} following the procedure described by Pelusio et al. (2022). After the 2-h crowding stress exposure, the water level was restored to its original state, allowing the animals a 24-h recovery period. Sampling occurred at three time points: immediately before the challenge test (time 0, T0), at the end of the 2-h exposure (time 2, T2), and 24 h after the high-density confinement (time 24, T24). This process aimed to evaluate the ability of different diets to confer advantages or disadvantages when faced with a stressful event.

Table 2
Proximate composition and amino acid profile of SCP from *Paecilomyces variotii* included in experimental diets.

	SCP	CTRL	SCP 5	SCP 7.5	SCP 10
Proximate composition (% on wet basis)					
Moisture	5.25	7.67	7.58	7.84	7.07
Crude protein	46.5	48.5	48.2	48.2	48.8
Crude fat	8.13	14.8	14.6	14.1	14.7
Ash	5.98	5.67	5.40	5.22	5.19
Gross energy (MJ/Kg)	4612.8	5249.3	5235.0	5231.2	5227.3
Amino Acid Profile (% on wet basis)					
Lysine	2.76	2.40	2.41	2.41	2.42
Methionine	0.67	1.25	1.29	1.32	1.35
Threonine	1.86	1.90	1.88	1.87	1.86
Valine	2.13	2.25	2.22	2.21	2.19
Isoleucine	1.71	1.90	1.88	1.88	1.87
Arginine	2.45	2.75	2.71	2.69	2.67
Leucine	2.91	4.79	4.75	4.73	4.71
Histidine	0.94	1.17	1.16	1.16	1.16
Phenylalanine	1.63	2.47	2.45	2.45	2.44
Tyrosine	1.38	1.96	1.96	1.95	1.95
Glycine	2.01	2.30	2.20	2.15	2.10
Serine	1.92	2.39	2.38	2.37	2.37
Proline	1.68	3.15	3.10	3.08	3.06
Alanine	3.05	3.01	2.99	2.99	2.98
Aspartic acid	n.a.	4.04	4.02	4.01	4.01
Glutamic acid	n.a.	8.70	8.61	8.57	8.52

Single cell proteins (SCP) origin: RISE Processum (Sweden). SCP5 = 50 g kg^{-1} SCP. SCP7.5 = 750 g Kg^{-1} SCP. SCP10 = 100 g kg^{-1} SCP.

2.5. Sampling

At the beginning and end of the growth trial, fish were anesthetized with tricaine methanesulfonate (MS-222) at a concentration of 100 mg L^{-1} and individually weighed, while fish sacrificed for analyses were previously euthanized using an overdose of MS-222 at a concentration of 300 mg L^{-1}. At the end of the growth trial, specific growth rate (SGR), feed intake (FI), and FCR were calculated. Proximate composition analysis of the carcasses was conducted at the start of the growth trial using a pooled sample of 15 fish and at the end using a pooled sample of 5 fish per tank. Protein efficiency rate (PER), gross protein efficiency (GPE), and gross lipid efficiency (GLE) were calculated. Still, at the end of the growth trial, distal intestine content from 5 fish per tank was aseptically collected and stored at −80 °C for gut microbiome. Additionally, the wet weight of the viscera, liver, and perivisceral fat was individually recorded for 5 fish per tank to determine the viscerosomatic index (VSI), hepatosomatic index (HSI), and mesenteric fat index (MFI).

At each time point: T0, T2, and T24, 5 fish per tank (15 fish per treatment) were anesthetized, and their blood was collected from the caudal vein. Samples were then centrifuged (3000 x*g*, 10 min, 4 °C) and plasma shares were stored at −80 °C until analysis (Parma et al., 2023). Thereafter, from the same fish batch sampled, distal intestine samples (20–30 mg) were aseptically collected from 3 fish per tank and stored in RNA later 1:5 *w/v*. Then, samples were maintained at +4 °C overnight and subsequently stored at −32 °C. Samples have been processed for RNA extraction and gene expression analysis.

2.6. Analytical methods

The proximate composition analysis was performed on diets and whole bodies. Moisture content was determined by measuring the weight loss resulting from overnight drying of samples in an oven at 105 °C. Crude protein was quantified by calculating total nitrogen (multiplied by 6.25) using Kjeldahl's method as per AOAC International standards (AOAC, 2010). Total lipids were extracted according to the Bligh and Dyer's method (Bligh and Dyer, 1959). Ash content was estimated by incinerating samples in a muffle oven at 450 °C overnight. Crude energy was assessed using an adiabatic calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, United States) (Busti et al., 2024).

2.7. Metabolic parameters in plasma

The analysis of plasma biochemistry was conducted on 500 μL aliquots previously collected at T0, T2, and T24. Glucose (GLU), lactate, total protein (TP), triglycerides (TRIG), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), phosphorus (P), magnesium (Mg), creatinine (CREA), uric acid, urea, total bilirubin (Tot Bil), cholesterol (CHOL), high-density lipoprotein (HDL), albumin (ALB), alanine aminotransferase (ALT), alkaline phosphatase (ALP), calcium (Ca^{2+}), potassium (K^{+}), sodium (Na^{+}), iron (Fe), chloride (Cl), and ALB/globulins (A/G) were detected by an automated analyzer (AU 480; 220 Olympus/Beckman Coulter, Brea, CA, United States) according to the manufacturer's instructions (Parma et al., 2023).

2.8. Gene expression analysis

Total RNA was extracted from distal intestine samples of all the fish using Trizol reagent and the PureLink™ RNA Mini Kit from Thermo Fisher Scientific. To remove any DNA contamination, RNA was further purified using a DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's instructions. The purity and concentration of the total RNA were measured using the Epoch Microplate Spectrophotometer by BioTeK Instruments (Winooski, USA). The integrity of the RNA was verified using Agilent 2200 TapeStation system in combination with RNA Screen Tape (Agilent 210 Technologies, CA, USA) and gel agarose electrophoresis. For the synthesis of first-strand complementary DNA (cDNA), 1.0 μg of total RNA was utilized in a 20-μl reaction that included 4 μl of the SuperScript™ IV VILO™ Master Mix kit by Thermo Fisher Scientific. Negative control reactions were performed concurrently by either omitting RNA or enzyme, as appropriate.

The qPCR primers were designed from NCBI *S. aurata* Reference Sequences using the Primer-BLAST tool of NCBI. Primer information is shown in Table 3. The selected genes were related to different functions such as stress response (*glutathione reductase*, *gr*, *glucocorticoid receptor*, *gcr*, *heat shock protein 70*, *hsp70*), immunity (*interleukin 1β*, *il-1β*, *interleukin 6*, *il-6*, *interleukin 8*, *il-8*, *interleukin 10*, *il-10*, *tumor necrosis factor alfa*, *tnf-α*, *T-cell receptor beta*, *tcrβ*, *major histocompatibility complex I*, *mhc i*, *proliferating cell nuclear antigen*, *pcna*, *lysozyme*, *lyz*), nutrient absorption (*peptide transporter 1*, *pept 1*, *fatty acid binding protein*, *fabp2*, *sodium/glucose cotransporter 1*, *slc5a1*, *chemerin chemokine-like receptor 1*, *cmklr1*, *aquaporin8*, *aquap8*), and mucosal barrier (*claudin15*, *cldn15*, *aminopeptidase N*, *aminopn*).

To determine the optimal annealing temperatures, gradient PCR reactions were performed for all primer pairs. The specificity of amplification was verified through melting curve analysis and visual inspection of the PCR products using agarose gel electrophoresis. Additionally, the PCR efficiency for each gene assay was evaluated by conducting 2-fold serial dilutions of randomly pooled complementary DNA (cDNA). The expression of target genes was analyzed using the LightCycler 96 system by Roche Diagnostics in Basel, Switzerland. In a 10-μl DNA amplification reaction, each included 2 μl of PCR grade water, 2 μl of a 1:10 diluted cDNA template, 5 μl of LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), and 0.5 μl of each forward and reverse primer at a concentration of 10 μM. Each sample was assayed in duplicate, and a no-template control was included. The three-step qPCR run consisted of an enzyme activation step at 95 °C (5 min), followed by 40 cycles at 95 °C (10 s), the annealing temperature (10 s), and 72 °C (15 s), and concluded with a melting curve step. The mean normalized expression of the target genes was calculated from the raw Cq values as described by Muller et al. (2002) using the formula: Etar^CqTar / Eref^CqRef. *Glyceraldehyde-3-phosphate dehydrogenase 2* (*gapdh2*), *β-actin* (*β-act*), and *ribosomal protein s18* (*rps18*) were evaluated for use as reference genes based on their overall coefficient of variation and interspecific variance (Kortner et al., 2011). Ultimately, due to its inter- and intraspecific stability, Rps18 was chosen as the reference gene.

2.9. Gut bacterial community DNA extraction and sequencing

Microbial DNA was extracted and analyzed from the individual distal intestine content of five fish per tank, following the methodology described by Marchi et al. (2024). Subsequently, the 16S rRNA gene analysis was conducted by amplifying the V3-V4 hypervariable region using KAPA HiFi HotStart ReadyMix (from KAPA Biosystems), along with 341F and 785R primers modified to include Illumina adapter overhang sequences. In summary, the thermal cycling process involved an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. As per the recommendations in the Illumina protocol "16S Metagenomic Sequencing Library Preparation" for the MiSeq system, PCR products were purified using Agencourt AMPure XP magnetic beads. A limited-cycle PCR was performed to create an indexed library using Nextera technology, followed by a second purification with AMPure XP magnetic beads. The sequencing process took place on the Illumina MiSeq platform, utilizing a 2 × 250 bp paired-end protocol as per the manufacturer's instructions in San Diego, CA. Following the sequencing, raw data underwent processing through a combination of PANDAseq and QIIME2 pipelines (Bolyen et al., 2019). High-quality reads were obtained after filtering based on length (ranging from 350 to 550 bp) and quality, using default parameters. These reads were then cleaned and grouped into

Table 3
Primer pairs and related information for real-time PCR assays.

Gene symbol	5′ -3′ primer sequence		Amplicon size (bp)	Anneling temperature (°C)	Efficency	Gene bank accession no.
	Forward	Reverse				
rps18	AGGGTGTTGGCAGACGTTAC	CTGCCTGTTGAGGAACCAGT	161	60	2.09	XM_030410417.1
gr	TCCTCCCGATGTCACTCAAC	TGGAAGTTGTCACGTCGGAG	123	60	1.81	XM_030396244.1
gcr	GCAGTTGAAGGCCAGCATAAC	GAAGACATTTCCTGAAGCGGC	106	60	1.97	XM_030437676.1
hsp70	TCATCAACGAGCCAACTGCT	GATGGGTATCTCCGGCAGTG	168	60	1.86	XM_030438845.1
il-1β	AGATTGCCCACCATCCCATC	TGAATCCAACACGTTGCCCT	86	60	1.82	XM_030416076.1
il-6	ATACGACAGCTCCCTCCGAC	ACAGCCTGCACAGCACTTAC	121	60	1.82	XM_030412636.1
il-8	GCCACTCTGAAGAGGACAGG	AGCACATCTCTTCCAGTCAGC	120	60	2.01	XM_030413378.1
il-10	TTTGTGAGCTTTCTGAGCCG	ATGTGTAGGACGCAATATGGTCT	99	60	1.94	XM_030418889.1
tnfα	CAGGCGTCGTTCAGAGTCTC	GATCCTGTGGCTGAGAGGTG	81	60	1.94	XM_030392876.1
tcrβ	AAGTGCATTGCCAGCTTCTT	GTAGACGCAGCCCTTGATGA	171	60	2.11	XM_030391829.1
mhc-i	CCCTCAGTGTCTCTCCTCCA	CAGACGAGTGAGGCTCTGTG	89	60	1.78	XM_030412951.1
pcna	CCTCCGTCTTGCAGTGAGAG	CTTGTAGCGTCACTCCGTCA	147	60	2.11	XM_030418162.1
lyz	CCTGCTACAGTCTGCCAATCT	CTGTGGCTCGTCTGACTGAT	160	60	1.87	XM_030424503.1
pept 1	CGTCGTTTCTCAGAGTGCCT	TCTGTGTTCGTCCACCTTCG	143	60	2.02	XM_030410196.1
fabp2	CGAGCACATTCCGCACCAAAG	CGGGTGAATTTGCCTTTCAGC	130	60	1.85	XM_030430852.1
slc5a1	TTGCTTTCAACGAGGTGGGA	GCAGTCTGCGCTGATGTTTC	92	60	1.92	XM_030434664.1
cmklr1	TGTCTCTCTGTACGGCAACG	CCCAGATGACCACTCCGTTC	161	60	1.92	XM_030393359.1
aquap8	ATTGCCATCTACCTGTGCGG	CAGTTGGCTCTCCGACTTGA	174	60	1.92	XM_030399323.1
cldn15	CTTTGAATCGATGCTCGCCC	TGGCTGAGCCGATCTTGATG	134	60	1.81	XM_030401871.1
aminopn	ACATCTTTACTGGGTTGTTGCT	CCAGCATCGTGGATCAGAGT	123	60	2.00	XM_030425309.1

gr, glutathione reductase, gcr, glucocorticoid receptor, hsp70, heat shock protein 70, il-1β, interleukine 1β, il-6, interleukine 6, il-8, interleukine 8, il-10, interleukine 10, tnf-α, tumor necrosis factor alfa, tcrβ, t-cell receptorbeta, mhc i, major histocompatibility complex i, pcna, proliferating cell nuclear anti-gen, lyz, lysozime, pept 1, peptide transporter 1, fabp2, fatty acid binding protein, slc5a1, sodium/glucose cotransporter 1, cmklr1, chemerin chemokine-like receptor 1, aquap8, aquaporin8, cldn15, claudin15, aminopn, aminopeptidase n.

amplicon sequence variants (ASVs) with the assistance of DADA2 (Callahan et al., 2016). Taxonomy was assigned using the VSEARCH algorithm (Rognes et al., 2016) against the SILVA database.

2.10. Calculations

The calculations were performed as follows: SGR (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). FI (% ABW^{-1} day^{-1}) = ((100 * total ingestion)/(ABW))/days)) (where average body weight, ABW = (IBW + FBW)/2; FCR = feed intake/weight gain. PER = (FBW – IBW) / protein intake. GPE (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake $fish^{-1}$. GLE (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake $fish^{-1}$. VSI (%) = 100 * (viscera weight/body weight). HSI (%) = 100 * (liver weight/body weight). MFI (%) = 100 * (mesenteric fat weight/body weight).

2.11. Statistical analysis

The data were subjected to a normality test using the Shapiro-Wilk test and a homogeneity of variance test using Bartlett's test. All data are presented in tables as mean ± standard deviation (SD). As experimental unit single tank was used to evaluate growth performance and nutritional indices. Five individual fish per tank were used for analysing somatic indices, blood biochemistry and gut microbiota community profiles. Growth, proximate composition results, and nutritional indices were analyzed by one-way analysis of variance (ANOVA). To evaluate the effect of diets at T0, T2, and T24, plasma biochemistry results were analyzed using a two-way ANOVA, with sampling points and diets as factors. When a statistically significant interaction or main effect was found in the two-way ANOVA ($p \leq 0.05$), Tukey's post hoc test was performed to determine pairwise differences among experimental groups using GraphPad Prism 9.1.1 for Windows (GraphPad Software, San Diego, CA, USA). Effects of different levels of SCP on gene expression were quantified through polynomial regression. At each time, linear and quadratic models were fit to the data. Then, for each gene, the model providing the best compromise between goodness of fit and complexity, according to the adjusted R^2, is reported. An F test was also performed to assess whether any significant effect of SCPs percentage in diet has been detected. Computations were carried out on R version 4.2.1. Gene expression analysis results of the three time points (T0, T2, T24) were analyzed by two-way variance analysis (ANOVA) followed by Tukey's multiple comparisons tests.

The R packages “Stats” and “Vegan” were used to perform gut microbiota statistical analysis. In particular, data separation in the PCA was tested using a permutation test with pseudo-F ratios (function “Adonis” in the “Vegan” package). *P* values were appropriately corrected for multiple comparisons using the Benjamini-Hochberg method. False discovery rate (FDR) ≤ 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA) and the Rstudio interface for R (HTTP://www.r-proje ct.org). All *p* values are shown in supplementary tables 2 and 3.

3. Results

3.1. Growth, nutrient utilization and morphometric indices

Data on growth performances (FBW and SGR), FI, FCR, HSI, VSI, and MFI at the end of the trial are summarized in Table 4. No significant differences ($p > 0.05$) were observed in FBW, SGR, FCR, and FI during the overall period. No significant differences ($p > 0.05$) were observed in VSI and MFI. According to the ANOVA analysis, HSI values were significantly higher in the SCP7.5 group compared to the other dietary groups ($p = 0.024$).

Table 4
Growth and nutritional indices values of gilthead sea bream fed the experimental diets over 104 days.

Diet	CTRL	SCP5	SCP7.5	SCP10	*p* value
IBW (g)	120.3 ± 0.55	120.3 ± 0.31	120.3 ± 0.22	120.4 ± 0.22	*0.998*
FBW (g)	312.3 ± 4.16	308.7 ± 7.18	318.3 ± 3.99	311.4 ± 6.73	*0.281*
SGR	0.92 ± 0.02	0.91 ± 0.02	0.94 ± 0.01	0.91 ± 0.02	*0.247*
FI	1.32 ± 0.07	1.34 ± 0.05	1.32 ± 0.05	1.34 ± 0.03	*0.968*
FCR	1.58 ± 0.12	1.66 ± 0.09	1.52 ± 0.06	1.57 ± 0.07	*0.395*
PER	1.33 ± 0.05	1.21 ± 0.12	1.20 ± 0.00	1.20 ± 0.03	*0.116*
GPE	25.8 ± 8.05	25.7 ± 1.76	26.9 ± 0.70	25.3 ± 1.46	*0.537*
GLE	75.9 ± 5.37	79.0 ± 8.02	84.6 ± 6.30	83.3 ± 4.45	*0.352*
HSI	1.31 ± 0.20[a]	1.32 ± 0.08[a]	1.64 ± 0.11[b]	1.35 ± 0.08[a]	***0.024***
VSI	4.73 ± 0.36	4.59 ± 0.34	5.17 ± 0.33	5.06 ± 0.47	*0.139*
MFI	1.28 ± 0.19	1.20 ± 0.29	1.51 ± 0.28	1.65 ± 0.44	*0.382*

Data are given as the mean ($n = 3$) ± SD except for HSI, VSI and MFI ($n = 15$). Different superscript letters indicate statistical significance (One-way Anova $p < 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day-1) = 100 × (ln FBW- ln IBW) / days.
FI = Feed intake (% ABW − 1 day−1) = ((100*total ingestion)/(ABW))/days)).
FCR = Feed conversion rate = feed intake/weight gain.
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.
GLE (%) Gross lipid efficiency = 100 × [(% final body lipid × FBW) − (% initial body lipid × IBW)]/total lipid intake fish.
HSI = Hepatosomatic index (%) = 100 × (liver weight / FBW).
VSI = Viscerosomatic index (%) = 100 × (viscera weight / FBW).
MFI = Mesenteric fat index (%) = 100 × (mesenteric fat weight / FBW).

3.2. Gut bacterial community

In order to assess the possible effect that increasing dietary SCP level from PV could have on gut microbial community during the on-growing stage of gilthead sea bream, at the end of the trial the 16S rRNA gene sequencing was performed. The gut microbiota variations between samples (alpha and beta-diversity) were assessed by the principal coordinates Analysis (PCoA) based on weighted and unweighted Unifrac (Fig. 1). Results showed that dietary SCP levels significantly affected the gut microbiota in terms of overall microbiome composition, in fact, SCP5, SCP7.5, and SCP10 showed a significant variation compared the CTRL which resulted to be clearly separated from other diets (CTRL vs SCP5/SCP7.5/SCP10; “pairwise Adonis permutation test”, $p = 0.001$). Concerning the internal ecosystem diversity (alpha-diversity), a significant reduction of diversity was observed in the dietary groups SCP7.5 and SCP10 compared to the CTRL (Fig. 1). All *p* values are shown in supplementary Tables 2 and 3. To highlight the gut microbiota composition of gilthead sea bream in the different dietary groups, the overall composition was investigated at phylum and family phylogenetic levels (Fig. 2). Specifically, at the phylum level, all treatments presented Firmicutes as dominant phyla, followed by Proteobacteria and Actinobacteriota. At a family level, Bacillaceae (~ 20%) and Lactobacillaceae (~ 17%) were dominant families in SCP5, SCP7.5, and SCP10, followed by Paenibacillaceae (~ 12%). In the CTRL group, the dominant bacterial families were Lachnospiraceae (7.64%), Rhodobacteraceae (7.60%), and Stappiaceae (7.33%). Considering a lower taxonomic level, several compositional differences in terms of bacterial genera relative abundance among dietary groups were observed (Fig. 3). According to our data, significantly higher relative abundance was presented by genera *Bacillus, Brevibacillus, Weissella*, (Wilcoxon $p \leq 0.001$), *Lactiplantibaccillus,* and *Paenibacillus* (Wilcoxon $p \leq 0.05$) in all SCP groups compared to the CTRL group.

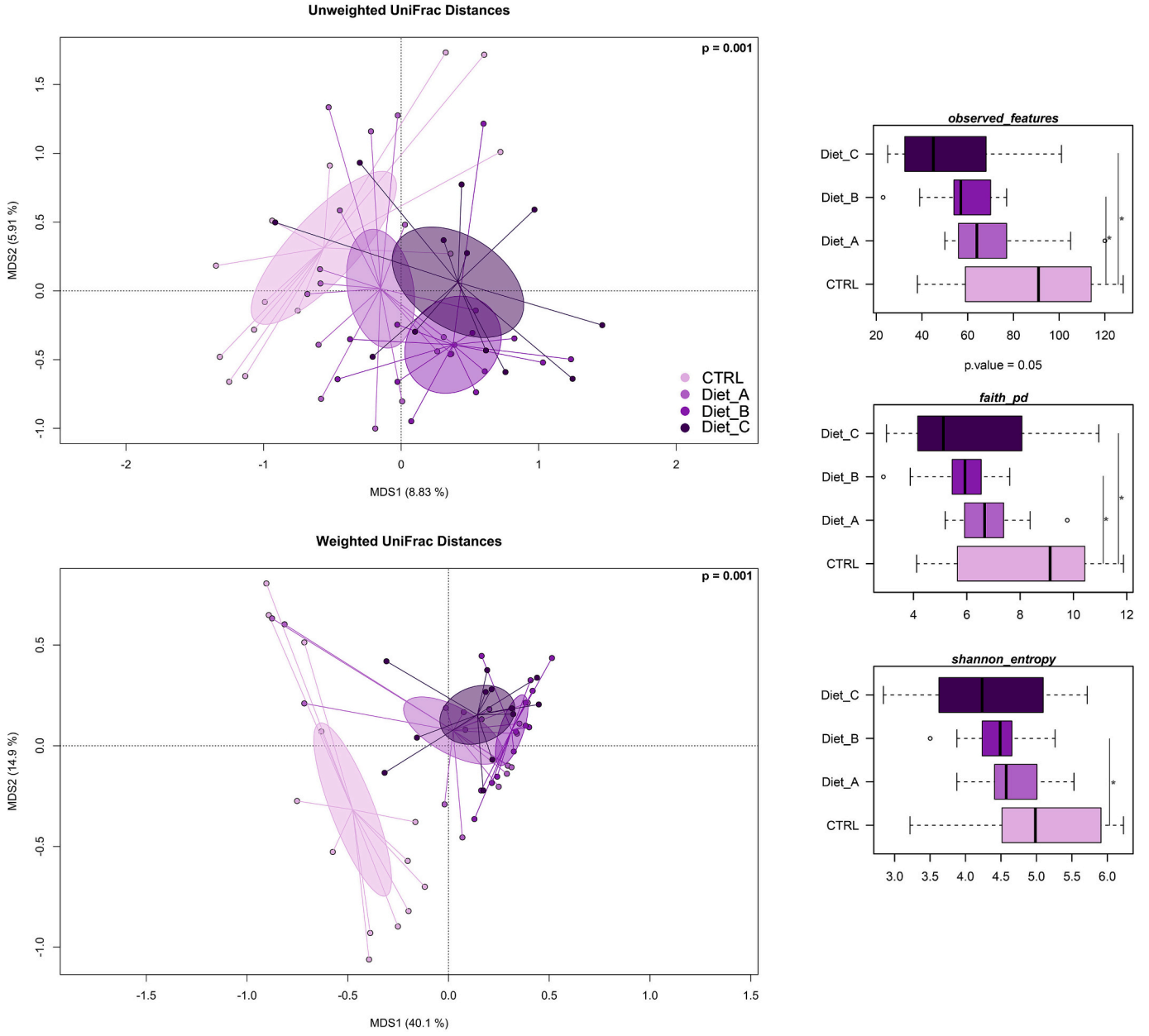

Fig. 1. Beta and alpha diversity of gilthead sea bream's gut microbiota. Fish were fed increasing dietary levels of SCP meal from *Paecilomyces variotii*. Diet A (SCP5) = 50 g kg^{-1} SCP, Diet B (SCP7.5) = 750 g Kg^{-1} SCP, Diet C (SCP10) = 100 g kg^{-1} SCP. On the left, principal Coordinates Analysis (PCoA) based on weighted and unweighted UniFrac distances between gut microbiota structure of animals fed diets CTRL, SCP5, SCP7.5, and SCP10. Barplots on the right present alpha diversity values, measured by Faith's Phylogenetic Diversity (PD_whole_tree), Shannon index, and amplicon sequence variants (observed_ ASVs).

3.3. Plasma biochemistry

The results of the two-way ANOVA analysis on plasma parameters are summarized in Table 5. Urea levels were affected by diet (p = 0.0002), with higher concentrations in the SCP7.5 and SCP10 groups compared to the CTRL group. Lactate also showed a significant dietary effect ($p = 0.0002$), with consistently higher values in the SCP7.5 group than the other treatments. Mg and P concentrations were influenced by diet ($p = 0.002$ and $p = 0.019$, respectively), showing higher values in SCP7.5 with respect to the CTRL diet. Among enzymatic markers, AST and CK were significantly affected by diet ($p = 0.009$ and $p = 0.021$), with lower values observed in SCP10 compared to CTRL diet, while LDH showed higher values in CTRL concerning diets containing SCP. In addition to these main effects, several parameters exhibited significant diet × time interactions, including TP ($p = 0.002$), CREA ($p = 0.0250$), HDL ($p = 0.0175$), Tot bil ($p < 0.0001$), ALB ($p = 0.0059$), Na^+ (p = 0.025), Ca^{2+} ($p < 0.0001$) and Cl ($p = 0.0078$). Some blood parameters showed a time effect. In particular, GLU tended to increase after crowding challenge, while CHOL decreased. Other parameters, such as Uric acid, K^+ and Fe, also varied significantly over time (Table 5).

3.4. Gene expression analysis

The expression of target genes involved in the immune and stress response and in nutrient absorption and mucosal barrier functions is reported in Tables 6, 7, 8, 9. In order to quantify the diet effect cleared of any stress factor, regression models were fit to each gene expression. At T0, *aquap8* average expression increased linearly by more than 0.003 for each additional 1% of SCP ($p < 5 \times 10^{-5}$). A significant positive linear effect was also observed for *gr*. A more complex trend was detected for

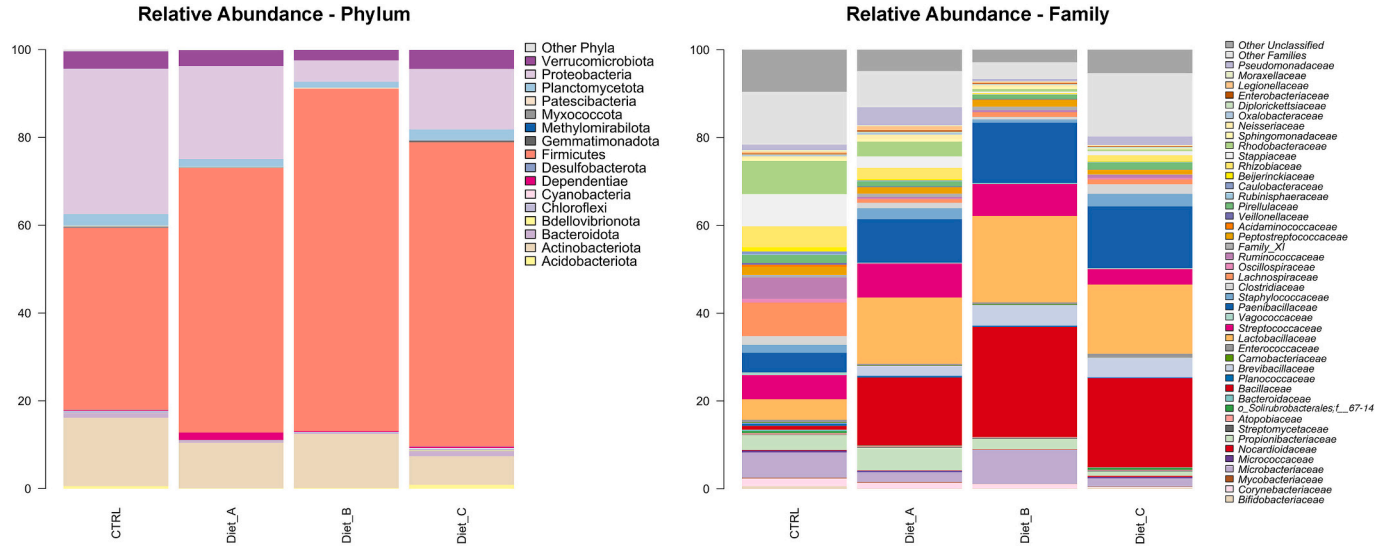

Fig. 2. Microbiota composition (%) of distal gut content of gilthead sea bream fed increasing dietary levels of SCP meal from *Paecilomyces variotii.* Diet A (SCP5) = 50 g kg^{-1} SCP, Diet B (SCP7.5) = 750 g Kg^{-1} SCP, Diet C (SCP10) = 100 g kg^{-1} SCP. Bar plot summarizing the microbiota composition at phylum and family level. Only phyla and families with a relative abundance >0.5% in at least 1 sample are represented.

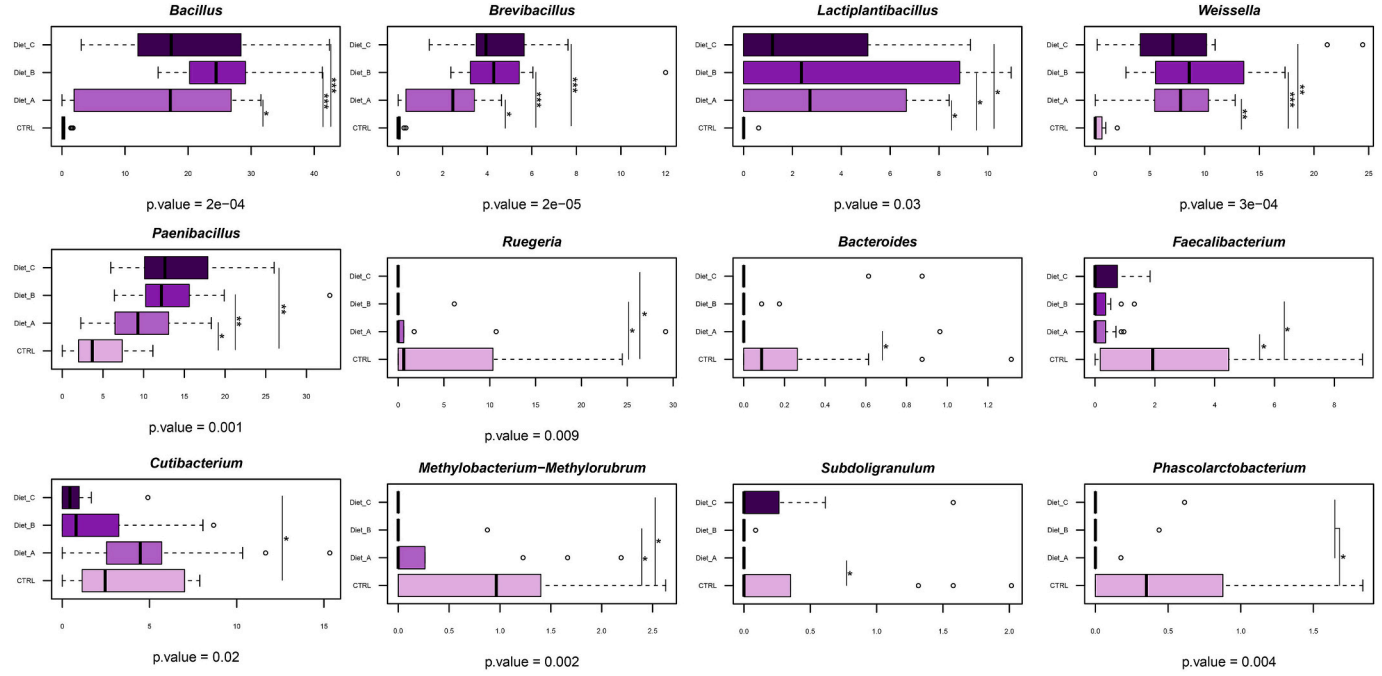

Fig. 3. Taxonomic composition of bacterial communities of distal gut content of gilthead sea bream fed increasing SCP meal from *Paecilomyces variotii* dietary levels. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05). CTRL = 0 g kg^{-1} SCP, Diet A (SCP5) = 50 g kg^{-1} SCP, Diet B (SCP7.5) = 750 g Kg^{-1} SCP, Diet C (SCP10) = 100 g kg^{-1} SCP. Only genera with a mean relative abundance ≥0.5% in at least 5 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.

gcr ($p = 0.001$), *pept1* ($p = 0.017$), *fabp2* ($p = 0.028$), *tnfα* ($p = 0.033$), and *slc5a1* ($p = 0.042$), for which a polynomial model was preferred. Among these genes, *gcr* showed a nonlinear decrease in expression with increasing SCP inclusion, whereas the remaining genes were equally or more expressed in almost all SCP diets compared to the CTRL.

Results at T2 are reported in Table 7. Regression analysis revealed a significant negative linear effect of SCP inclusion on *il-6* expression ($p = 0.0029$). A similar decrease was observed for *aminopn* ($p = 0.0278$), although with a nonlinear pattern, resulting in a marked difference between SCP10, SCP7.5, and SCP5 or lower inclusion levels. No significant effects were detected for the other genes investigated. The two-way ANOVA analysis (Table 9) highlighted additional diet-stress interactions at T2. Specifically, *gr* was significantly downregulated in SCP7.5 ($p = 0.0184$). Moreover, *mhc-i* was downregulated in SCP5, while *slc5a1* expression decreased in SCP5. Finally, *aminopn* was downregulated at T2 in both SCP7.5 and SCP10 ($p = 0.0312$).

At T24, regression results are shown in Table 8. A significant positive linear effect of SCP inclusion was observed for *mhc-i* ($p = 0.0385$). Although a sigmoidal pattern was visually suggested, the linear model was preferred over the quadratic one. Polynomial models described the

Table 5
Plasma biochemistry values for gilthead sea bream fed the experimental diets and subjected to 2 h crowding challenge.

Analyte	Time	CTRL	SCP5	SCP7.5	SCP10	*p value*		
						Diet	Time	Interaction
GLU (mg dL^{-1})	T0	101.5 ± 49.4	96.7 ± 40.3	75.3 ± 43.1	74.3 ± 28.2	0.3987	**<0.0001(B/C/A)**	0.1620
	T2	127.3 ± 38	101.2 ± 44.8	141.8 ± 25.1	143.6 ± 62.9			
	T24	78.1 ± 33	57 ± 9.97	57.1 ± 17.7	64.8 ± 10.6			
Lactate (mmol L^{-1})	T0	59.6 ± 14.1	69.3 ± 18.4	74.8 ± 33.3	48.8 ± 9.4	**0.0002(A/A/B/A)**	**<0.0001(B/B/A)**	0.0914
	T2	54 ± 12.6	53.2 ± 10.3	76 ± 16.2	52.1 ± 9.7			
	T24	41.5 ± 17	33 ± 10.6	55.6 ± 21	50.2 ± 11			
TP (g dL^{-1})	T0	4.75 ± 0.42	5.22 ± 0.31^{b}	4.99 ± 0.56	4.98 ± 0.23^{b}	0.0841	**0.0284(B/A/AB)**	**0.0029**
	T2	$4.54 \pm 0.36^{*}$	$5.17 \pm 0.55^{b\#}$	$4.70 \pm 0.29^{*\#}$	$4.46 \pm 0.47^{a*}$			
	T24	$4.78 \pm 0.54^{*\#}$	$4.58 \pm 0.36^{a*}$	$4.95 \pm 0.27^{*\#}$	$5.12 \pm 0.24^{b\#}$			
Urea (mg dL^{-1})	T0	14.1 ± 1.69	14.9 ± 1.49	16.1 ± 2.27	15.8 ± 2.75	**0.0002(A/AB/B/B)**	**<0.0001(B/A/A)**	0.5946
	T2	10.7 ± 0.90	11.9 ± 2.67	12.6 ± 2.21	12.7 ± 2.25			
	T24	11.0 ± 1.58	13.0 ± 2.22	15.2 ± 1.76	12.9 ± 2.28			
CREA (mg dL^{-1})	T0	0.21 ± 0.03	0.22 ± 0.02^{b}	0.21 ± 0.03	0.20 ± 0.01	0.7590	**0.0181(B/AB/A)**	**0.0250**
	T2	0.20 ± 0.03	0.19 ± 0.02^{a}	0.20 ± 0.02	0.19 ± 0.02			
	T24	0.20 ± 0.02	0.18 ± 0.02^{a}	0.21 ± 0.01	0.21 ± 0.02			
Uric acid (mg dL^{-1})	T0	0.36 ± 0.28	0.36 ± 0.22	0.34 ± 0.28	0.17 ± 0.24	0.2852	**<0.0001(B/B/A)**	0.0552
	T2	0.24 ± 0.21	0.28 ± 0.21	0.53 ± 0.18	0.43 ± 0.29			
	T24	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.02	0.03 ± 0.03			
Tot bil (mg dL^{-1})	T0	$0.08 \pm 0.02^{b\#}$	$0.09 \pm 0.02^{b\#}$	$0.07 \pm 0.01^{※\#}$	$0.05 \pm 0.03^{a※}$	**0.0248(A/B/AB/AB)**	0.8312	**<0.0001**
	T2	$0.05 \pm 0.02^{a※}$	$0.09 \pm 0.02^{ab\#}$	$0.07 \pm 0.02^{※\#}$	$0.07 \pm 0.02^{b※\#}$			
	T24	$0.06 \pm 0.03^{ab※}$	$0.07 \pm 0.02^{a※\#}$	$0.08 \pm 0.02^{※\#}$	$0.09 \pm 0.02^{b\#}$			
CHOL (mg dL^{-1})	T0	303.2 ± 49.0	335.4 ± 35.7	297.2 ± 47.0	291.7 ± 23.4	0.6114	**0.0002(B/A/B)**	0.0576
	T2	272.0 ± 30.6	279.4 ± 31.2	284.9 ± 23.4	262.7 ± 25.1			
	T24	296.1 ± 48.0	294.1 ± 26.9	313.0 ± 18.7	326.1 ± 49.9			
HDL (mg dL^{-1})	T0	116.2 ± 18.7	128.3 ± 14.8^{b}	111.2 ± 18.9	113.0 ± 11.3^{ab}	0.8496	**<0.0001(B/A/B)**	**0.0175**
	T2	102.9 ± 11.6	104.7 ± 4.5^{a}	110.3 ± 8.0	101.1 ± 7.9^{a}			
	T24	116.3 ± 24.4	111.6 ± 15.5^{a}	122.8 ± 8.7	129.4 ± 10.6^{b}			
TRIG (mg dL^{-1})	T0	332 ± 53.6	349.6 ± 76.8	376 ± 60.5	354.7 ± 36.9	0.1338	**0.0003(B/A/A)**	0.8217
	T2	279.3 ± 58.8	306.1 ± 40.5	319.8 ± 26.1	317.5 ± 37.9			
	T24	316.2 ± 41.7	302.7 ± 25.1	318.1 ± 30	328.1 ± 30.1			
ALB (g dL^{-1})	T0	1.18 ± 0.08	1.31 ± 0.16^{b}	1.21 ± 0.09	1.21 ± 0.03	0.0698	0.3662	**0.0059**
	T2	$1.16 \pm 0.09^{※}$	$1.32 \pm 0.22^{b\#}$	$1.15 \pm 0.08^{※}$	$1.10 \pm 0.09^{※}$			
	T24	1.21 ± 0.15	1.13 ± 0.06^{a}	1.26 ± 0.18	1.19 ± 0.06			
ALT (U L^{-1})	T0	3.62 ± 0.91	4.22 ± 1.85	6.12 ± 8.98	3.75 ± 1.16	0.0580	0.4513	0.5550
	T2	4.62 ± 2.72	3.12 ± 1.45	4.62 ± 1.40	2.37 ± 2.32			
	T24	6.62 ± 2.92	3.37 ± 0.91	5 ± 1.92	3 ± 0.92			
ALP (U L^{-1})	T0	166 ± 70	174 ± 50.6	193 ± 82.1	161 ± 135	0.5924	0.1675	0.5864
	T2	99.8 ± 32.6	170.5 ± 87.2	170.8 ± 110.3	126.7 ± 41.3			
	T24	149.6 ± 64.2	146.4 ± 78.5	122.1 ± 35.8	149 ± 65.8			
AST (U L^{-1})	T0	47.1 ± 22.4	20 ± 8.53	47.7 ± 50.2	38.1 ± 19.4	**0.0096(B/A/AB/A)**	**0.0042(AB/A/B)**	0.3999
	T2	39.5 ± 23.4	16 ± 6.37	40.5 ± 25.4	13.7 ± 9.22			
	T24	68 ± 39.2	55.1 ± 43.8	48.2 ± 24.3	33.5 ± 15.6			
CK (U L^{-1})	T0	692.8 ± 554.2	140 ± 98.9	375.2 ± 382.5	367.8 ± 288.9	**0.0211(B/AB/AB/A)**	**<0.0001(A/A/B)**	0.4129
	T2	502.1 ± 318.3	444.3 ± 391.3	421.6 ± 350.9	100.7 ± 59.7			
	T24	2154.7 ± 1918.2	1406.8 ± 1406.3	1095.1 ± 735.9	758.3 ± 316.9			
LDH (U L^{-1})	T0	1080.4 ± 721.5	180.2 ± 127.1	356.8 ± 199.7	418.4 ± 233.6	**<0.0001(B/A/A/A)**	**0.0116(AB/A/B)**	0.5466
	T2	575 ± 511.6	221.2 ± 131.5	418.6 ± 587.5	96 ± 57.6			
	T24	1071.2 ± 877.5	621.1 ± 532	695.3 ± 510.2	401.6 ± 180.5			
Na^{+} (mEq L^{-1})	T0	$195 \pm 3.29^{b*\#}$	$194.2 \pm 1.66^{b*\#}$	$198.3 \pm 7.11^{b\#}$	$192.6 \pm 3.74^{*}$	**0.0036(A/A/B/AB)**	**<0.0001(B/A/A)**	**0.0257**
	T2	186.2 ± 4.46^{a}	189.2 ± 3.95^{a}	191 ± 2.82^{a}	189.3 ± 3.62			
	T24	$187.7 \pm 3.77^{a*}$	$186.7 \pm 1.75^{a*}$	$191 \pm 4.63^{a*\#}$	$193 \pm 1.85^{\#}$			
Ca^{2+} (mg dL^{-1})	T0	$14.5 \pm 0.53^{*}$	$15.9 \pm 0.97^{b\#}$	$16.3 \pm 0.91^{\#}$	$14.8 \pm 0.65^{*\#}$	**0.0354(A/AB/B/AB)**	0.0612	**<0.0001**
	T2	$14.2 \pm 1.3^{*}$	$15.7 \pm 1.4^{b\#}$	$15 \pm 0.8^{*\#}$	$14.2 \pm 0.9^{*}$			
	T24	$15.3 \pm 1.8^{\#}$	$13.9 \pm 0.8^{a*}$	$15.5 \pm 1.0^{\#}$	$16.3 \pm 0.4^{\#}$			
P (mg dL^{-1})	T0	19 ± 1.24	20.1 ± 3.23	24.6 ± 7.38	22.7 ± 3.92	**0.019(A/AB/B/AB)**	**0.0001(B/AB/A)**	0.1843
	T2	18.1 ± 1.56	21.5 ± 1.91	19.7 ± 2.05	19.6 ± 3.3			
	T24	16.8 ± 3.78	15.6 ± 2.85	18.6 ± 2.14	19.2 ± 3.58			
Mg (mg dL^{-1})	T0	5 ± 0.35	5.42 ± 0.49	5.76 ± 1.04	5.30 ± 0.46	**0.0017(A/AB/B/AB)**	**<0.0001(B/B/A)**	0.4711
	T2	4.65 ± 0.52	5.14 ± 0.42	5.53 ± 0.3	5.46 ± 0.88			
	T24	4.27 ± 0.66	4.04 ± 0.37	4.60 ± 0.56	4.48 ± 0.48			
Cl (mEq L^{-1})	T0	$155.9 \pm 3.21^{b※}$	$154.8 \pm 2.94^{b※}$	$161.2 \pm 2.65^{c\#}$	$156.0 \pm 2.08^{b※}$	0.1110	**<0.0001(B/A/B)**	**0.0078**
	T2	151.9 ± 2.27^{a}	150.3 ± 3.72^{a}	151.4 ± 2.97^{a}	152.4 ± 3.36^{a}			
	T24	156.6 ± 3.21^{b}	158.4 ± 2.46^{b}	157.0 ± 2.11^{b}	158.0 ± 3.40^{b}			
K^{+} (mEq L-1)	T0	2.70 ± 0.44	2.71 ± 0.35	2.68 ± 0.42	3.03 ± 0.51	0.6249	**<0.0001(B/A/A)**	0.0839
	T2	2.54 ± 0.32	2.14 ± 0.35	2.21 ± 0.27	2.39 ± 0.16			
	T24	2.48 ± 0.57	2.54 ± 0.32	2.46 ± 0.42	2.21 ± 0.34			
Fe (μg dL^{-1})	T0	77.5 ± 21.4	83.8 ± 18.6	87.0 ± 19.1	75.1 ± 15.7	0.4967	**<0.0001(B/A/A)**	0.2844
	T2	57.5 ± 15.3	59.0 ± 14.3	58.4 ± 6.61	51.1 ± 11.8			
	T24	70.9 ± 29.2	57.0 ± 22.5	45.5 ± 27.0	55.4 ± 17.4			
A/G	T0	0.33 ± 0.02	0.32 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	**0.0161(B/AB/AB/A)**	0.0642	0.0811
	T2	0.34 ± 0.02	0.35 ± 0.02	0.32 ± 0.01	0.33 ± 0.01			
	T24	0.35 ± 0.02	0.32 ± 0.02	0.33 ± 0.04	0.32 ± 0.02			

Data are presented in means (n = 15 diet $^{-1}$) ± standard deviation SD. Bold bracketed letters indicate significant differences in two-way ANOVA. Bracketed letter sets correspond to time (T0/T2/T24) or diet (CTRL/SCP5/SCP7.5/SCP10) groups, respectively, and data values corresponding to "A" are lower than those corresponding to "B". Different symbols represent significant differences between treatments at the same time, while lower case letters represent significant differences between times for the same diet. CTRL = 0 g kg^{-1} single cell protein (SCP); SCP5 = 50 g kg^{-1} SCP; SCP7.5 = 750 g Kg^{-1} SCP; SCP10 = 100 g kg^{-1} SCP. T0, time point before crowding challenge; T2 time point after 2 h of crowding stress; T24, time point after 24 h from crowding stress. GLU, glucose; TP, total proteins; CREA, creatinine; Tot bil, total bilirubin; CHOL, cholesterol; HDL, High-density lipoprotein; TRIG, triglycerides; ALB, albumin; ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase; Na^+, sodium; Ca^{2+}, calcium; P, inorganic phosphorus; Mg, magnesium; Cl, chloride; K^+, potassium; Fe, iron; A/G, albumin/globulins.

Table 6
Results of the regression analysis of increasing levels of SCP on gene expression at T0. Models showing significant effects are highlighted in bold.

Genes	*p* value	R^2	R^2adj	Intercept	SCP	SCP^2
gr	**0.0108**	**0.49**	**0.44**	**0.0000181**	**0.0000218**	**.**
gcr	**0.001**	**0.78**	**0.73**	**0.0080871**	**−0.0008907**	**0.0000521**
hsp70	0.7922	0.01	−0.09	0.6639132	−0.0037206	.
il-1β	0.8588	0	−0.10	0.0010421	0.0000123	.
il-6	0.6043	0.11	−0.09	0.000034	0.0000062	0.0000006
il-8	0.545	0.13	−0.07	0.0029783	0.0008136	−0.000074
il-10	0.4863	0.05	−0.05	0.0017683	0.0000986	.
tnfα	**0.0334**	**0.53**	**0.43**	**0.000249**	**−0.0000382**	**0.000005**
tcrβ	0.5367	0.13	−0.06	0.0719474	0.0061864	−0.0005562
mhc-i	0.1518	0.34	0.2	3.1461534	0.7917658	−0.0847956
pcna	0.1607	0.19	0.11	0.0253114	0.0016118	.
lyz	0.308	0.23	0.06	0.001181	−0.00023	0.0000214
pept1	**0.0171**	**0.6**	**0.51**	**0.0018067**	**0.0092869**	**−0.0007121**
fabp2	**0.0282**	**0.55**	**0.45**	**0.2200243**	**0.3250585**	**−0.0288985**
slc5a1	**0.0423**	**0.5**	**0.39**	**0.2823892**	**0.1410502**	**−0.0123616**
cmklrl	0.1621	0.33	0.18	0.0003754	−0.0001077	0.0000142
aquap8	**0.0000**	**0.86**	**0.85**	**0.0080329**	**0.0032522**	**.**
cldn15	0.1917	0.18	0.09	0.4080492	0.0193682	.
aminopn	0.3401	0.21	0.04	0.0113861	0.0021367	−0.0001681

gr, glutathione reductase; gcr, glucocorticoid receptor; hsp70, heat shock protein 70; il-1β, interleukine 1β; il-6, interleukine 6; il-8, interleukine 8; il-10, interleukine 10; tnfα, tumor necrosis factor alfa; tcrβ, t-cell receptorbeta; mhc-i, major histocompatibility complex i; pcna, proliferating cell nuclear anti-gen; lyz, lysozime; pept 1, peptide transporter 1; fabp2, fatty acid binding protein; slc5a1, sodium/glucose cotransporter 1; cmklr1, chemerin chemokine-like receptor 1; aquap8, aquaporin 8; cldn15, claudin 15; aminopn, aminopeptidase n; SCP, single cell protein.

Table 7
Results of the regression analysis of increasing levels of SCP on gene expression at T2. Models showing significant effects are highlighted in bold.

Genes	*p* value	R^2	R^2adj	Intercept	X	X^2
gr	0.1759	0.18	0.09	0.000255	−0.000018	.
gcr	0.0958	0.41	0.27	0.004265	0.000250	-0.000049
hsp70	0.2154	0.15	0.06	0.528313	0.041450	.
il-1β	0.0896	0.29	0.21	0.001876	−0.000096	.
il-6	**0.0029**	**0.61**	**0.57**	**0.000034**	**−0.000002**	**.**
il-8	0.3789	0.09	−0.01	0.002166	0.000039	.
il-10	0.2116	0.29	0.13	0.002358	−0.000388	0.000034
tnfα	0.4930	0.15	0.04	0.000451	−0.000057	0.000005
tcrβ	0.8047	0.01	−0.09	0.063014	0.000459	.
mhc-i	0.2214	0.31	0.14	2.307685	−0.320980	0.029322
pcna	0.5850	0.11	−0.08	0.034470	0.006272	−0.000586
lyz	0.3425	0.09	0.00	0.000833	−0.000027	.
pept1	0.1305	0.36	0.22	0.008324	−0.005998	0.001409
fabp2	0.5107	0.04	−0.05	1.812626	-0.055726	.
slc5a1	0.0889	0.26	0.19	0.191194	0.020310	.
cmklrl	0.1019	0.40	0.26	0.000123	0.000109	−0.000008
aquap8	0.2202	0.15	0.06	0.047719	−0.002420	.
cldn15	0.0569	0.47	0.35	0.475210	0.035380	−0.006265
aminopn	**0.0278**	**0.55**	**0.45**	**0.009683**	**0.000422**	**−0.000107**

gr, glutathione reductase; gcr, glucocorticoid receptor; hsp70, heat shock protein 70; il-1β, interleukine 1β; il-6, interleukine 6; il-8, interleukine 8; il-10, interleukine 10; tnfα, tumor necrosis factor alfa; tcrβ, t-cell receptorbeta; mhc-i, major histocompatibility complex i; pcna, proliferating cell nuclear anti-gen; lyz, lysozime; pept 1, peptide transporter 1; fabp2, fatty acid binding protein; slc5a1, sodium/glucose cotransporter 1; cmklr1, chemerin chemokine-like receptor 1; aquap8, aquaporin 8; cldn15, claudin 15; aminopn, aminopeptidase n; SCP, single cell protein

expression patterns of *gcr* ($p = 0.0005$), *cldn15* ($p = 0.0009$), *slc5a1* ($p = 0.007$), *aminopn* ($p = 0.0164$), and *tcrβ* ($p = 0.0349$). In all cases, the estimated linear coefficients were negative and the quadratic coefficients positive, indicating expression trends reaching a minimum at SCP inclusion levels higher than 0. According to the two-way ANOVA (Table 9), *tcrβ* exhibited a contrasting regulation at T24, being downregulated in SCP7.5 but upregulated in SCP10 ($p < 0.0001$). *Mhc-i* remained downregulated in SCP5, while SCP7.5 showed an alternating up- and downregulation pattern ($p = 0.0414$). *Slc5a1* expression increased at T24 in SCP10 ($p = 0.0034$), whereas *aminopn* was downregulated in SCP5.

Table 8
Results of the regression analysis of increasing levels of SCP on gene expression at T24. Models showing significant effects are highlighted in bold.

Genes	*p* value	R^2	R^2adj	Intercept	X	X^2
gr	0.6514	0.02	−0.08	0.0000839	−0.0000019	.
gcr	**0.0005**	**0.81**	**0.77**	**0.0052497**	**−0.0009311**	**0.0000639**
hsp70	0.0984	0.40	0.27	0.4735939	−0.0514322	0.0062495
il-1β	0.7179	0.01	−0.09	0.0028925	−0.0000867	.
il-6	0.3953	0.07	−0.02	0.0000382	−0.0000013	.
il-8	0.3227	0.22	0.05	0.0041304	0.0018562	−0.0001950
il-10	0.3356	0.22	0.04	0.0013021	0.0001640	−0.0000176
tnfα	0.9119	0.00	−0.10	0.0004602	0.0000013	.
tcrβ	**0.0349**	**0.53**	**0.42**	**0.0926230**	**−0.0342872**	**0.0038734**
mhc-i	**0.0385**	**0.36**	**0.30**	**1.6514004**	**0.2320626**	**.**
pcna	0.4413	0.17	−0.02	0.0322076	0.0054327	−0.0006036
lyz	0.8391	0.00	−0.10	0.0007704	0.0000057	.
pept1	0.1271	0.22	0.14	0.1390982	−0.0114762	.
fabp2	0.2394	0.14	0.05	5.9107260	−0.3773595	.
slc5a1	**0.0070**	**0.67**	**0.59**	**0.1199636**	**−0.0742314**	**0.0163619**
cmklrl	0.2295	0.28	0.12	0.0002202	−0.0000434	0.0000057
aquap8	0.4004	0.18	0.00	0.0239758	0.0061057	−0.0005439
cldn15	**0.0009**	**0.79**	**0.74**	**0.4073473**	**−0.1011695**	**0.0140198**
aminopn	**0.0164**	**0.60**	**0.51**	**0.0082230**	**−0.0013093**	**0.0002358**

gr, glutathione reductase; gcr, glucocorticoid receptor; hsp70, heat shock protein 70; il-1β, interleukine 1β; il-6, interleukine 6; il-8, interleukine 8; il-10, interleukine 10; tnfα, tumor necrosis factor alfa; tcrβ, t-cell receptorbeta; mhc-i, major histocompatibility complex i; pcna, proliferating cell nuclear anti-gen; lyz, lysozime; pept 1, peptide transporter 1; fabp2, fatty acid binding protein 2; slc5a1, sodium/glucose cotransporter 1; cmklr1, chemerin chemokine-like receptor 1; aquap8, aquaporin 8; cldn15, claudin 15; aminopn, aminopeptidase n; SCP, single cell protein

4. Discussion

In the present study, feeding gilthead sea bream with increasing levels of dietary SCP (up to 10%, corresponding to a 36.5% reduction in FM) did not result in significant differences in growth or feed utilization. These findings suggest that SCP can support partial FM replacement in diets with a low FM content. Similarly, in Atlantic salmon, it was possible to include 16% *Paecilomyces variotii* SCP in the diet as a partial replacement for FM, wheat gluten, and soy protein concentrate, although the FM level (31% dietary FM inclusion) was higher than that tested in the present study (Hooft et al., 2024). No significant differences in FBW and weight gain were observed in a study on rainbow trout, where 30% of FM was replaced by the filamentous fungi *Neurospora intermedia* (Singh et al., 2021). Furthermore, when fungi were used as a substitute for vegetal protein sources, such as in the species Reba carp (*Cirrhinus reba*), or added as a dietary supplement to a plant-based diet in Gibel carp and common carp (*Cyprinus carpio*), higher growth rates were observed (Noor-Ul et al., 2020; Chakraborty et al., 2022; Jasim et al., 2022). These results affirm that filamentous fungi, whether employed as substitutes for traditional protein sources in existing feed formulations or as dietary supplements, represent a valuable protein source, demonstrating the potential to promote growth and, in certain instances, even enhance it. Specifically, gilthead sea bream appears to be a suitable species for incorporating microbial single-cell ingredients as a replacement for FM or other high-value protein sources. In fact, Torula yeast (*Candida utilis*), included at up to 7.5% in a low-FM diet, successfully replaced approximately 30% of FM without negatively affecting growth or feed efficiency in juveniles (25–110 g) (Busti et al., 2025). Similarly, in fingerlings (7–20 g), the inclusion of 18% of a single-cell protein mixture composed of *Methylococcus capsulatus, Saccharomyces cerevisiae*, and *Schizochytrium* spp.- along with 5% krill meal, reduced FM from trimmings by 35% and soybean concentrate by approximately 58%, while also enhancing growth and protein efficiency (Tampou et al., 2024). Moreover, in the same species, during the ongrowing phase, dietary inclusion of single-cell protein from *Corynebacterium glutamicum* at levels up to 20% enabled the complete replacement of soy protein concentrate and the partial replacement of corn gluten, without adversely affecting growth or feed efficiency (Marchi et al., 2023).

In the present study, 16S rRNA gene sequencing revealed that dietary PV significantly altered the overall gut microbiota, reducing internal diversity. Generally, the most abundant phyla in the gut microbiota of fish are Firmicutes and Proteobacteria (Hao et al., 2022). This finding is consistent with the present study. However, it is particularly noteworthy that diets incorporating SCP from PV promoted a higher prevalence of Firmicutes alongside a decrease in Proteobacteria and other phyla, resulting in distinct microbial compositions between PV-fed and control groups. While shifts in microbiota diversity are often linked to gut health dynamics, they do not necessarily indicate negative outcomes. Recent studies showed that specific dietary interventions can lead to changes in microbiota composition. These changes, in turn, may create specialized microbial environments that support gut function and resilience, fostering beneficial metabolic activities while reducing potential pathogens. Therefore, the observed alterations in microbiota composition could reflect a tailored, functional ecosystem that enhances intestinal health, depending on the balance and activity of dominant taxa (Rimoldi et al., 2018; Souza et al., 2020). In the present study, the CTRL diet presented a varied and well-distributed abundance of bacteria, while the PV diets showed a lower diversity but also an increased abundance of families *Bacillaceae* and *Lactobacillaceae.* According to previous studies, these families may enhance the immune system, increase disease resistance, improve digestion, and inhibit the growth of harmful microorganisms (Marchi et al., 2023). Our findings show an increased abundance of two LABs, *Lactiplantibacillus* and *Weissella*. Species of *Lactiplantibacillus spp*, particularly *Lactiplantibacillus plantarum*, have recently gained significant attention as probiotics in aquaculture due to their beneficial effects on maintaining the intestinal mucosal barrier, reducing pathogen colonization, and enhancing digestive function (Iorizzo et al., 2022; Liu et al., 2024a). Similarly, *Weissella* has been associated with improved performance in gilthead sea bream fed a low-lipid diet under temperature fluctuations and is considered a promising candidate for use in aquaculture (Pelusio et al., 2021; Ringø et al., 2020). As reported in rainbow trout fed the filamentous fungus *Neurospora intermedi*a, the promotion of lactic acid bacteria is likely due to components of the fungal cell wall, such as β-glucans, which can serve as fermentable substrates (Singh et al., 2021).

Among the bacterial genera of interest, *Bacillus*, *Brevibacillus*, and *Paenibacillus* showed increased abundance in response to higher levels of PV. In particular, *Bacillus* spp. was found to increase in gilthead sea bream fed single-cell protein (SCP) derived from *Corynebacterium glutamicum* (Marchi et al., 2023). *Brevibacillus,* although less commonly recognized as a fish gut microbial component, has recently been shown to enhance disease resistance by inhibiting pathogenic bacteria,

Table 9
Relative expression levels of target genes in gilthead sea bream fed experimental diets and exposed to a 2-h crowding challenge.

	Gene ID	Time	CTRL	SCP5	SCP7.5	SCP10	*P-value*		
Function							Diet	Time	Interaction
	gr	T0	0.000030 ± 0.000014*	0.000053 ± 0.000042$^{*\#}$	0.000283 ± 0.000084$^{b\#}$	0.000198 ± 0.000052$^{*\#}$	0.1714	0.8086	**0.0184**
		T2	0.000220 ± 0.000047	0.000259 ± 0.000319	0.000064 ± 0.000068^{a}	0.000063 ± 0.000049			
		T24	0.000081 ± 0.000046	0.000067 ± 0.000048	0.000098 ± 0.000067^{ab}	0.000048 ± 0.000048			
Stress responce	*gcr*	T0	0.0081 ± 0.0015	0.0051 ± 0.0009	0.0041 ± 0.0002	0.0045 ± 0.0009	**<0.0001 (B/A/A/A)**	**<0.0001 (B/A/A)**	0.111
		T2	0.0042 ± 0.0016	0.0045 ± 0.0016	0.0031 ± 0.0016	0.0020 ± 0.0007			
		T24	0.0053 ± 0.0014	0.0020 ± 0.0003	0.0021 ± 0.0004	0.0022 ± 0.0003			
	hsp70	T0	0.641 ± 0.202	0.667 ± 0.197	0.685 ± 0.217	0.579 ± 0.104	0.2464	**0.0374 (AB/B/A)**	0.3064
		T2	0.552 ± 0.212	0.552 ± 0.212	1.114 ± 0.709	0.829 ± 0.171			
		T24	0.473 ± 0.023	0.374 ± 0.131	0.438 ± 0.056	0.585 ± 0.177			
	il-1β	T0	0.0012 ± 0.0007	0.00063 ± 0.00031	0.0016 ± 0.0016	0.00108 ± 0.00033	0.3656	0.4171	0.2262
		T2	0.0019 ± 0,0002	0.00142 ± 0.00118	0.0011 ± 0.0002	0.00093 ± 0.00017			
		T24	0.0037 ± 0.0012	0.01121 ± 0.00743	0.0035 ± 0.0003	0.00453 ± 0.00294			
	il-6	T0	0.000033 ± 0.000027	0.000025 ± 0.000025	0.000011 ± 0.000007	0.000035 ± 0.000026	0.0723	0.4424	0.8418
		T2	0.000035 ± 0.000003	0.000021 ± 0.000003	0.000015 ± 0.000013	0.000014 ± 0.000005			
		T24	0.000039 ± 0.000023	0.000038 ± 0.000018	0.000013 ± 0.000008	0.000033 ± 0.000021			
	il-8	T0	0.0029 ± 0.0013	0.0058 ± 0.0047	0.0041 ± 0.002	0.0040 ± 0.0020	0.0725	**0.0406 (AB/A/B)**	0.3143
		T2	0.0022 ± 0.0007	0.0024 ± 0.0006	0.0022 ± 0.0003	0.0027 ± 0.0005			
		T24	0.0037 ± 0.0012	0.0112 ± 0.0074	0.0035 ± 0.0003	0.0045 ± 0.0029			
	il-10	T0	0.0016 ± 0.0007	0.0029 ± 0.0028	0.0018 ± 0.0002	0.0029 ± 0.0029	0.7119	0.2007	0.7293
		T2	0.0023 ± 0.0012	0.0014 ± 0.0010	0.0012 ± 0.0003	0.0020 ± 0.0003			
		T24	0.0013 ± 0.0005	0.0018 ± 0.0007	0.0014 ± 0.00003	0.0012 ± 0.0001			
Immunology	*tnfα*	T0	0.00025 ± 0.00007	0.00017 ± 0.000005	0.00026 ± 0.00013	0.00037 ± 0.00004	0.4679	**0.005 (A/AB/B)**	0.2591
		T2	0.00044 ± 0.00024	0.00036 ± 0.00011	0.00019 ± 0.00016	0.00040 ± 0.00017			
		T24	0.00047 ± 0.00007	0.00039 ± 0.00016	0.00056 ± 0.00018	0.00044 ± 0.00012			
	tcrβ	T0	0.0717 ± 0.009	0.090 ± 0.035	0.0853 ± 0.0195^{b}	0.079 ± 0.014^{a}	**0.0098 (AB/A/A/B)**	0.3012	**<0.0001**
		T2	0.065 ± 0.018	0.052 ± 0.006	0.0843 ± 0.0375^{b}	0.061 ± 0.009^{a}			
		T24	0.086 ± 0.025$^{\#}$	0.057 ± 0.021$^{\#}$	*0.0011 ± 0.0001*^{a*}	0.157 ± 0.050$^{b\bullet}$			
	mhc-i	T0	3.071 ± 0.793	5.438 ± 2.637^{b}	3.710 ± 1.301^{ab}	2.811 ± 0.279	0.3913	**0.0197 (B/A/AB)**	**0.0414**
		T2	2.358 ± 0.394	0.979 ± 0.010^{a}	1.955 ± 0.362^{a}	1.878 ± 0.824			
		T24	1.913 ± 0.459$^{*\#}$	1.664 ± 0.461^{a*}	4.642 ± 1.464$^{b\#}$	3.608 ± 0.775$^{*\#}$			
	pcna	T0	0.024 ± 0.004	0.036 ± 0.011	0.036 ± 0.025	0.041 ± 0.012	0.4088	0.4601	0.6862
		T2	0.033 ± 0.015	0.059 ± 0.012	0.038 ± 0.023	0.043 ± 0.032			
		T24	0.033 ± 0.007	0.039 ± 0.024	0.046 ± 0.025	0.024 ± 0.006			
	lyz	T0	0.00119 ± 0.00051	0.00053 ± 0.00031	0.00071 ± 0.00080	0.00101 ± 0.00053	0.2459	0.5948	0.7187
		T2	0.00083 ± 0.00041	0.00064 ± 0.00018	0.00076 ± 0.00051	0.00050 ± 0.00030			
		T24	0.00087 ± 0.00033	0.00049 ± 0.00020	0.00106 ± 0.00013	0.00080 ± 0.00044			
	pept 1	T0	0.0027 ± 0.0022	0.025 ± 0.004	0.038 ± 0.020	0.021 ± 0.003	0.9284	0.1277	0.1466
		T2	0.0047 ± 0.0028	0.035 ± 0.052	0.014 ± 0.014	0.100 ± 0.076			
		T24	0.1507 ± 0.1588	0.055 ± 0.068	0.060 ± 0.085	0.032 ± 0.023			
	fabp2	T0	0.206 ± 0.104	1.207 ± 0.352	0.921 ± 0.649	0.623 ± 0.257	0.7128	**0.0212 (A/AB/B)**	0.8332
		T2	1.697 ± 0.327	1.874 ± 2.145	1.177 ± 0.226	1.249 ± 0.672			
		T24	5.912 ± 7.451	3.748 ± 2.341	3.626 ± 3.560	1.866 ± 0.535			
	slc5a1	T0	0.263 ± 0.155	0.795 ± 0.174^{b}	0.490 ± 0.125	0.515 ± 0.130^{a}	**0.0022 (A/AB/B)**	0.0655	**0.0034**
		T2	0.210 ± 0.057	0.206 ± 0.132^{a}	0.443 ± 0.140	0.363 ± 0.164^{a}			
		T24	0.121 ± 0.044	0.152 ± 0.021^{a}	0.492 ± 0.535	1.011 ± 0.307^{b}			
	cmklr1	T0	0.00037 ± 0.00021*	0.00020 ± 0.00004$^{\#}$	0.00035 ± 0.00036$^{*\#}$	0.00072 ± 0.00052$^{*\#}$	0.1074	0.2879	0.4658
Transporters		T2	0.00012 ± 0.00003	0.00046 ± 0.00034	0.00048 ± 0.00008	0.00038 ± 0.00025			
		T24	0.00022 ± 0.00009*	0.00013 ± 0.00006$^{*\#}$	0.00023 ± 0.00022*	0.00035 ± 0.00017$^{\#}$			
	aquap8	T0	0.0082 ± 0.0035	0.025 ± 0.004	0.030 ± 0.009	0.042 ± 0.004	0.473	0.4589	0.062
		T2	0.0519 ± 0.0217	0.020 ± 0.018	0.044 ± 0.031	0.021 ± 0.018			
		T24	0.0245 ± 0.0085	0.038 ± 0.010	0.043 ± 0.022	0.029 ± 0.024			
	cldn15	T0	0.431 ± 0.181	0.535 ± 0.026	0.327 ± 0.124	0.700 ± 0.164	0.5186	0.1211	0.0854
		T2	0.480 ± 0.180	0.469 ± 0.131	0.424 ± 0.193	0.189 ± 0.009			
Barrier		T24	0.407 ± 0.020	0.252 ± 0.065	0.438 ± 0.181	0.797 ± 0.166			
	aminopn	T0	0.0114 ± 0.0024	0.0176 ± 0.0026^{b}	0.0183 ± 0.0114^{b}	0.0158 ± 0.0039^{b}	0.5729	**0.0006 (B/A/AB)**	**0.0312**
		T2	0.0096 ± 0.0044	0.0097 ± 0.0028^{ab}	0.0061 ± 0.0013^{a}	0.0035 ± 0.0012^{a}			
		T24	0.0084 ± 0.0020$^{*\#}$	0.0064 ± 0.0011^{a*}	0.0132 ± 0.0072$^{ab*\#}$	0.0181 ± 0.0039$^{b\#}$			

gr, glutathione reductase; gcr, glucocorticoid receptor; hsp70, heat shock protein 70; il-1β, interleukine 1β; il-6, interleukine 6; il-8, interleukine 8; il-10, interleukine 10; tnfα, tumor necrosis factor alfa; tcrβ, t-cell receptorbeta; mhc-i, major histocompatibility complex i; pcna, proliferating cell nuclear anti-gen; lyz, lysozime; pept 1, peptide transporter 1; fabp2, fatty acid binding protein; slc5a1, sodium/glucose cotransporter 1; cmklr1, chemerin chemokine-like receptor 1; aquap8, aquaporin 8; cldn15, claudin 15; aminopn, aminopeptidase n.

supporting gut microbial balance, and stimulating immune responses (Liu et al., 2024b). *Paenibacillus*, known for its chitin-degrading capabilities, significantly increased in sea bream fed *Hermetia illucens* meal at inclusion levels of 5% and 15% (Busti et al., 2024). Notably, Domenech et al. (1994) identified β-glucan–chitin complexes representing 42.7% to 47.3% of the cell wall polysaccharides in *Paecilomyces variotii*, suggesting a potential source of chitin (Hooft et al., 2024). Moreover, in gilthead sea bream fed insect meal, the presence of *Paenibacillus* in the gut microbiome was associated with increased levels of volatile short-chain fatty acids (SCFAs), particularly acetic and propionic acids, which are known for their anti-inflammatory properties and key roles in modulating the immune system.

Plasma values analyzed at T0 were consistent with those expected for gilthead sea bream under optimal nutritional and unstressed conditions (Peres et al., 2013; Parma et al., 2020; Busti et al., 2020). In fish, previous research has demonstrated that factors such as feeding, diet, stress, and disease can alter blood biochemistry making it a valuable diagnostic tool (Peres et al., 2013). Parameters such as GLU, TRIG, and TP have slow variations under normal conditions but respond to perturbations with potential clinical relevance (Peres et al., 2014). Under the current experimental conditions, glucose (GLU) levels increased immediately following stress, a common metabolic response associated with energy mobilization. After 24 h of recovery, GLU levels decreased below the initial T0 values, in all diets while remaining within the basal range for sea bream, in line with previous observations in European sea bass (*Dicentrarchus labrax*) (Di Marco et al., 2008; Pelusio et al., 2022). Interestingly, TRIG showed an opposite trend to GLU, decreasing after stress and failing to recover at T24. This reduction can be attributed to the use of triglycerides as an energy source during stressful events, combined with a decrease in lipid production, as suggested in prior studies (Pérez-Jiménez et al., 2007). Plasma lactate levels can be considered an indicator of the secondary stress response, reflecting metabolic and hormonal responses (Saccol et al., 2018). In this study, lactate values of fish fed SCP7.5 were higher compared to the others during all time points. This may indicate a stronger response to crowding through increased anaerobic muscle activity, as reported in European sea bass fed high FM/FO diets compared to those with low FM content and similarly exposed to crowding (Pelusio et al., 2022). Starvation or elevated stocking densities cause a significant reduction in P, confirming its importance as a general indicator of stress in fish (Pelusio et al., 2022). Accordingly, in this study P levels followed the expected stress-induced pattern, decreasing significantly at T24, yet remaining generally higher in the SCP7.5 group. The electrolyte Mg followed a similar pattern, indicating acid-base and ion transport disturbances after stress. Non-specific plasma enzymes ALT, AST, ALP, LDH and CK are plasma indicators that can provide important information on the health status of fish. Elevated plasma levels of these enzymes can be an indication of tissue damage in several organs, such as the liver, muscle, spleen, and kidney (Pelusio et al., 2022). AST showed higher values in the CTRL diet compared to SCP5 and SCP10 at all time points. Considering that AST is specifically associated with liver damage, this data suggests that although acute stress promote increase of AST in all treatments, SCP diets, and particularly SCP5 and SCP10, counteracted this effect. Similar results were illustrated in Nile tilapia (Noor-Ul et al., 2020), where lower levels of AST in the serum were observed in response to a diet containing the filamentous fungi *Geotrichum candidum*. Increased plasma LDH is a signal of muscle, renal and liver damage (Peres et al., 2013). In this study, LDH values showed a slight decrease at T2 and returned to basal levels by T24 in SCP diets, indicating the potential benefits of SCP for the above-mentioned organs. CK is mainly expressed in muscle and heart tissue and plays an important role in cellular energy homeostasis (Peres et al., 2013). CK levels were found to be similarly elevated for all treatments at 24 h post-stress, probably due to muscle damage. Generally, at all-time points, CK values were lower in SCP10, pointing to a potential improvement in enzyme functioning. The inclusion of SCP in the diet influenced the stress response and overall plasma biochemistry, particularly in mitigating tissue damage and enhancing metabolic resilience. SCP diets, especially SCP5 and SCP10, demonstrated antioxidant and liver-protective properties, as evidenced by lower AST and CK levels post-stress. These effects are likely linked to components such as β-glucans and chitosan, which are present in fungal cell walls. β-glucans are known for their antioxidant properties, stabilizing and neutralizing reactive radicals like hydroxyl (OH•) and superoxide (O2-•) (Fernandes and Coimbra, 2023). Chitosan oligosaccharides, on the other hand, have demonstrated the ability to reduce oxidative damage and serum AST levels in other models, supporting their hepatoprotective role (Xiang et al., 2021; Busti et al., 2024).

In this study, based on the gene expression results at T0, several differences were detected in genes related to antioxidant and immune regulation among treatments. Glutathione reductase is essential for maintaining intracellular levels of reduced glutathione by catalyzing the reduction of oxidized glutathione. An increase in *gr* expression indicates an enhanced antioxidant enzyme response in fish, which may be associated with specific components of the PV, including chitin. Similarly, chitin derived from insects has been shown to enhance intestinal antioxidant defenses in rainbow trout fed yellow mealworm larvae meal (Henry et al., 2018). These findings support the plasma data observed in this study: the concomitant increase in *gr* expression and the reduction in CK and LDH levels suggest that antioxidant protection is active and effective in preventing muscle and tissue damage. Reactive oxygen species can compromise cellular components such as lipids, proteins, and membranes, leading to the leakage of intracellular enzymes like CK and LDH into the plasma (Henry et al., 2018). Regarding the stress response, intestinal *gcr* expression was significantly reduced by the dietary PV both before and 24 h after the stress challenge, with a decreasing trend also observed 2 h post-stress ($p < 0.1$). This down-regulation may indicate a reduced need for cortisol-mediated immunosuppression, potentially due to the presence of anti-inflammatory compounds in the yeast, such as β-glucans, mannan oligosaccharides, and nucleotides. In previous studies, *gcr* expression in the intestine of gilthead sea bream was shown to increase 24 h after acute air exposure, confirming its role in the local stress response (Vallejos-Vidal et al., 2022). The pro-inflammatory cytokine *tnfα* was notably upregulated in SCP10. *tnfα* is a versatile cytokine that plays a crucial role in the immune system. It is involved in a wide range of cellular processes, including cell proliferation, differentiation, apoptosis, and the regulation of homeostasis (Güroy et al., 2022). Considering its properties, upregulation of *tnfα* indicates an enhanced immune response, which can be advantageous in bolstering the fish's ability to combat pathogens. These findings align with previous research indicating an increased expression of *tnfα* in rainbow trout fed a diet supplemented with a multi-strain yeast fraction product (*Saccharomyces cerevisiae* and *Cyberlindnera jadinii*) (Rawling et al., 2021). Similarly, an up-regulation of *tnfα* was observed also in largemouth bass (*Micropterus salmoides*) fed SCP from *Methylococcus capsulatus* (Zhang et al., 2022). On the contrary, SCP from yeast decreased *tnfα* expression in Atlantic salmon (Sahlmann et al., 2019) and catfish (*Ictalurus punctatus*) (Hao et al., 2022). These studies underscore that yeast and fungi supplements can enhance the immune response by upregulating pro-inflammatory cytokines like *tnfα*. However, the impact may vary depending on the type of SCP used and the specific fish species observed. Additionally, the genes involved in nutrient absorption, *pept 1,*

slc5a1, and fabp2, presented higher expression in diets containing PV, and the water channel gene *aquap8* involved showed a clear dose-response effect. Nevertheless, our findings suggest that the inclusion of SCP from PV have a discernible impact on the expression of genes associated with nutrient transport, resulting in increased expression. This observation may signify an enhanced ability for nutrient absorption and potentially reflects a natural adaptation of the fish to dietary changes.

The transcriptional profiles observed in this study indicate that dietary SCP influenced the ability of fish to cope with crowding stress. Indeed, the two-way ANOVA revealed significant interactions between diet and sampling time in several transcripts. Antioxidant enzymes, such as *gr*, are known to increase or be modulated in response to environmental challenges, including pollutants and stressors (Formicki et al., 2025; Hossen et al., 2023). For instance, rainbow trout exposed to high stocking densities showed elevated antioxidant activities as part of the neuro-endocrine-immune response (Hou et al., 2019). In our experiment, a transient downregulation of *gr* expression was observed at T2 in the SCP7.5 group, which may indicate a reduced activation of the stress axis and a more efficient antioxidant response at this inclusion level. Environmental stressors are known to influence the regulation of *mhc* genes in fish, thereby modulating their immune responses. For instance, downregulation of *mhc-I* has been reported under cold stress (Abram et al., 2017), whereas high temperatures have shown variable effects depending on species and tissue (Rogozynski et al., 2024). In our trial, *mhc-I* expression was generally reduced at T2, but only in the SCP7.5 group it showed a subsequent increase at T24. Given that *mhc-I* molecules are essential for presenting endogenous antigens to cytotoxic T lymphocytes (Johnstone and Chaves-Pozo, 2022), these results support the immunomodulatory potential of SCP at the tested inclusion level. Similarly, tcrβ is a critical component of the T cell receptor complex, mediating antigen recognition and activation of cytotoxic T lymphocytes. The expression of *tcrβ* is known to increase following stressor events, such as pathogen exposure in fish (Shi et al., 2025). To our knowledge, no previous studies have directly examined the combined effects of SCP-based diets, crowding stress, and *tcrβ* expression in fish. In our study, *tcrβ* was significantly modulated at T24 only in the SCP7.5 and SCP10 groups. Taken together, these complementary trends suggest that SCP inclusion may support both antigen presentation (via *mhc-I*) and T cell activation (via t*crβ*). This coordinated modulation points toward a potential protective role of SCP in sustaining adaptive immune competence under crowding stress.

Concerning intestinal absorptive capacity under stress condition, an upregulation of *slc5a1* was observed in fish fed the SCP10 diet after stress (T24). Conversely, *aminopn*, a marker of gut barrier function, tended to be downregulated across treatments shortly after the crowding challenge (T2). These patterns are consistent with previous reports indicating that crowding stress impairs gut barrier integrity and enzyme activities in fish (Zhang et al., 2025), while diets supplemented with SCP may help preserve intestinal structure and function even when FM is partially replaced (Woolley et al., 2023).

5. Conclusion

In conclusion, these findings validate the filamentous fungus *Paecilomyces variotii* as a promising protein source for gilthead sea bream, not only as a replacement for conventional protein sources in current feed formulations but also for its potential as a functional ingredient.

CRediT authorship contribution statement

Serena Busti: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Trond M Kortner:** Writing – review & editing, Methodology, Investigation. **Elin Christine Valen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Elisa Benini:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Alessio Bonaldo:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Charilaos Xiros:** Methodology, Conceptualization. **Fabio Brambilla:** Writing – review & editing, Investigation, Conceptualization. **Daniel Scicchitano:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Giorgia Palladino:** Writing – review & editing, Methodology, Investigation. **Marco Candela:** Methodology, Conceptualization. **Marco Berrettini:** Writing – review & editing, Methodology, Investigation. **Francesco Dondi:** Writing – review & editing, Validation, Methodology. **Maria Giulia Ferrari:** Writing – review & editing, Methodology, Investigation. **Pier Paolo Gatta:** Writing – review & editing, Conceptualization. **Luca Parma:** Writing – review & editing, Writing – original draft, Methodology, Investigation, 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 entitled.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2026.743729.

Data availability

Data will be made available on request.

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