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# Effects of dietary curcumin in growth performance, oxidative status and gut morphometry and function of gilthead seabream postlarvae

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ABSTRACT

An imbalance in the production and detoxification of reactive oxygen species and other oxidants can disrupt all types of cellular compounds, and lead to a state of oxidative stress. Preventing this state is essential to improve growth and health in animal production. Therefore, the aim of this work was to assess if the dietary supplementation of curcumin could improve the antioxidant status and intestine structure and functionality of gilthead seabream postlarvae, with the global objective of improving fish growth performance and robustness. Two experimental diets supplemented with different doses of curcumin (LOW and HIGH diets), and a commercial diet were fed to quadruplicate groups of postlarvae, for 20 days. At the end of the feeding trial fish fed the supplemented diets significantly improved their antioxidant status compared to CTRL fed fish. LOW and HIGH fed fish presented lower protein oxidative damage ($P < 0.05$) and higher total antioxidant capacity ($P < 0.05$). Moreover, postlarvae fed curcumin supplemented diets also presented an upregulation of nuclear factor erythroid 2 – related factor 2 (*nrf2*) and glutathione-disulfide reductase (*gr*) in HIGH ($P < 0.05$) and heat shock protein 70 (*hsp70*) in LOW treatments ($P < 0.05$). No differences were observed in growth performance, intestine morphometry, and digestive enzymes activities among treatments ($P > 0.05$). In conclusion, dietary curcumin supplementation was able to enhance gilthead seabream postlarvae robustness through a modulation of the oxidative status, increasing total antioxidant capacity and decreasing protein oxidative damage. This data provide evidence that curcumin can be a suitable feed additive to promote heath status and robustness of fish at early stages of development, therefore contributing for the development and sustainability of marine fish hatchery production.

## 1. Introduction

Marine fish larvae presents higher growth rates when compared to juveniles and adults, with rates of 10–30%/day being common (Conceição et al., 2007). Moreover, oxygen consumption is also higher at early stages and consequently larvae presents high metabolic rates. Hence, this period in fish development is considered highly susceptible to stress (Betancor et al., 2012). The generation of reactive oxygen species (ROS) is a by-product inherent to metabolism. It mainly occurs in the mitochondria through the leakage of electrons from the electron transport chains, leading to a partial reduction of oxygen (Munro and Treberg, 2017). The main ROS produced at mitochondrial level are superoxide radicals which can be rapidly dismutased into non-radical hydrogen peroxide that are easily diffuse to all cellular compartments. These oxygen - derived free radicals can lead to oxidation of several cellular constituents like proteins, lipids, or DNA (Halliwell and Gutteridge, 2015). However, the potential deleterious effects of ROS can be counteracted with endogenous antioxidants, which comprises a variety

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of enzymatic and non-enzymatic scavenger molecules. At low or moderate levels, ROS acts as secondary messengers for several vital functions, such as cell proliferation, differentiation, immune response, cell senescence and death (Sokolova, 2018). Nevertheless, a state of oxidative stress occurs when there is an imbalance between the formation of ROS and antioxidant defences (Hoseini et al., 2022).

The intestinal tract is an organ highly prone to oxidative stress, due to multiple internal (eg., high metabolic rate and mitochondria content) (Taylor et al., 2010) and external triggers (eg., prooxidants/toxic substances present in the feed and water) (Hua-Tao et al., 2019; Hoseini et al., 2019, 2022; Wang et al., 2019; Hoseini and Yousefi, 2019; Hoseini, et al., 2021). The intestinal mucosa acts as a physiological barrier, and it can adapt and morphologically react (altering villus length and height, and number of goblet cells) in response to external factors (Rajput et al., 2013; Yusuf et al., 2017). The structure and function of the intestine plays a vital role in fish digestive capacity and efficiency, therefore, has a direct impact on growth performance and aquaculture production (Midhun et al., 2016).

The use of additives in fish diets has been widely studied as promoters of growth and health status, hence improving aquaculture production efficiency. Curcumin (diferuloylmethane), a polyphenol extracted from the rhizome of the Indian spice *Curcuma longa* L. has received increasing attention as a good candidate for fish diet supplementation. Curcumin has recognized roles as antioxidant, anti-inflammatory, immunostimulant and antimicrobial (Alagawany et al., 2020). This pigment can activate the translocation of the transcript factor *nrf2* which is involved in the regulation of the cellular antioxidant defences (Giri et al., 2019). A recent study reported that dietary curcumin supplementation positively modulated antioxidant capacity and enhanced growth of Senegalese sole postlarvae (*Solea senegalensis*) (Xavier et al., 2021a, 2020). Additionally, inclusion of curcumin in the diets for juvenile rainbow trout (*Oncorhynchus mykiss*) (Akdemir et al., 2017; Yonar et al., 2019), Nile tilapia (*Oreochromis niloticus*) (Abd El-Hakim et al., 2020; Cui et al., 2013; Mahmoud et al., 2017) and common carp (*Cyprinus carpio*) (Giri et al., 2019) improved fish immune response and disease resistance. Due to the curcumin multiple action pathways, dietary curcumin also led to a wide range of effects in the gastrointestinal tract of fish species. Improved growth was observed in crucian carp (*Carassius auratus*) and grass carp (*Ctenopharyngodon idella*) juveniles fed 0.5% and 0.01–0.05% dietary curcumin, respectively, through a promotion of digestive and absorptive capacity (Jiang et al., 2016; Li et al., 2020). Similarly, in Nile tilapia, curcumin supplementation at 0.5% and 1% improved digestive enzymes activity (α-amylase, protease and lipase) and the expression of growth factors in the muscle (Midhun et al., 2016). Moreover, curcumin (0.5%) and turmeric (1%) modulated gut morphometry in climbing perch (*Anabas testudineus*) and tilapia, respectively (Manju et al., 2013; Yusuf et al., 2017). In gilthead seabream (*Sparus aurata*) dietary curcumin 0.3% was able to enhance proteolytic capacity in larvae and in juveniles addition of 2–3% curcumin in the diet was able to modulate the fish gut microbiota (Ashry et al., 2021; Xavier et al., 2021b).

The aim of this work was to assess if the dietary supplementation of curcumin could improve the antioxidant status and intestinal structure and functionality of gilthead seabream postlarvae, with the global objective of improving fish growth performance and robustness.

## 2. Material and methods

### 2.1. Husbandry and experimental set-up

This experiment was carried out by trained scientists and followed the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes and was approved by the Committee of Ethic and Animal Experimentation of the Centre of Marine Sciences of Algarve (CCMAR). The CCMAR (Faro, Portugal) facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinaria', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Gilthead seabream postlarvae were supplied by MARESA - Mariscos de Estero S.A. (Huelva, Spain) and transferred to a recirculating aquaculture system at Centre of Marine Sciences facilities (CCMAR, Faro, Portugal). The experimental feeding period lasted 20 days, from 42 days after hatching (DAH) to 62 DAH, under optimal environmental and zootechnical conditions. Postlarvae dry weight (DW) and standard length (SL) at 42 DAH were in average 0.70 ± 034 mg and 7.78 ± 1.03 mm, respectively. Postlarvae were kept in 100 L cylindro-conical tanks, at an initial density of 2200 individuals (22 postlarvae/L). The dietary treatments (CTRL, LOW and HIGH diets) were randomly assigned to replicate tanks ($n = 4$ tanks per treatment). The recirculating aquaculture system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer and UV sterilizer. Abiotic parameters were measured, and mortality was recorded daily; dead postlarvae were removed, and the tanks carefully cleaned with minimal disturbance. Dissolved oxygen in water was 97.1 ± 2.1% of saturation, temperature was 19.9 ± 0.2 °C and salinity was 35.3 ± 0.8 ppt. A 12 h: 12 h light/dark photoperiod cycle was maintained. Inert diet was supplied by hand to visual satiety during the first 8 h and semi-continuously with automatic feeders during the rest of the day (16 h) (cycles of 2 h of feeding followed by 1 h resting), feeding plan provide in Table 1.

### 2.2. Experimental diets

Three diets were tested in this study; two experimental diets supplemented with curcumin (LOW and HIGH diets) and a commercial diet used as control (CTRL diet, WINFast, SPAROS Lda., Portugal). According to manufacturer's data, the diet contained ingredients such as squid meal, wheat gluten, fish meal, crustacean meal, fish protein hydrolysate, gelatin, fish oil, lecithin and a micronutrient premix comprising vitamins, minerals and other additives. The two experimental diets were prepared by supplementing the CTRL diet with curcumin extract at 0.8 g/kg feed (LOW diet) and at 1.5 g/kg of feed (HIGH diet). The selected doses of curcumin were based on previous work in Senegalese sole postlarvae (Xavier et al., 2020) and were modified base on larvae feed intake and developmental stage (Xavier et al., 2021b). The

**Table 1**
Feeding plan of gilthead seabream postlarvae throughout the experimental period (41–62 DAH).

| Feeding plan | |
|---|---|
| Age (DAH) | Inert diet |
| 41 | 4.0 |
| 42 | 5.0 |
| 43 | 6.0 |
| 44 | 6.0 |
| 45 | 6.0 |
| 46 | 7.0 |
| 47 | 7.0 |
| 48 | 8.0 |
| 49 | 9.0 |
| 50 | 9.0 |
| 51 | 9.5 |
| 52 | 9.5 |
| 53 | 9.5 |
| 54 | 10.0 |
| 55 | 10.0 |
| 56 | 11.0 |
| 57 | 11.0 |
| 58 | 11.0 |
| 59 | 11.0 |
| 60 | 11.0 |
| 61 | 11.0 |
| 62 | 11.0 |

Inert diet daily ration is expressed as 'g/tank/day'.

curcumin (95.34% purity) used in the supplemented diets were provided by Denk Ingredients (Germany). All diets were manufactured by SPAROS Lda. (Olhão, Portugal). Feed samples were grounded and analysed for dry matter (105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 150 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). Diets proximal composition was identical for all three diets, with 66.3% Dry matter (DM) crude protein, 17.4% DM crude fat, and 21.1 MJ $kg^{-1}$ gross energy.

### 2.3. Growth performance

Before any sampling the postlarvae were euthanized with an overdose of 2-phenoxyethanol (1000 ppm; Prolabo, VWR International LLC, Radnor, USA), washed twice in distilled water. For dry weight (DW; mg) and body length (Standard length (SL); mm) determination, individual postlarvae were sampled at the beginning ($n = 30$) and at the end of the experiment ($n = 15$ per replicate; $n = 60$ per treatment). Individual SL was determined using Leica Application Suite LAS (Leica Microsystems, Germany) for digital image analysis and DW was obtained from freeze-dried postlarvae using a high precision microbalance ( ± 0.001 mg; MSA36S-000-DH, Sartorius, Germany). The condition factor (K) was calculated according to Fulton's condition factor formula: K= final body weight (mg)/[final body length (mm)]$^3$. To determine the inter-individual variation of weight, length, and condition among fish of the same treatment, the coefficient of variation (CV, %) was calculate as: CV = treatment standard deviation/treatment mean × 100. Growth was expressed as relative growth rate (RGR, % $day^{-1}$) was calculated, at the end of the experiment, using the formula: ($e^{g-1}$) × 100, with g = [(ln final weight−ln initial weight)/time] (Ricker, 1958). Survival rate (%) was calculated as the percentage of the live postlarvae at the end of the trial relative to the initial number.

### 2.4. Proximal composition

At the end of the growth trial, 20 pooled-postlarvae per replicate tank ($n = 4$ pools per treatment) were sampled for proximal composition analysis. Samples were freeze-dried and then finely ground using an Ultra-Turrax IKA T8 (IKA-WERG, Germany). Total protein quantification was analysed following the method of Lowry adapted by Rutter W. J. (Rutter, 1967) and total lipid quantification was determined by Bligh and Dyer method (Bligh and Dyer, 1959).

### 2.5. Antioxidant status

The antioxidant status of the postlarvae was assessed by measuring the following oxidative stress biomarkers: total glutathione (GSH), total antioxidant capacity (TAC), protein carbonylation (PC), lipid peroxidation (LPO) and mitochondrial reactive oxygen species production (mtROS).

#### 2.5.1. Sample preparation for biomarkers analysis

For the analyses of GSH, TAC, PC and LPO, three pools of 5 postlarvae per replicate tank ($n = 12$ pools per treatment) were sampled at the end of growth trial (62 DAH), and snap-freeze in liquid nitrogen. Samples were kept at − 80 ºC until further analysis. Samples were homogenized using a TissueLyser (Star-Beater, VWR, USA) in 1500 µl of ultra-pure water. From each sample, two aliquots of the supernatant were taken. One aliquot of 200 µl containing 4 µl of 4% butylated hydroxytoluene (BHT) in methanol was used for the determination of endogenous LPO. The second aliquot of 500 µl was diluted (1:1) in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 10 min at 10,000 g (4 ºC). The post-mitochondrial supernatant (PMS) was divided into microtubes and kept in –80 ºC for the analysis of total glutathione (GSH), total antioxidant capacity (TAC) and protein carbonylation (PC).

For mtROS determination, two pools of 5 postlarvae per replicate tank ($n = 8$ pools per treatment) were sampled at the end of growth trial (62 DAH), and snap-freeze in liquid nitrogen. Samples were kept at − 80 ºC until further analysis. Samples were homogenized through a TissueLyser (Star-Beater, VWR, USA) in 1 ml of ice-cold mitochondria isolation buffer containing 225 mM manitol, 75 mM sucrose, 1 mM EDTA and 4 mM HEPES (pH 7.2) following the protocol described by Da Silva et al. (2015). The homogenate was centrifugated for 10 min at 1200 g and 4 °C. The supernatant was carefully removed and centrifugated for 10 min, at 16,500 g and 4 °C. The pellet was re-suspended in a buffer containing 250 mM sucrose and 5 mM HEPES (pH 7.2). The samples were kept on ice during the assay and then maintained in – 80 ºC until further analysis.

The protein content of PMS (GSH, TAC and PC determination), LPO and mtROS samples was determined according to the Bradford method (Bradford, 1976), using bovine γ-globulin as a standard. All biomarkers were determined in 96 well flat bottom microplates using a temperature-controlled microplate reader (Synergy 4 BioTek, USA).

#### 2.5.2. Oxidative stress biomarkers measurement

Total glutathione content (GSH) was determined using a recycling reaction of reduced glutathione with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (Baker et al., 1990; Tietze, 1969). Briefly, 250 µl of a reaction buffer composed by Na-K phosphate buffer, NADPH, DTNB and GR was mixed with 50 µl of the sample in the microplate; kinetic was measured spectrophotometrically at 412 nm during 3 min. GSH content was calculated as the rate of $TNB^{2-}$ formation with an extinction coefficient of DTNB chromophore formed, $\varepsilon = 14.1 \times 103\ M^{-1}cm^{-1}$ (Baker et al., 1990; Rodrigues et al., 2017). Results were expressed in mmol GSH per mg protein.

Total antioxidant capacity (TAC) was assessed following the protocol described by Erel (2004), using coloured 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+}$). This method is based on the colourless molecule ABTS, which is oxidized to a characteristic blue-green $ABTS^{+}$. When the coloured $ABTS^{+}$ is mixed with any substance that can be oxidized, it is reduced to its original colourless ABTS form again; in contrast, the reacted substance is oxidized. This change in colour was measured as a change in absorbance at 660 nm and the assay was calibrated with Trolox. Briefly, 5 µl of sample was mixed in a microplate with 200 µl of acetate buffer solution (0.4 mol/l, pH 5.8) and 20 µl of $ABTS^{+}$ (in acetate buffer at 30 mmol/l, pH 3.6). The first reading was taken just after adding all the reagents (as sample blank) while the last absorbance was taken at the end of the incubation period (5 min after the mixing). Results were expressed in mmol Trolox equivalent per mg protein.

Protein carbonylation (PC) was measured based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl groups, according to the DNPH alkaline method described by Mesquita et al. (2014). Briefly, DNPH (120 µl, 10 mM in 2 M HCl) was added to 120 µl of sample and after 10 min of incubation, 60 µl of NaOH (6 M) were added. The amount of carbonyl groups was quantified spectrophotometrically at 450 nm at room temperature against a blank where the sample was substituted by an equal volume of buffer solution (22,308 $mM^{-1}cm^{-1}$extinction coefficient). Results were expressed in nmol carbonyl per mg protein.

Lipid peroxidation (LPO) was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) (Bird and Draper, 1984). Briefly, 100 µl of cold tricholoroacetic acid (TCA) 100% were added to the samples, followed by the addition of 1000 µl of 2-thiobarbituric acid (TBA) 73%. Samples were incubated at 100 °C for 1 h. After this period, samples were kept for up to 16 h in the dark. Samples were centrifuged at 10,350 g for 5 min at 25 °C and 300 µl of resulting supernatant were loaded into a microplate and absorbance was read at 535 nm. Results were expressed in nmol TBARS/mg protein.

Mitochondrial reactive oxygen species (mtROS) production was assessed by the dihydrodichloro-fluorescein diacetate –H(2)DCF-DA (Da

Silva et al., 2015; van der Toorn et al., 2007). This dye is non-fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent (García-Ruiz et al., 1995). The mitochondrial suspension (0.5 mg protein) was incubated in the presence of 1 μM DCFDA and fluorescence was monitored over 5 min of gentle shaking at 28 °C, with excitation and emission wavelengths of 503 and 529 nm, respectively. Under the described conditions the linear increment of fluorescence indicated the rate of ROS formation. Results are expressed as Relative Fluorescence Units (RFU) per mg mitochondrial protein.

### 2.6. Reverse transcription–quantitative real-time PCR (qPCR)

For gene expression, two pools of 3 postlarvae per replicate tank ($n = 8$ pools per treatment) were analysed at 62 DAH, and snap-freeze in liquid nitrogen. Samples were kept at − 80 ºC until further analysis. Gene expression was determined in fish visceral cavity, hence, head, body section above the vertebral column and tail fin were removed from all postlarvae. This process was done individually without thawing. Samples were homogenised using a TissueLyser II (Star-Beater, VWR, USA) with 1 ml Tri Reagent (Sigma-Aldrich) for 60 s. Chloroform (200 μl) was added to each sample before centrifuging at 11,000 g for 15 min. The supernatant content was transferred to columns of the Isolate II RNA Mini Kit (Bioline) and total RNA was treated twice for 30 min with DNase I following the manufacturer's protocols. Total RNA quality and concentration was determined by Nanodrop OneC (Thermo Scientific). One μg of total RNA was reverse-transcribed using the M-MLV Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's protocol.

The qPCR assays were performed in triplicates in a 10 μl volume containing cDNA generated from 10 ng of the original RNA template, 300 nM of each specific forward and reverse primers, and 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad). The expression level of genes involved in the oxidative stress defence was analysed: superoxide dismutase [Cu-Zn] (*sod1*), catalase (*cat*), glutathione peroxidase 1 (*gpx1*), glutathione peroxidase 4 (*gpx4*), glutathione-disulfide reductase (*gr*), nuclear factor erythroid 2 – related factor 2 (*nrf2*); cellular stress proteins: heat shock protein 70 (*hsp70*), heat shock protein 90 alpha (*hsp90aa*) and gut digestive capacity: enterocyte membrane peptide (*pept1*), intestinal alkaline phosphatase (*ialp*). Primers for *Sparus aurata* were previously published (Cerezuela and Meseguer, 2013; Espinosa et al., 2018; Pérez-Sánchez et al., 2013; Vieira et al., 2012) and are presented in Table 2. The qPCR amplification protocol was set as follows: 7 min for denaturation and enzyme activation at 95 ºC followed by 40 cycles of 30 s at 95 ºC and 1 min at 60 ºC. Expression data were normalised using the geometric mean of two reference genes, β-actin (*actin*) and elongation factor 1 alpha (*ef1a*) (Estruch et al., 2018) and the relative mRNA expression calculated using the comparative Ct method (Livak and Schmittgen, 2001).

### 2.7. Digestive enzymes activity

At 62 DAH, two pools of 5 postlarvae per replicate tank ($n = 8$ pools per treatment) were freeze-dried and analysed to assess the activity of leucine-alanine peptidase (Leu-Ala) and alkaline phosphatase (Alk phosphatase). Samples were mechanically homogenized using an Ultra-Turrax® Homogenizer T-18 (IKA®-Werke). Extracts were prepared following the protocol described by Gisbert et al. (2018), consisting in two steps; a first one to extract intestinal cytosolic enzymes, and a second step for brush border (BB) membranes purification. Briefly, samples were homogenized in 50 mM mannitol - 2 mM Tris (pH 7) and centrifuged at 9000 g, 10 min, 4 °C. The supernatant was recovered and used for the analysis of leu-ala activity. To the remaining pellet of cell debris 10 mM $CaCl_2$ were add and a second centrifugation was performed at 16, 000 g, 40 min, at 4 °C. The supernatant was discarded, and the pellet (containing BB) was dissolved in 50 mM mannitol - 2 mM Tris (pH 7). This fraction was used for the analysis of alk phosphatase activity. All samples were kept in ice during the process described above to avoid enzymes denaturation and/or damage. Enzyme extracts were kept at − 20 °C until analysis.

The analysis of leu–ala was performed using the method described by Nicholson and Kim (1975), using leucine–alanine as substrate in 100 mM Tris–HCl buffer (pH 8.0), and time of incubation of samples with the substrate was 20 min (end point measurement). One unit of enzyme activity (U) was defined as 1 nmol of the hydrolysed substrate per min per ml of homogenate at 37 ºC and at 530 nm. Alk phosphatase activity was quantified for 5 min at 37 ºC (kinetics measurement) using 4-nitrophenyl phosphate (PNPP) as substrate in 100 mM Tris-HCl buffer, pH 8. One unit (U) was defined as the hydrolysis of 1 μmol of p-nitrophenyl phosphate per min per ml of BB homogenate at 407 nm (Bessey, 1946). The activity of both enzymes was expressed as units per milligram of dry weight (U/mg DW).

### 2.8. Gut morphometry

At the end of the trial eight individual postlarvae per treatment were photographed for SL determination and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, USA) in phosphate buffered saline (PBS tablets, Sigma-Aldrich, St. Louis, USA) for 24 h. Samples were then washed with PBS and stored in ethanol 70% at 4 ºC. Samples were dehydrated in a graded ethanol (AGA, Prior Velho, Portugal) series, cleared in xylol (Prolabo, VWR International LLC, Radnor, USA) and included in paraffin Histosec® (Merck, Whitehouse Station, USA). Each postlarva was sectioned (5 μm) transversely to the body axis, from the mouth to the anal opening level, to obtain the anterior intestine sections; sections were then mounted on adhesive slides, and stained with Alcian blue/PAS (Merck, Whitehouse Station, USA) (pH 2.5) before placing a cover slip.

The following morphometric values were measured in transversal body sections of the anterior intestine of each individual fish: the cross-section area [CSA ($mm^2$)]; the *villus* length and the *villus* width (μm)

**Table 2**
Primers used in qPCR.

| Gene | Fwd sequence (5′ → 3′) | Rev sequence (5′ → 3′) | Acession nr (GenBank) |
|---|---|---|---|
| *sod1* | TCACGGACAAGATGCTCACTCTC | GGTTCTGCCAATGATGGACAAGG | JQ308832 |
| *Cat* | TGGTCGAGAACTTGAAGGCTGTC | AGGACGCAGAAATGGCAGAGG | JQ308823 |
| *gr* | TGTTCAGCCACCCACCCATCGG | GCGTGATACATCGGAGTGAATGAAGTCTTG | AJ937873 |
| *gpx1* | GAAGGTGGATGTGAATGGAAAAGATG | CTGACGGGACTCCAAATGATGG | DQ524992 |
| *gpx4* | TGCGTCTGATAGGGTCCACTGTC | GTCTGCCAGTCCTCTGTCGG | AM977818 |
| *nrf2* | GTTCAGTCGGTGCTTTGACA | CTCTGATGTGCGTCTCTCCA | FP335773 |
| *hsp70* | TGTGAGAGGGCCAAGAGAAC | CCCTGGTGATGGAGGTGTAG | DQ524995.1 |
| *hsp90aa* | CGACGACACATCAAGAATGG | GAAATGTCGATTTGAAGAAGCA | Contig08719 |
| *pept1* | TTGAACATAACGTCGGGTGA | AATTTTGCATTTCCCTGTGG | GU733710 |
| *ialp* | TTACTGGGCCTGTTTGAACC | ATCCTTGATGGCCACTTCCAC | AY266359 |

Gene name, sequences, Accession numbers at GenBank are indicated.

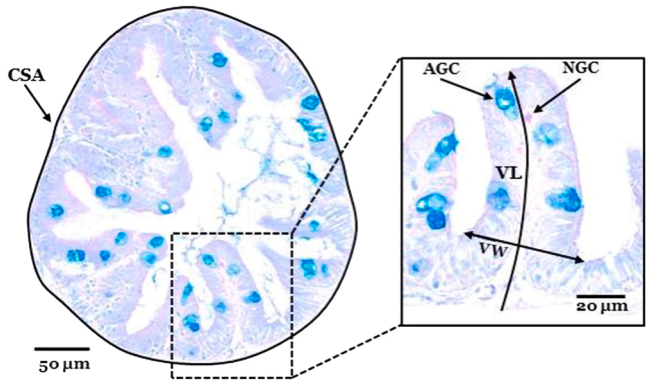


**Fig. 1.** Schematic representation of the different parameter measured in anterior intestine of gilthead seabream postlarvae at the end of the growth trial (62 DAH). CSA - Cross-section area; VL - Villus length; VW - Villus width; AGC and NGC - Acid and neutral goblet cells, respectively. Magnification: 20x and 40x. Scale bar: 50 μm and 20 μm.

(Fig. 1). The percentage of analysed *villi* corresponded to at least 80% of the total existing and were chosen based on their integrity and higher length. Total number of goblet cells present in intestinal sectional area are divided into acid and neutral cells (blue and magenta cells, respectively), and are only considered when they exhibited a round and defined cytoplasm. During the present analysis, two fish per treatment were discarded, due to the low quality of the samples. This morphometric study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Olympus cellSens Standard Desktop imaging software and photos were capture with CCD-video camera (Olympus SC50).

### 2.9. Statistical analysis

Data of growth performance, oxidative status, intestine morphology, and physiology from gilthead seabream postlarvae are means ± standard deviation (S.D.) of treatment replicates (n = 4). The data were tested for normality using a Kolmogorov-Smirnov (whenever n > 30) or Shapiro-Wilk test (whenever n < 30) and for homogeneity of variance using a Levene's test. Data were log transformed whenever required and percentages were arcsin transformed prior analysis. Comparisons between groups fed different diets were made using one-way ANOVA followed by a Tukey post-hoc test for growth performance, gut morphometry and functionality and redox status biomarkers. In all cases, significant levels were set at $P < 0.05$. All tests were carried out using IBM SPSS Statistics v19 software.

## 3. Results

### 3.1. Growth performance and proximal composition

At the end of the experiment gilthead seabream postlarvae presented a 5.9-fold increase in weight relative to the initial DW. The supplementation of curcumin (LOW and HIGH diets) did not have a significative impact in growth performance parameters of the fish, neither for DW ($P = 0.170$) or SL ($P = 0.091$) when compared to CTRL fish (Table 3). Moreover, the dietary treatments presented similar coefficient of variation for DW ($P = 0.652$) and SL ($P = 0.707$). The condition factor of the fish was not affected by the dietary treatments. A significant increase in the variation of condition factor of CTRL postlarvae was observed when compared to the fish fed the supplemented diets ($P = 0.027$). The relative growth rate (RGR) of gilthead seabream from 42 to 62 DAH was in average 9.26 ± 1.11%/day, regardless of the dietary treatments ($P = 0.771$). At the end of the trial the survival rate was 36.63 ± 4.77% ($P = 0.380$). The protein and lipid composition of the whole-body postlarvae were 80.83 ± 1.46% ($P = 0.774$) and 6.12 ± 0.45% ($P = 0.668$), respectively, and no differences were observed between dietary treatments.

**Table 3**
Gilthead seabream postlarvae key performance indicators the end of the growth trial (62 DAH).

| | Treatments | | | 1-way Anova |
|---|---|---|---|---|
| | CTRL | LOW | HIGH | P value |
| DW (mg) | 4.37 ± 1.63 | 3.92 ± 1.46 | 4.25 ± 1.36 | 0.17 |
| $CV_{DW}$ (%) | 26.92 ± 7.98 | 31.09 ± 5.94 | 27.93 ± 4.93 | 0.65 |
| SL (mm) | 11.54 ± 1.20 | 11.10 ± 1.00 | 11.36 ± 0.89 | 0.09 |
| $CV_{SL}$ (%) | 8.25 ± 3.28 | 7.87 ± 1.47 | 6.98 ± 1.38 | 0.71 |
| K | 1.42 ± 0.26 | 1.33 ± 0.17 | 1.41 ± 0.17 | 0.05 |
| $CV_k$ (%) | 16.81 ± 4.33$^{a}$ | 11.51 ± 1.87$^{ab}$ | 9.97 ± 1.86$^{b}$ | 0.03 |
| RGR (% $day^{-1}$) | 9.52 ± 1.44 | 8.92 ± 1.26 | 9.42 ± 0.95 | 0.77 |
| Survival (%) | 39.08 ± 4.02 | 37.35 ± 2.56 | 34.08 ± 6.57 | 0.38 |

Values are presented means ± SD, n = 120 per treatment at 42 DAH, n = 60 per treatment at 62 DAH. Different superscript letters (a, b) indicate significant differences ($P > 0.05$, 1-way ANOVA) between treatments at 62 DAH. DW – dry weight; CVDW – coefficient of variation dry weight; SL – standard length; K – condition factor; CVk – coefficient of variation condition factor; RGR – specific growth rate.

### 3.2. Oxidative status

All treatments presented similar contents of GSH (947.03 ± 395.09 μM/min/mg protein; $P = 0.112$) and LPO (1.66 ± 0.80 nmol TBARS/mg protein; $P = 0.418$) at the end of the growth trial. The dietary supplementation of curcumin promoted TAC of LOW and HIGH postlarvae compared to CTRL fish (Fig. 2; $P = 0.002$). This increase was 28% higher in the fish fed the supplemented diets than CTRL group. Additionally, the fish fed LOW and HIGH diet decreased PC content when compared to the CTRL treatment (Fig. 3; $P = 0.002$), showing a reduction of 47%. Postlarvae mtROS production was in average 1055.49 ± 369.02 (RFU/ mg protein) and no differences were observed among dietary treatments ($P = 0.108$).

All fish, regardless of the treatment, showed no differences in the expression of endogenous antioxidant defences (*sod1, cat, gpx1, gpx3*) and the cellular stress protein *hsp90aa* (Table 4; $P > 0.05$). In contrast, the activity of *gr* was up-regulated in HIGH and LOW postlarvae,

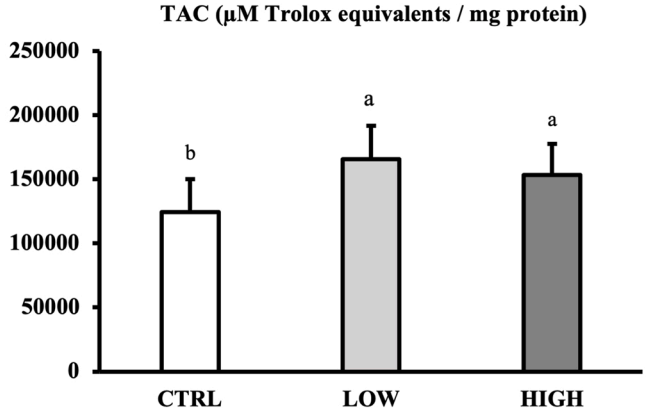


**Fig. 2.** Total antioxidant capacity (TAC) of gilthead seabream postlarvae from CTRL, LOW and HIGH treatments at the end of the growth trial (62 DAH). Values are presented means ± SD, $n = 12$ pools per treatment. Different superscript letters (a, b) indicate significant differences ($P < 0.05$, 1-way ANOVA) between treatments.

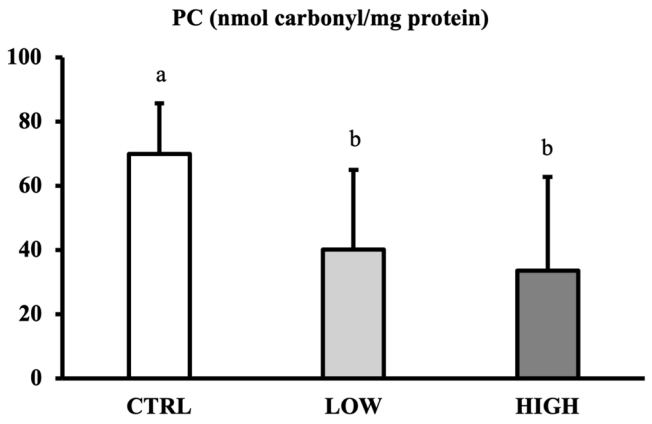


**Fig. 3.** Protein carbonylation (PC) content of gilthead seabream postlarvae from CTRL, LOW and HIGH treatments at the end of the growth trial (62 DAH). Values are presented means ± SD, $n = 12$ pools per treatment. Different superscript letters (a, b) indicate significant differences ($P < 0.05$, 1-way ANOVA) between treatments.

**Table 4**
Expression level of genes encoding for antioxidative status, cellular stress and gut maturation in 62 DAH gilthead seabream postlarvae from CTRL, LOW and HIGH treatments.

| | Treatments | | | 1- way Anova |
|---|---|---|---|---|
| | CTRL | LOW | HIGH | P value |
| Gene expression | | | | |
| *sod1* | 1.01 ± 0.13 | 1.02 ± 0.08 | 1.06 ± 0.10 | 0.86 |
| *cat* | 1.01 ± 0.15 | 0.96 ± 0.16 | 0.95 ± 0.10 | 0.98 |
| *gr* | 1.03 ± 0.25[b] | 1.17 ± 0.28[ab] | 1.44 ± 0.30[a] | 0.03 |
| *gpx1* | 1.00 ± 0.09 | 1.04 ± 0.13 | 0.89 ± 0.14 | 0.14 |
| *gpx4* | 1.01 ± 0.15 | 1.06 ± 0.21 | 0.90 ± 0.15 | 0.21 |
| *nrf2* | 1.00 ± 0.10[b] | 1.06 ± 0.14[b] | 1.15 ± 0.04[a] | 0.00 |
| *hsp70* | 1.04 ± 0.31[b] | 1.29 ± 0.15[a] | 1.17 ± 0.14[ab] | 0.03 |
| *hsp90aa* | 1.02 ± 0.20 | 1.00 ± 0.22 | 0.97 ± 0.26 | 0.74 |
| *pept1* | 1.02 ± 0.22 | 1.13 ± 0.31 | 0.89 ± 0.24 | 0.21 |
| *ialp* | 1.01 ± 0.14 | 1.07 ± 0.15 | 0.99 ± 0.18 | 0.54 |

Values are presented means ± SD, n = 8 pools per treatment. Different superscript letters (a, b) indicate significant differences ($P < 0.05$, 1-way ANOVA) between the dietary treatments. sod1 – superoxide dismutase; cat – catalase; gr – glutathione-disulfide reductase; gpx1 – glutathione peroxidase 1; gpx4 – glutathione peroxidase 4; nrf2 – nuclear factor erythroid 2 – related factor 2; hsp70 – heat shock protein 70; hsp90aa – heat shock protein 90 alpha; pept1 – enterocyte membrane peptide; ialp – intestinal alkaline phosphatase

however, only the HIGH treatment was significantly higher compared to the CTRL group ($P = 0.031$). In addition, the expression of *nrf2* was also higher in HIGH fish than in LOW and CTRL fish ($P = 0.002$). An up-regulation in the expression of *hsp70* was apparent in LOW and HIGH postlarvae. However, only the LOW treatment significantly differed from the CTRL ($P = 0.034$).

### 3.3. Gut morphometry and function

The supplementation of curcumin did not present any significant effect on the expression of *pept1* and *ialp* genes in gilthead postlarvae compared to the CTRL ($P > 0.05$) (Table 4). Furthermore, gilthead seabream fed the different diets presented similar activities of leu-ala and alk phosphatase ($P > 0.05$), with values of 28.80 ± 5.05 (U / DW) and 75.26 ± 16.04 (U / DW), respectively. The mean ratio between the activity of these two enzymes (leu-ala / alk phosphatase) was 0.38 ± 0.02 in 62 DAH gilthead postlarvae ($P = 0.509$).

The fish anterior intestinal structure was measured by the area and perimeter of the intestinal cross-section, the villi length and width and the total number of goblet cells (acid and neutral) per cross-section, did not present significant differences among treatments ($P < 0.05$) (Table 5).

## 4. Discussion

Feed additives present a variety of biological compounds that might promote growth performance and health status of the fish. It is well described that the dietary supplementation of curcumin stimulates growth performance in juveniles of different freshwater species such as Nile tilapia, wuchang bream (*Megalobrama amblycephala*), snakehead fish (Channa argus), rainbow trout, common and grass carp (Akdemir et al., 2017; Cui et al., 2013; Giri et al., 2019; Li et al., 2022; Ming et al., 2020; Xia et al., 2015). However, only recently this supplement has been studied in marine fish species (Ashry et al., 2021; Ji et al., 2020; Xavier et al., 2020). In 70 DAH Senegalese sole postlarvae, a growth promoter effect of dietary curcumin was observed after 25 days of feeding (Xavier et al., 2020, 2021a). Similarly, in juveniles of gilthead seabream (average initial weight: 20.00 ± 0.37 g) the same positive effect was observed with the supplementation of curcumin in the diets. However, in the present work dietary curcumin could not influence growth performance, RGR or condition factor of gilthead seabream postlarvae at 62 DAH. Nevertheless, fish fed LOW and HIGH diets presented a more homogeneous condition factor when compared to CTRL fish. In hatcheries, homogeneous cohorts have a positive impact on fish biomass production. A higher homogeneity in fish condition factor might suggest a positive contribution of curcumin supplementation in the promotion of fish robustness. Similarly, a study performed in gilthead seabream larvae fed curcumin-supplemented diets from mouth opening did not show significative differences in growth performance, however, these larvae presented a better condition factor when compared with larvae without any supplementation (Xavier et al., 2021b). The effects of dietary curcumin supplementation have been shown to vary within fish species (Li et al., 2020), with inclusion dose (Mahmoud et al., 2017) and with the duration of the trial (Manju et al., 2012). The present results may indicate that for gilthead seabream postlarvae a higher dietary curcumin supplementation level might be required to display a positive effect on growth performance, as previously observed in lasted stages of development (Ashry et al., 2021).

The antioxidant effect associated with curcumin is due to its chemical structure that allows it to act as a direct scavenger of radicals or

**Table 5**
Anterior intestine morphology of gilthead seabream postlarvae from CTRL, LOW and HIGH treatments at the end of the growth trial (62 DAH).

| | Treatments | | | 1 - way Anova |
|---|---|---|---|---|
| | CTRL | LOW | HIGH | P value |
| Area ($\mu m^2$) | 57,981.00 ± 7241.68 | 46,146.62 ± 15,172.05 | 47,352.88 ± 10,356.32 | 0.36 |
| Perimeter (μm) | 921.94 ± 54.66 | 810.62 ± 138.66 | 847.13 ± 129.54 | 0.37 |
| Villus lenght (μm) | 86.98 ± 22.36 | 72.85 ± 20.43 | 73.33 ± 8.26 | 0.40 |
| Villus width (μm) | 54.97 ± 9.34 | 55.24 ± 18.25 | 48.80 ± 1.25 | 0.77 |
| Total number of goblet cells (*n*) | 40.25 ± 21.60 | 30.00 ± 7.07 | 31.88 ± 8.27 | 0.56 |
| Acid goblet cells (*n*) | 40.00 ± 21.64 | 28.88 ± 6.76 | 31.50 ± 8.01 | 0.52 |
| Neutral goblet cells (*n*) | 0.25 ± 0.50 | 1.13 ± 0.85 | 0.38 ± 0.48 | 0.16 |

Values are presented means ± SD, n = 6 fish per treatment. Absence of superscript letters indicates no significant differences ($P > 0.05$, 1-way ANOVA) between treatments at 62 DAH.

indirectly by inducing an antioxidant response (Kidd, 2009). In fact, this propriety has been related with the growth promoter effect of curcumin in tilapia, rainbow trout and Senegalese sole (Akdemir et al., 2017; Cui et al., 2013; Xavier et al., 2021a). To understand the effects of dietary antioxidant curcumin in gilthead postlarvae oxidative status the present study analysed the products of ROS-induced modification of cellular constituents, low molecular weight antioxidants, and mtROS production. The present results showed that dietary supplementation of curcumin (LOW and HIGH diets) enhanced antioxidant status in gilthead seabream postlarvae compared to CTRL fed fish. Both doses of curcumin, LOW and HIGH, were able to increase postlarval antioxidant capacity and decreased protein oxidative damage, which might allow fish to cope with their physiological needs and with the surrounding environmental, thus improving fish robustness. In fact, previous studies also showed that curcumin supplementation promoted antioxidant status, through a reduction of oxidative damage and improvement of several antioxidant defences; these mechanisms allowed different juvenile fish species to cope with different adverse conditions compare to fish fed no supplemented diets (Giri et al., 2019, 2021; Hoseini et al., 2022; Mahmoud et al., 2017; Ming et al., 2020; Yonar et al., 2019).

A holistic view of the biochemical and molecular oxidative stress-related biomarkers results suggests that the two doses of dietary curcumin tested in the present study might affect the redox status of the fish by different modes of action. Although both promoted TAC and decreased PC in the postlarvae, HIGH fish showed an upregulation of *nrf2*, a transcript factor known to be the major regulator of antioxidant and cellular protective genes, which was reflected in the up-regulation of *gr* that encode the enzymatic antioxidant glutathione reductase responsible for the restorage of GSH into its reduced form. In addition, LOW postlarvae increased *hsp70* expression levels, belonging to the chaperones family, which are able to protect proteins from degradation. Therefore, beyond the species-specificity and duration of the exposure, the multiple actions of curcumin seems to be also dose dependent, as previously described for gilthead seabream larvae and juveniles of rainbow trout and Nile tilapia (Akdemir et al., 2017; Mahmoud et al., 2017; Xavier et al., 2021b). Similar effects on the oxidative status regulatory pathways have been also reported in other fish species fed curcumin-supplemented diets. In genetically improved farmed tilapia (GIFT tilapia) juveniles fed different supplementation levels of curcumin, only fish fed at the higher inclusion level showed a decrease in the oxidative damage of lipids (measured as MDA) and an increase of TAC, as well as an upregulation of *hsp70* (Cui et al., 2013). In common carp, dietary supplements of curcumin at 15 g $Kg^{-1}$ led to a decrease in MDA content linked to an upregulation of *nrf2* and *sod*, but no effects on *hsp70* expression levels were observed (Giri et al., 2019). Similarly, in crucian carp, a decrease of PC and MDA levels together with an up-regulation of *gr* expression were observed in fish fed a curcumin-supplemented diet (Jiang et al., 2016). There are several mechanisms of ROS generation in biological systems, and the majority are produced as side-products of oxygen metabolism in the mitochondria. The use of mtROS as a biomarker of oxidative stress in fish is still poorly studied. However, there are studies in juveniles of rainbow trout, common carp and lake sturgeon (*Acipenser fulvescens*) supporting that mitochondria are responsible, at least in part, for the ROS production that leads to oxidative stress in these species (Banh et al., 2016). Our results showed that dietary supplementation with curcumin did not significantly decreased the production of mtROS in gilthead seabream postlarvae. Similar, no differences were registered in mtROS production in 31 DAH gilthead seabream fed with curcumin compared to non-supplemented diet. However, larvae fed curcumin were able to maintain the mtROS production along development in contrast to non-supplemented larvae (Xavier et al., 2021b). The present results on the effect of curcumin in fish mtROS differ from previous observations in rats supplemented with curcumin that showed a positive modulation on mitochondria production (biogenesis) and degradation (mitophagy) (Hamidie et al., 2015; Lu et al., 2021). Overall, our data seems to indicate that dietary curcumin acts as promoter of gilthead seabream antioxidant status as a direct scavenger and modulator of antioxidant defences rather than decreasing mtROS production. However, more detailed studies regarding the electron transport system activity (ETS) might bring different perspectives about the effects of curcumin in the generation of endogenous ROS.

The intestinal morphology plays an important role in the digestive and absorptive functions of the alimentary tract, thereby showing a direct correlation with growth and well-being in animal production. Longer *villi* are associated with activated cell mitoses and improved intestinal surface area which might allow a more efficient nutrient absorption (Rajput et al., 2013). In the present work, gilthead seabream postlarvae from the different treatments did not differ in the morphometry of the anterior intestine. Contrarily to what was observed in the present study, the use of dietary curcumin was able to modulate the intestinal mucosa by improving villus length and crypt depth in in other animals: hens, broilers (Gallus gallus domesticus) and piglets (Sus scrofa domesticus) (Arshami et al., 2013; Hady et al., 2013; Xun et al., 2015). In fact, the expression of *nrf2* and *hsp70* was associated with the protective effects of curcumin in the jejunum of growing pigs (Yan et al., 2020). In fish, studies on the effect of curcumin in the gut morphology are scarce and not consensual. In the climbing perch (average initial weight: $40 \pm 5$ g) fed dietary curcumin for six months, the number of goblet cells in the *villi* decreased, which was related to an improved digestive capacity by longer retention time of the food in the gut (Manju et al., 2013). Tilapia juveniles (average initial weight: $13 \pm 0$ g) fed dietary curcumin for two months showed to increase the intestinal folds lengths, even though a higher supplementation resulted in a degeneration of the *villi* and in a decrease in the number of goblet cells (Yusuf et al., 2017). The present results indicate that the higher inclusion of curcumin (HIGH diet) can be further increased, as no negative side effects were observed in postlarvae gut morphology, and previous studies showed that this antioxidant can positively modulate fish gut morphometry and improve digestion. This can be an advance in the development of diets for early stages of development, that present higher growth rates when compared to juveniles. A supplement that can promote redox homeostasis and absorptive capacity, will allow a significative enhancement of larvae and postlarvae robustness.

In general, fish postlarvae already present a fully developed intestine tract that is characterised by abundant intestine folds and a thick brush border membrane of the enterocytes. Concomitantly, a decrease in the activity of cytosolic enzyme activities (leu-ala peptidase) and an increase in the activities of the brush border membrane (alk phosphatase and aminopeptidase N), indicates the transition to the adult mode of digestion by enterocytes (Infante and Cahu, 2001). In this study, dietary curcumin supplementation did not improve postlarval digestive capacity, showing no differences between dietary treatments in the activity of neither leu-ala, alk phosphatase or their ratio. Moreover, the expression of *ialp* and *pept1* were similar between treatments. Previous works showed that dietary curcumin supplementation may improve digestion by increasing the activity of different digestive enzymes (e.g trypsin, chymotrypsin lipase and amylase), as described in juveniles of Nile tilapia (Midhun et al., 2016) and crucian carp (Jiang et al., 2016) and gilthead seabream larvae (Xavier et al., 2021b). In fact, contrarily to the present results, curcumin improved activity and expression levels of *ialp* in juveniles of crucian and grass carp (Jiang et al., 2016; Li et al., 2020). The lack of effect of curcumin supplementation in gilthead postlarvae gut morphometry and function might be explained by the fact that in postlarvae the majority of the energy is invested in growth and to counteract the negative impacts of higher metabolism this supplement might be diverted to maintain allostasis. Again, it would be interesting to verify whether a higher dose of curcumin supplementation may promote digestive maturation and / or efficiency, leading to faster growing fish.

## 5. Conclusions

Curcumin supplemented diets were able to promote oxidative status of gilthead seabream postlarvae through a higher antioxidant capacity and a lower oxidative damage. Moreover, dietary curcumin at the tested doses had no impact in fish intestine health status. Overall, this work provides new insights into the effects of a dietary antioxidant: curcumin in marine fish postlarvae concerning both biochemical and physiological indicators, as well as morphological parameters. Therefore, these findings can contribute for the development of functional diets as promoters of growth and health status of aquaculture fish species at early stage of development.

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## CRediT authorship contribution statement

**Maria J. Xavier:** Formal analysis, Investigation, Visualization, Writing – original draft. **Carmen Navarro-Guillén:** Supervision, Writing – review & editing. **André Lopes:** Investigation. **Rita Colen:** Investigation. **Rita Teodosio:** Investigation. **Rodrigo Mendes:** Investigation. Beatriz Oliveira: Investigation. **Luisa M.P. Valente:** Conceptualization, Supervision, Writing—reviewing & editing, funding acquisition. **Luís E.C. Conceição:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Sofia Engrola:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

## Declaration of Competing Interest

The two experimental diets (LOW and HIGH) are included in the patent pending application PCT/IB2020/056001.

## Data Availability

The datasets supporting the conclusions of this article are included within the article.

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