

Dietary Natural Plant Extracts Modulate Growth Performance and Antioxidant Status of Senegalese Sole Postlarvae

Maria J. Xavier (✉ mmxavier@ualg.pt)

Centro de Ciencias do Mar <https://orcid.org/0000-0003-4353-9586>

Luís E.C. Conceição

Sparos Lda

Luisa M.P. Valente

UP ICBAS: Universidade do Porto Instituto de Ciencias Biomedicas Abel Salazar

Rita Colen

CCMAR: Centro de Ciencias do Mar

Andreia C.M. Rodrigues

CESAM: Universidade de Aveiro Centro de Estudos do Ambiente e do Mar

Rui J.M. Rocha

CESAM: Universidade de Aveiro Centro de Estudos do Ambiente e do Mar

Luísa Custódio

CCMAR: Centro de Ciencias do Mar

Carlos Carballo

IFAPA: Instituto Andaluz de Investigacion y Formacion Agraria Pesquera Alimentaria y de la Produccion Ecologica

Manuel Manchado

IFAPA: Instituto Andaluz de Investigacion y Formacion Agraria Pesquera Alimentaria y de la Produccion Ecologica

Sofia Engrola

CCMAR: Centro de Ciencias do Mar

Research

Keywords: Plant extracts, Nutrition, Senegalese sole, Oxidative stress, Growth performance

Posted Date: February 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-117940/v2>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background

Oxidative stress has a direct impact on fish production, affecting both growth and health status. Plant based extracts, such as those from green tea, curcumin and grape seeds, are known for their abundant content and diversity of polyphenols. These bioactive compounds have a high antioxidant capacity making such extracts good additives to include in fish diets potentially improving the oxidative status of fish, and therefore enhancing growth and stress resistance of farmed fish.

A growth trial with Senegalese sole postlarvae (45 days after hatching) fed with four experimental diets, a control (CTRL) and three supplemented with natural antioxidants, namely curcumin (CC), green tea (GT) and grape seed (GS) extracts, was performed during 25 days to check if these supplements could improve growth performance and fish oxidative status. Moreover, a thermal stress was applied at the end of the growth trial, to assess the effect of these supplemented diets in stress resistance of sole postlarvae.

Results

Sole growth was improved by the dietary inclusion of CC and GS compared to CTRL. Postlarvae from CC and CTRL present the lowest values of oxidative damage (lipid peroxidation and protein carbonylation). Moreover, the fish fed CC showed a decrease in stress related biomarkers (heat shock protein 70 and glutathione-S-transferase) compare to CTRL, which might be due to direct antioxidant capacity. In contrast the supplementation of GT and GS increased the content of oxidative damage in sole reared in standard conditions. However, after a thermal stress exposure both GT and GS treatment seem to prevent the increase of protein carbonylation content and the decrease in the levels of antioxidant glutathione in sole, depending on the time of exposure.

Conclusions

Overall, dietary supplementation with these natural extracts modulates oxidative status and stress response after a short/long term temperature increase. The incorporation of curcumin seems to be a safe additive to include in sole diets, enhancing growth and oxidative status. The supplementation of the studied doses of green tea and grape seed extracts in fish diets needs further research as they may act as pro-oxidant depending of the culture conditions.

1. Background

Natural extracts from plants are an important source of bioactive compounds such as polyphenols, alkaloids and terpenoids. These compounds are produced as secondary metabolites to protect the cells against the microbial infections exerting several biological properties, mainly antioxidant and anti-inflammatory (1-5). Polyphenols are the most abundant and diverse group and are categorised according to the number of phenolic rings and the structural elements bounded to the rings. The two main classes are represented by phenolic acids and flavonoids (6).

Several plants extract rich in polyphenolic compounds are considered as promising feed additives in fish nutrition with a high potential to enhance weight gain, feed efficiency, and/or disease resistance in cultured fish (7, 8). Curcumin is a lipophilic polyphenol extracted from the rhizome of turmeric (*Curcuma longa* L.) - commonly used as a spice (9). Recently it was reported that 1 and 1.5 % of curcumin supplementation in carp (*Cyprinus carpio*) diets significantly improved growth performance, oxidative status and skin immune response (10). A similar effect on growth and non-specific immune response was also observed in Wuchang bream (*Megalobrama amblycephala*) fed with a diet supplemented with 0.01 % curcumin (11). In tilapia (*Oreochromis mossambicus*), the dietary administration of 0.5 and 1 % curcumin modulated the expression of growth factors (insuline-like growth factors 1 and 2) in muscle and increased the activity of digestive enzymes (12). Moreover, the inclusion of curcumin (between 0.01 - 0.04 %) in diets for tilapia and rainbow trout (*Oncorhynchus mykiss*) improved oxidative status and disease resistance in response to stressful conditions (13-15).

Green tea (*Camellia sinensis* L.) and grape seed (*Vitis vinifera* L. *ssp sativa*) are also two plants rich in polyphenols. The green tea is one of the most popular beverages worldwide and it contains high levels of flavanols (e.g. catechin, epigallocatechin gallate) that represents 25 – 35 % of the leaves dry weight. In aquaculture production, the inclusion at 5 % of green tea in juvenile grass carp (*Ctenopharyngodon idellus*) diets positively modulated myogenic regulator factors and stress related genes (16) and at 0.01 % of dietary inclusion enhanced the antioxidant and immune system in rainbow trout (17). On the other hand, the grape is a by-product of

the winery and grape juice industry. Polyphenols represent 5 – 8 % of the constituent of grapes depending on the variety, and the most abundant are flavonoids, tannins and phenolic acids (18, 19). Dietary grape seed extract at 0.1 % was beneficial for growth performance and meat quality in juvenile rainbow trout (20) and in postlarvae of Senegalese sole (*Solea senegalensis*), the dietary administration of grape seeds supplemented in the mineral premix at 1.2 % also showed an increase in growth performance and modulation of gene expression relate to muscle growth development (21).

All the modulatory effects observed in growth and health of these fish species seem to be related with the antioxidant activity exhibited by polyphenol-rich extracts, that reduce the damage caused by the oxygen reactive species (ROS), either by the presence of the phenol group that direct scavenge these molecules into stable radicals or by enhancing the activity of endogenous antioxidant enzymes through an up-regulation of transcription factor Nrf2 signalling pathway (7, 22, 23).

A high concentration of free radicals or other pro-oxidants that were not detoxified and removed from cells, or a deficiency in the antioxidant system, may cause an imbalance in the redox homeostasis of the cell leading to a state of oxidative stress (24). To counteract the high levels of ROS all aerobic organisms possess endogenous antioxidant molecules capable to neutralise them, thus preventing membrane cell damage, enzyme inactivation and nucleic acid alterations (25). The first line of the antioxidant defence is constituted by three enzymes: superoxide dismutase (SOD); catalase (CAT) and glutathione peroxidase (GPx) (26). Glutathione-S-transferase (GST), acts in the second line of antioxidant defences, and is also an antioxidant enzyme family that inactivates secondary metabolites and lipid peroxidation (LPO) products, into excretable molecules by catalysing the conjugation with glutathione (GSH). Other endogenous non-enzymatic antioxidant defences are also vital to maintain oxidative balance such as vitamins E, C and A, ubiquinol and GSH a peptide with an important role in the neutralisation of radicals, cofactor for several detoxifying enzymes (such as GPx and GST) and converting vitamin C and E into their active forms (24, 27).

Many factors can influence the antioxidant defence response in fish. Biotic factors such as age, species and feeding behaviour and abiotic conditions such as temperature, diet, dissolved oxygen, and toxins present in the water can modulate the antioxidant defences and as a consequence, the oxidative status of the animals (28, 29). It is crucial to assess the fish oxidative status, as it has a direct impact in health, flesh quality and growth, in order to improve fish welfare and promote cost-effectiveness in aquaculture. Therefore, the aim of this work was to evaluate if dietary plant extracts supplementation (curcumin, green tea and grape seed) modifies growth performance and antioxidant status of Senegalese sole postlarvae, and help fish to cope with a stressful event.

2. Methods

2.1 Experimental diets

Four diets were tested in this study, including a commercial diet (WINFlat, SPAROS Lda., Portugal) that was used as the control (CTRL diet). This diet contains ingredients such as krill meal, squid meal, wheat gluten, fish meal, shrimp meal, fish hydrolysate, pea protein concentrate, fish gelatine, fish oil, lecithin and a micronutrient premix comprising vitamins, minerals, and other additives. Moreover, three experimental diets were prepared by supplementing the CTRL diet with an extract of either curcumin (CC diet) at 46 g/kg of the micronutrient premix, green tea (GT diet) at 12g/kg of the micronutrient premix or grape seed (GS diet) at 12 g/kg of the micronutrient premix. The extract of curcumin (95.34 % purity), green tea ([≈] 50 % polyphenols) and grape seed ([≈] 70 % polyphenols) used in the supplemented diets were provided by Denk Ingredients (Germany). These selected doses of each antioxidant extract are under a patent pending application (PCT/IB2020/056001) and were chosen based on previous trials conducted in the Centre of Marine Science of Algarve (CCMAR) (21). All diets were prepared by SPAROS Lda. (Portugal). Feed samples were freeze-dried, ground and analysed for dry matter (DM; 105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco; N × 6.25), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt; 150 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA). Diets proximal composition was identical for all 4 diets (Table 1), and they only changed in the supplementation with extracts, and this supplementation did not exceed 1 % of the diets.

2.2 Senegalese sole husbandry and experimental set-up

Senegalese sole postlarvae were reared for 25 days, starting at 45 days after hatching (DAH), in a recirculation aquaculture system at CCMAR (Portugal), under optimized environmental and zotechnical conditions. Sole postlarvae were kept in flat-bottom tanks (21 L), each tank stocking 630 individuals (corresponding to a 3,000 ind m⁻²). The dietary treatments (CTRL, CC, GT and GS) were randomly assigned to replicate tanks (*n* = 3 tanks per treatment). Abiotic parameters were measured, and mortality was recorded daily. Dead

fish were removed, and the rearing units were carefully cleaned with minimal disturbance. Dissolved oxygen in water was maintained at 96.6 ± 7.2 % of saturation, temperature at 19.6 ± 0.5 °C and salinity at 35.4 ± 0.7 g.L⁻¹. A 10:14 h light:dark photoperiod was maintained, and the light intensity was 400 lx at the water surface. Inert diet was delivered semi-continuously with automatic feeders. The amount of feed distributed to each tank was based on predicted maximum growth and daily adjustments were done based on visual inspection to avoid a large excess of uneaten food (30).

2.3 Thermal stress test

A thermal stress test was conducted at the end of the experimental period (70 DAH) to assess how the dietary treatments modify the animal physiological responses. Two challenging periods were analysed in fish response: an acute stress (at 72 DAH) and a chronic stress (at 78 DAH). The seawater temperature of the rearing system was raised from 19.6 °C until 24.0 ± 0.5 °C (~5 °C over the experimental temperature) during a 24 h period. For the acute stress challenge fish were sampled after remaining at this temperature during 24 h (72 DAH), whilst for the chronic stress test fish were maintained at this temperature for one week (78 DAH). At the end of each thermal stress fish from the different dietary treatments were sampled for biomarkers response assessment.

2.4 Key performance indicators

At the beginning (45 DAH, $n = 60$) and at the end of the experiment (70 DAH, $n = 120$) fish were killed by using an overdose of anaesthetic 2-phenoxyethanol (1000 ppm; Prolabo) then individually collected for dry weight (DW, mg) and body standard length (SL, mm) determination. These postlarvae were frozen at -80 °C, photographed for measuring SL using Axio Vision L.E. 4.8.2.0 (Carl Zeiss Micro Imaging GmbH) and freeze-dried for DW determination (Denver Instrument, 0.001 mg precision). Survival rate (%) was calculated as the percentage of fish counted at the end of the trial relative to their initial number in each replicate. Growth, expressed as relative growth rate (RGR, %/day), was calculated, at the end of the experiment, using the formula: $(e^{g-1}) \times 100$, with $g = [(\ln \text{ final weight} - \ln \text{ initial weight})/\text{time}]$ (31). The condition factor (K) was calculated as $(\text{fish wet weight}/\text{total length}^3) \times 100$.

2.5 Preparation of diet extracts for antioxidant capacity assessment

Methanol extracts were prepared from the four diets CTRL, CC, GT and GS. For that purpose, the diets were freeze-dried, mixed with methanol (1:40, w/v) and maintained in an ultrasonic bath for 30 min. Then, samples were extracted overnight, with stirring, at room temperature (RT, approximately 20 °C) (32). The extracts were then filtered (Whatman n° 4) to remove solid debris, and methanol was removed by using a rotary evaporator (60 °C; 337 mbar). The obtained dried extracts were weighed, dissolved in methanol at 50 mg/mL and stored at -20 °C.

4.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS)

Methanol extracts from diet samples were tested for radical scavenging activity against the DPPH and ABTS radicals at concentrations of 50 mg/mL, as described previously (32). Ascorbic acid was used as a positive control at the same concentrations of the samples. Results were expressed as a percentage of inhibition, relative to a control containing methanol in place of the sample.

4.6.2. Total phenolic (TPC) and Flavonoids (TFC) content

The TPC and TFC were determined in the methanol extracts at the concentration of 50 mg/mL and absorbance was measured in a microplate reader (Biotek Synergy 4). The TPC was assessed by the Folin-Ciocalteu assay and TFC was estimated by the aluminium chloride colorimetric method adapted to 96-well microplates. Results were expressed respectively as gallic acid equivalents (GAE) and quercetin equivalents (QE) in milligrams per gram of diet (dry weight, DW). All methods were performed as previously described (32).

2.6 Preparation of fish sample for biomarkers response assessment

For biomarkers analysis, 3 pools ($n = 3$ postlarvae/pool) per replicate ($n=9$ per dietary treatment) were sampled at the end of growth trial (70 DAH) and thermal stress (72 and 78 DAH). The region from the operculum cavity until the end of the visceral cavity was selected in each fish. Samples were homogenised through sonication (Branson Sonifier 250) on ice using 1500 µl of ultra-pure water. From each sample, 2 aliquots of the supernatant were taken. One aliquot of 200 µl containing 4 µl of 4 % butylated hydroxytoluene (BHT) in methanol, to prevent oxidation of lipids, was used for the determination of endogenous lipid peroxidation (LPO). The other

aliquot of 500 µl was diluted (1:1) 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 10 min at 10,000 g (4 °C). The post-mitochondrial supernatant (PMS) was divided into microtubes and kept in -80 °C until further analyses. All biomarkers were determined spectrophotometrically, in micro-assays set up in 96 well flat bottom plates, with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific).

2.6.1 Antioxidant biomarkers assessment

Protein concentration of PMS was determined according to the Bradford method (33), using bovine γ -globulin as a standard. Catalase (CAT) activity was determined in PMS by measuring the decomposition of the substrate 30 % H₂O₂ at 240 nm (34). Glutathione-S-transferase (GST) activity was determined following the conjugation of GSH with 1-chloro-2,4- dinitrobenzene (CDNB) at 340 nm ($\epsilon = 9.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) (35). Total glutathione (GSH) content was determined at 412 nm using a recycling reaction of reduced GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (36, 37). GSH content was calculated as the rate of TNB²⁻ formation with an extinction coefficient of DTNB chromophore formed ($\epsilon = 14.1 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) (37, 38). Lipid peroxidation (LPO) was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) (39). Briefly, 100 µL of cold trichloroacetic acid (TCA) 100% were added to the samples, followed by 1000 µL of 2-thiobarbituric acid (TBA) 73% and were incubated at 100 °C for 1 h. After this period, samples were kept for up to 16 h in the dark, then 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. Protein carbonylation (PC) was measured by the quantification of carbonyl groups based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl groups, according to the DNPH alkaline method (40). The amount of carbonyl groups was quantified spectrophotometrically at 450 nm ($\epsilon = 22,308 \text{ mM}^{-1}\text{cm}^{-1}$).

2.6.2 Total Antioxidant Capacity (TAC)

The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS^{•+}) is decolorised by antioxidants according to their concentrations and antioxidant capacities. This change in colour was measured as a change in absorbance at 660 nm and the assay was calibrated with Trolox (41).

2.6.3 Heat shock proteins (HSP70) response

HSP70/HSC70 content was assessed by ELISA, adapted from Rosa (42). Samples were added to coated 96 well microplates and allowed to incubate overnight at 4 °C. Microplates were then washed (3 \times) with 0.05 % PBS-Tween-20, the blocking solution (1% BSA, Sigma-Aldrich) was added and left to incubate at RT for 2 h. Microplates were washed and 5 µg mL⁻¹ primary antibody (1° Anti-HSP70 mouse mAB (C92F3A-5) Millipore), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible hsp and hsc70, was added to each well and then incubated overnight at 4 °C. The non-linked antibodies were removed by washing the microplates again, which were then incubate for 2 h at RT with 1 µg mL⁻¹ of the secondary antibody, anti-mouse IgG (2° Anti-mouse IgG (fab specific) Sigma). After another wash, the substrate p-nitrophenyl phosphate was added and incubated for 30 min at RT. Then, the stop solution (3 mol L⁻¹ NaOH) was added to each well and the absorbance was read at 405 nm, using as a standard the purified HSP70 active protein (HSP70 protein Millipore).

2.7 Reverse transcription–quantitative real-time PCR (qPCR)

Gene expression analysis was performed in 70, 72 and 78 DAH soles from each dietary treatment. Fish were kept at -80 °C until analysis. A region from the operculum cavity until the end of the visceral cavity was select in each larva ($n = 4$ per dietary treatment). This process was realised in each larva without thawing. Selected genes for oxidative stress defences and cellular stress proteins are described in detail in [Table 2](#).

Samples were homogenised using a Fast-prep FG120 instrument (Bio101) and Lysing Matrix D (Q- Bio- Gene) with 1 mL Tri Reagent (Sigma-Aldrich) for 60s at speed setting 6. Chloroform (0.2 mL) was added to each sample before centrifuging at 14,000 rpm 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 was checked by agarose gel electrophoresis and a Nanodrop ND-8000 (Thermo Scientific) was used to determine its concentration. One µg of total RNA was reverse-transcribed using the iScriptTM cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol.

The qPCR assays were performed in duplicate 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 genes analysed were involved in the oxidative stress defences: catalase (*cat*), glutathione peroxidase 1 (*gpx1*), glutathione peroxidase 3 (*gpx3*), superoxide dismutase [Cu-Zn] (*sod3*) and cellular stress proteins (heat shock protein 70 (*hsp70*), heat shock protein 90 alpha (*hsp90aa*), heat shock protein 90 beta (*hsp90ab*). Primers for Senegalese sole *hsp70*, *hsp90aa* and *hsp90ab* were previously published (43, 44) and new species-specific primers for qPCR were designed for remaining genes (Table 2). The qPCR amplification protocol was as follows: 7 min for denaturation and enzyme activation at 95 °C followed by 40 cycles of 30 s at 95 °C and 1 min at 60 °C. Expression data were normalised using the geometric mean of two reference genes, ubiquitin (*ubi*) and glyceraldehyde-3-phosphate dehydrogenase 2 (*gadph2*) (45) and the relative mRNA expression calculated using the comparative Ct method (46).

2.8 Data analyses

All data were tested for normality using a Kolmogorov-Smirnov (whenever $n > 30$) or Shapiro-Wilk (whenever $n < 30$) test and for homogeneity of variance using a Levene's test using STATISTICA v13 (StatSoft). Data were log transformed when 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 and oxidative stress biomarkers at the end of the growth trial. To assess the response of oxidative stress biomarkers to thermal stress exposure by each treatment group a two-way ANOVA was made and the analysis of the delta variation between pre- and post-stress indicators was performed by a one-way ANOVA, both followed by a Tukey post-hoc test. Significance levels were set at $P < 0.05$.

3. Results

3.1. Dietary antioxidant properties

Main features for Radical scavenging activity (RSA) of the methanol extracts made from the experimental diets are summarized in Table 3. Overall, the extracts had low to moderate RSA. Regarding the ABTS assay, the GS diet presented the highest activity (28.6 % for ABTS), while in the DPPH method, the utmost activity (50.2 %) was recorded for the CC diet. The TPC ranged from 1.3 to 6.4 mg GAE/g diet, and the three supplemented diets had higher levels than CTRL ($P < 0.001$). The content of flavonoids varied from 0.7 to 4.4 mg QE/g diet. The GS diet presented the highest amounts of flavonoids, similar to the CC diet ($P < 0.001$). As average, the total phenols and flavonoids content in the supplemented diets was 4-fold higher than in the CTRL diet.

3.2. Key performance indicators

Postlarvae fed with CC and GS diets were heavier than CTRL diet at the end of the feeding trial ($P < 0.001$) (Table 4). All postlarvae fed with supplemented diets showed a higher length than CTRL group ($P < 0.001$). Condition factor (K) was higher than 1 in all treatments, however, fish fed the supplemented diets had significantly lower values than those fed with CTRL. The relative growth rate (RGR) remained unaffected by the dietary treatments. The average survival rate was 98.3 ± 0.6 % and did not differ between dietary treatments (Table 4).

3.3. Oxidative status indicators

The CAT activity and TAC were not statistically different among the different dietary treatments (Table 5). All postlarvae fed with the supplemented diets showed a lower GST activity than CTRL ($P < 0.01$). The content of GSH was significantly higher in soles from dietary CC and CTRL treatments ($P < 0.001$). When HSP70 proteins were analysed, soles fed with CC and GS had lower levels than CTRL and GT ($P < 0.001$). The oxidative damage measured as LPO was not significantly different between supplemented diets and the CTRL, and only a significant reduction in CC with respect to the GT diet was found. The postlarvae from CC and CTRL diets had the lowest values of oxidative damage observed at the protein level (PC) when compared with fish from GT and GS diets that presented the highest values ($P < 0.001$) (Fig. 1).

Expression of most of the genes related to oxidative stress was not modified by dietary treatments except for *cat* and *gpx1* gene ($P < 0.05$). The postlarvae fed with GT had lower *cat* mRNA levels than GS. An up-regulation of *gpx1* transcription was observed in fish fed with CC and GT compared to the other dietary treatments.

3.3.1. Thermal stress – acute exposure

The majority of physiological indicators, except LPO and TAC, showed a significant interaction between dietary treatments and response to thermal stress (Diet*Stress $P < 0.05$). The fish LPO content was only significantly modified by the dietary treatment (Diet $P = 0.001$) and the values of fish TAC was significantly influenced by diet and acute stress independently (Diet $P = 0.026$; Stress $P < 0.001$) (Table S1).

A significant decrease in the activity of CAT was observed in sole after acute stress ($P = 0.024$). Unlike CTRL and GT diets, CC and GS diets did not significantly decrease the postlarvae' GST activity after the acute stress ($P = 0.003$). Moreover, the CTRL and CC soles were unable to maintain high levels of GSH ($P < 0.001$) (Table S1). When the shift in GSH levels was compared after an acute stress, all dietary treatments revealed a reduction in GSH content, but the lowest decrease was observed in GS and GT treatments ($P < 0.001$) (Fig. 2a). The levels of HSP70 were similar after the acute stress conditions in soles fed with CTRL, CC and GS diets, however, in GT a 10-fold increase of HSP70 levels was observed after acute stress ($P < 0.001$) (Table S1). A slight increase was also observed in GS postlarvae compared to CTRL group that presented a decrease in the delta variation between standard conditions/acute stress of the HSP70 levels ($P < 0.001$) (Fig. 2b). TAC increased in postlarvae after acute stress conditions and in sole fed with CC a significant higher TAC was observed than postlarvae fed GS diet. The PC content was higher in soles fed with CTRL, CC and GT diets after the acute stress ($P < 0.001$) (Table S1). However, delta variation between pre/post stress showed that the fish fed with GT and GS were able to decrease PC content ($P < 0.001$) (Fig 2c). Fish from CC treatment had the lowest values of LPO when compared to those from GT treatment and did not differ from the remaining treatments.

Expression of most genes (*cat*, *gsr*, *gpx3*, *hsp70* and *hsp90b*) related to oxidative stress were affected by the acute stress (Stress $P < 0.05$) (Table S1). Postlarvae exposed to a thermal acute stress reduced *gpx3* and *gsr* mRNA levels and increased *hsp90b* and *hsp70* transcript amounts independently of the dietary treatment ($P > 0.05$). On the other hand, the expression levels of *cat* decreased after acute stress, however, the fish fed with GS up-regulated the expression of *cat* compared to CTRL and GT (Diet $P < 0.013$) (Table S1). The expression of *sod3* and *gpx1* were not modified by the thermal stress and dietary treatments ($P > 0.05$). A significant interaction between stress and the diets (Diet*Stress $P = 0.007$) was observed in the *hsp90aa* expression showing that the dietary treatments modulated differently sole response to a stressful situation. Both CC and GT soles downregulated *hsp90aa* mRNA levels after the acute stress contrarily to CTRL and GS fish that up-regulated the expression of this gene (Table S1).

3.3.2. Thermal stress – chronic exposure

A significative interaction between treatments and chronic stress was observed in sole GSH, HSP70 and PC content (Diet*Stress $P < 0.001$). The fish physiological indicators (CAT and TAC) only responded to chronic stress (Stress $P < 0.001$), and the GST activity and LPO content were significantly influenced by diet and chronic thermal stress, independently (Diet $P < 0.05$; Stress $P < 0.05$) (Table S2).

After chronic stress the postlarvae from GS diet presented the highest content of GSH compared to CTRL and CC groups ($P < 0.001$). The delta variation of the levels of this tripeptide, under standard rearing and chronic stress conditions, decreased in all treatments, however in GT and GS sole this decline was smaller than in the remaining diets ($P < 0.001$) (Fig. 3a). After chronic stress, postlarvae from all dietary treatments showed similar levels of HSP70. However, regarding the delta variation between pre-post stress was visible a significant difference between the highest values of HSP70 content in GS fish compare to GT treatment ($P = 0.012$) (Fig. 3b). The PC content decreased in fish fed with GT after the chronic exposure; on the contrary, fish fed with GS presented the highest amounts ($P < 0.001$) (Table S2). The delta variation from chronic stress and standard condition showed that CC, GS and CTRL sole had improved PC content when compared to GT fish ($P = 0.001$) (Fig. 3c). The fish activity of CAT and TAC was higher after to a chronic thermal stress, in opposition the activity of GST and LPO content decrease after chronic stress exposure ($P < 0.05$). In addition, fish from CC, GS and GT diets presented lower GST than the CTRL fed fish ($P < 0.001$) (Table S2). The LPO content was lower in CTRL and CC fed fish that in fish fed GT diet. In fact, when the shift in LPO levels was compared after a chronic stress, the CTRL and CC treatments showed the lowest values compare to the other treatments ($P < 0.001$) (Fig. 3d).

The expression of *gpx3*, *gsr*, *hsp90b* and *hsp70* was only affected by the long exposure to higher temperature (Stress $P < 0.05$), regardless of the dietary treatments (Table S2). After 7 days all postlarvae up-regulated the expression of *hsp70* and *gpx3* and down-regulated *gsr*. Moreover, the *cat* mRNA levels increased after thermal chronic stress (Stress $P < 0.001$) and the fish fed with GS presented a significantly higher expression than those fed with GT (Diet $P < 0.05$). The expression of *sod1* was not affected by the dietary treatments or thermal exposure. ($P > 0.05$) The expression of *gpx1* and *hsp90aa* presented an interaction between chronic stress conditions and the dietary treatments (Diet*Stress $P < 0.05$). The long exposure to high temperature up-regulated *gpx1* mRNA

levels only in CTRL and GT postlarvae. After chronic temperature exposure, the transcription of *hsp90aa* in CC sole significantly decreased to values similar of those observed in the remaining treatments (Table S2).

4. Discussion

In the last decade there has been a growing interest in dietary inclusion of natural extracts with high biological activity as feed additives to enhance growth performance and welfare of farmed fish species. In this work, three experimental diets supplemented with antioxidants (extracts from turmeric, green tea and grape seed) were tested. To gain insight into the antioxidative potential of the dietary supplements, two radical scavenging assays (ABTS and DPPH) and the total phenolic and flavonoid contents were assessed in methanol extracts of the experimental diets. The extract from CC and GS diets exhibited the highest capacity to scavenge free radicals. As expected, the supplemented diets (CC, GT and GS) increased the levels of TPC and TFC when compared to CTRL diet. Phenolic compounds contain an aromatic ring bearing one or most hydroxy substituents, including functional derivatives (e.g., esters, methyl ethers, glycoside) and their content is usually associated with the antioxidant capacity of plant extracts, as they can act as hydrogen or electron donor in the presence of oxidants (47, 48).

The present results showed that diet supplementation with curcumin (CC diet) and grape seed (GS diet) extracts promote larger sole postlarvae. In fact, previous data showed that these supplemented diets improve sole growth performance through a modulation in the expression of genes related to muscle growth and development (21). The supplementation of curcumin started recently in diets for freshwater fish juveniles, such as in crucian carp (49), rainbow trout (14), common carp (10) and Nile tilapia (13, 15). Fewer information is available regarding grape seed extract and results are not always congruent regarding growth performance. In one experiment using juveniles of rainbow trout, an improvement of growth was registered (20). In another experiment on rainbow trout, only juveniles fed the highest doses of grape seed improved the final weight (50). On contrary, in grass carp, the supplementation was unable to show any effect on growth (51). Therefore, antioxidants supplementation may present different outputs depending on the compound dose and the species. In this study, CC and GS diets improved sole growth, but the same was not observed for green tea extract supplementation (GT diet). Similarly, in rainbow trout (17, 52), grass carp (16) and olive founder juveniles (53) fed with green tea supplemented diets no positive effect on growth performance (final body weight) or feed conversion ratio could be observed. In the case of grass carp juveniles, the green tea supplementation had a detrimental effect on growth (16). In Nile tilapia fed with five experimental diets containing graded levels of green tea, only two doses, namely 0.25 and 0.50 g/kg, were able to improve fish final body weight and specific growth rates (54). The negative effects of green tea on rat's growth have been related to the content of caffeine and the combination of polyphenols with polysaccharides that interact with the absorption of fatty acids and serum triglyceride levels (55). All these results suggest that the dose of green tea supplementation able to promote growth performance in fish still needs to be ascertained and is probably species and dose dependent.

The K factor decreased in sole fed with supplemented diets with respect to the CTRL. A high K is sometimes linked to a better nutritional state of the fish. However, this does not seem to be the case in our data. The decrease observed in fish supplemented with the dietary antioxidants does not mean that these fish were in a worse physical condition than those fed with the CTRL diet. In fact, this result indicates that fish had a faster growth in length than body mass provoking a shift in body shape (more elliptic). A similar study performed in tilapia fed with different curcumin supplementations also showed that the fish from the treatment with final higher body weight and growth had a significant lower K (13).

Growth and development are well orchestrated processes that depend on the balance of cellular proliferation, differentiation, and apoptosis. The cell fate depends on a variety of molecular pathways, gene expression and protein function that are sensitive to the cellular reduction potential. Measuring the levels of antioxidant defences and oxidative damage is highly relevant to assess fitness, because high oxidative stress levels may compromise survival and growth (56, 57). In order to understand the possible pathways by which different natural antioxidant modulates the antioxidant status of sole, several oxidative stress biomarkers were analysed in the present work (Fig. 1). The results from our study suggest that CC diet supplementation improved oxidative status in Senegalese sole postlarvae. Contradictory results were observed with the GT and GS diets that showed to increase sole protein oxidative damage content and decrease the levels of essential non-enzymatic antioxidant GSH at the end of the growth trial.

Both fish fed with CC and CTRL diet showed the lower levels of lipid and protein oxidation. However, in order to maintain these similar levels of LPO and PC the fish from CTRL needed to increase endogenous antioxidant defence GST and HSP70, which are linked to the biotransformation process of metabolites and to the protection of the cell during stress. So, it seems that postlarvae of CTRL

required a higher GST activity to detoxicate LPO products and higher content of protein chaperon HSP70 to prevents the accumulation of oxidized proteins and promote their degradation (58). Increasing antioxidant defences is an energy-consuming process and may deflect energy or nutrients that might be needed for other physiological functions. So, curcumin extract supplementation in diets may spare antioxidant defenses by directly scavenging the ROS. Consequently postlarvae, are able to allocate energy in other functions like growth.

Similarly, was reported in tilapia (59) and common carp (10) fed a lower dose of curcumin supplementation, an improvement in final weight. However, no differences were registered in the content of lipid peroxidation and activity of endogenous antioxidants defences when compared with the control treatment. Moreover, in a test performed with climbing perch (*Anabas testudineus*) fed with two doses of curcumin for 8 weeks, unaltered levels of endogenous enzymes (GR, CAT and GPx) and oxidative damage LPO were observed in treated fish compared to the control, also suggesting that curcumin have a direct free radical scavenging activity (60). In juveniles of tilapia the growth improvement observed in the juveniles fed the higher dose of curcumin was accomplish by a decrease of LPO, but with no differences in the activity of CAT and GSH content (15). Nevertheless, in other studies the supplementation of curcumin also reported enhanced growth of tilapia and trout juveniles but through a decrease in the LPO content and improvement in the activity of antioxidant defences (13, 61). Given the multitude of pathways that curcumin can act it is challenging to identify the key pattern of action of this antioxidant. In fact, the impact of curcumin in the redox status of fish is highly dependent on the duration of the experimental trial (60), doses (10, 15) and species (62).

It appears that the supplementation of both green tea (GT diet) and grape seed (GS diet) might have a pro-oxidant effect in sole postlarvae. Although there are just a few studies regarding the pro-oxidant effects of antioxidants, a variety of plant extracts showed to have both pro-oxidative and antioxidative properties, depending on their main bioactive molecules characteristics (e.g. metal-reducing potential, chelating behaviour), concentration and the microenvironment (e.g. pH and presence of metal ions and redox status) (63, 64). As far we know there are no previous studies regarding the pro-oxidant effect of either green tea or grape seeds extracts in the oxidative status of heath animal models. However, juveniles of rainbow trout fed with grape seed oil supplementation, at higher concentrations, also showed an improvement in the growth and a decreased activity of some endogenous antioxidant defenses (SOD, CAT and GST), which seems to corroborate the present results (50). Moreover, the inclusion of Epigallocatechin-3-gallate (catechin present in green tea) and green tea in diets for rainbow trout juveniles (52) and grass carp (16), respectively, did not affect the oxidative status parameters analysed. On the other hand, in rainbow trout juveniles fed green tea presented a higher SOD activity and lower LPO content compare to the control group (17) and similar was observed when fed dietary grape seed extracts, showing a reduction of LPO and upregulation of antioxidant defences (65). Hence, the inclusion of green tea and grape seed extracts in fish diets can improve the oxidative status and consequently improve the endogenous antioxidant defences in fish species. However, the dose of the antioxidant supplementation and the duration of the administration period still need tuning. Moreover, it is also important to highlight that postlarvae have higher growth rates and feed intake than juveniles and adults, which might have contributed to increased concentration of antioxidants per body weight unit possibly explaining the pro-oxidant effect observed in this study. Nevertheless, these results also corroborate previous concepts, including that polyphenols can modulate oxidative status biomarkers by different mechanisms of action (6).

High water temperatures increase oxygen consumption and mitochondrial respiratory rates leading to an increment of proton leakage rates that could lead to oxygen incomplete reduction and ROS formation (66). Our data suggest that sole fed with CC and CTRL diets were unable to maintain the lower PC and the highest GSH content after an acute increase of temperature. Moreover, fish fed with CTRL could not maintain the activity levels of GST after 24 h of thermal stress. On the other hand, sole fed with GS were able to maintain the activity of GST and GSH content after the acute stress and were even able to decrease the PC levels. It seems that the pro-oxidant effect under standard rearing condition, has changed to antioxidant under acute challenging conditions. In fact, dietary pro or antioxidant can have different impacts on oxidative status of fish depending on normal/stressful rearing condition (67). This may suggest that the supplementation dose needs to be tuned between promoting growth in standard rearing conditions and to help fish coping with environmental changes. In other fish species, a more evident effect of curcumin supplementation in response to a stressful situation was observed. For example, both rainbow trout (14) and tilapia (59) fed diets supplemented with CC showed a decrease in LPO content. Moreover, the expression of Nrf2 and antioxidant defences activity (SOD and GPx) were also promoted in these fish species, respectively. Similarly, previous reports corroborate our study and evidencing the protective effect of grape extracts (or grape related) on oxidative induced stress. In case of abalone fed a grape seed extract supplementation diet under a thermal stress (increase of water temperature) the expression of *cat* was up-regulated compared to control treatment and in juveniles of grass carp infected with *pseudomonas aeruginosa* and fed grape pomace flour supplementation had an increased CAT activity in serum and

splenic tissues (51). Moreover, tilapia fed diets with different doses of resveratrol were able to counteract some negative effects of intraperitoneal injection of H₂O₂ in the oxidative status of the fish, by decreasing the levels of LPO and enhancing CAT activity and TAC (68).

Depending on the severity and duration of heat stress, the antioxidant system and associated enzymes behave differently (69). After one week of thermal stress (chronical exposure), the fish might be adapted to the new conditions and restore the homeostatic values or reach an allostatic equilibrium. Overall, LPO content of the fish decreased independently of the dietary treatment and the values of PC of the postlarvae from GS raised again. On the other hand, the values of protein oxidation (PC) in the GT sole were not affected by the thermal chronic expose, which may indicate that a high content of HSP70, as a response to an acute stress, might have a long-term effect in the protection of proteins to oxidative stress. Similar adjustments of oxidative status and adaptive responses of antioxidant defences to stressful events were also reported in other fish species fed with green tea extracts. The antioxidant role of the green tea was reported to remedy the toxic effects of 4-nonylphenol in African catfish *Clarias gariepinus* (70) and the toxic effect of oxidised fish oil in sturgeon (71). In both studies, fish were able to decrease LPO content and increase the levels of some antioxidant biomarkers.

Overall, fish fed GS diet, after an acute thermal stress exposure, did not decrease the activity of GST and GSH content, and were even able to decrease the levels of PC, however, after a week of exposure to increased water temperature the PC content returned to higher values compared to fish from the other treatments. In sole fed GT, after 24 h of increased water temperature, the fish maintained the values of GSH and increased the content of HSP70, which suggests that after a chronic exposure these fish were able to protect protein oxidation which is supported by the decrease of PC content. The CC treatment did not show any significant improvement in the oxidative status of the fish after an acute/chronic increased of the water temperature.

5. Conclusions

The dietary supplementation of natural antioxidants was an effective way of modulating the oxidative status, antioxidant response and growth of Senegalese sole postlarvae. These antioxidants are known to interfere in numerous routes by acting as direct antioxidant as chelating metals, reducing via electron transfers or hydrogen atoms transference, and indirectly by upregulating the expression of endogenous antioxidants. The CC and GS diets improved growth of sole postlarvae, although the mechanism by which these supplements act seem different and still requires further investigation. It seems that curcumin extract improved the oxidative status of the sole, showing low levels of protein and lipid degradation and a reduction on GST activity and HSP70 content, allowing fish to invest more energy into growth rather than on endogenous oxidative defences. The GS diet, on the other hand, showed a negative impact in the oxidative status of the sole by increasing the protein damage, so the positive effect on growth may be reverted in the long-term. The inclusion of green tea extract did not improve growth performance nor the sole antioxidant status during the growth trial. Both green tea and grape seed extracts seem to act as pro-oxidants, by increasing the oxidative damage in sole postlarvae, under standard rearing conditions. However, in response to a thermal stress, both GT and GS diets increased the fish capacity to cope with the new stressful event. However, these antioxidants appear to act in distinct time manners and exert different responses: Grape seed extract seems to have an immediate action and result in a short-term improvement on fish oxidative status, while green tea extract has a long-term effect on the antioxidant capacity of the sole. Hence, curcumin extract seems a good candidate for long-term supplementation of young fish diets, as it improves welfare and growth of Senegalese sole. The use of green tea and grape seed extracts in diets for young fish still requires further evaluation to identify the most adequate inclusion level, although the short-term use of the tested doses seems a feasible solution before highly stressful short periods (e.g., transportation, handling and temperature rises). These results suggest that dietary natural plant extracts can improve young fish robustness and even promote growth when supplemented at the optimal doses.

Abbreviations

ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); BHT: Butylated hydroxytoluene; CAT: Catalase; CC: Dietary curcumin supplementation; CDNB: 1-chloro-2,4- dinitrobenzene; DHA: Days after hatching; DM: Dry matter; DNPH: 2,4-dinitrophenylhydrazine; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid); DW: Dry weight; gadph2: Glyceraldehyde-3-phosphate dehydrogenase 2; GAE: Gallic acid equivalents; GPx: Glutathione peroxidase; GR: Glutathione reductase; GS: Dietary grape seeds supplementation; GSH: Glutathione; GSSG: Glutathione disulfide; GST: Glutathione-S-transferase; GT: Dietary green tea supplementation; HSP: Heat shock protein; K: Condition factor; LPO: Lipid peroxidation; Nrf2: Nuclear factor E2-related factor 2; PC:

Protein carbonyl; PMS: Post-mitochondrial supernatant; QE: Quercetin equivalents; RGR: Relative growth rate; ROS: Reactive oxygen species; RSA: Radical scavenging activity; RT: Room temperature; SL: Standard length; SOD: Superoxide dismutase; TAC: Total Antioxidant Capacity; TBA: 2-thiobarbituric acid; TBARS: Thiobarbituric acid-reactive substances; TCA: Trichloroacetic acid; TFC: Total flavonoids content; TPC: Total phenolic content; ubi: Ubiquitin.

Declarations

Ethical statement

Animal handling and subsequent procedures complied with European laws (2010/63/EU) and Portuguese legislation for the use of laboratory animals (DL n°113/2013, 7 August). This study was approved by the ORBEA Animal Welfare Committee of Centre of Marine Science of Algarve (CCMAR). This experiment was performed 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 under authorization reference number 0421/000/000/2016. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this article are included within the article (and its additional files).

Competing interests

The authors declare no competing interests. The three experimental diets (CC, GT and GS) are included in the patent pending application PCT/IB2020/056001.

Funding

This work was funded by the Projects PATHAA and UIDB/04326/2020 from the Foundation for Science and Technology of Portugal (FCT), and by project VALORMAR [ref. 024517] through Compete 2020, Lisboa 2020, CRESC Algarve 2020, Portugal 2020 and the European Union's ERDF, and IFAPA project RTA2017-00054-C03-01 funded from MCIU/AEI/FEDER, UE. Maria J. Xavier was supported by Grant PDE/0023/2013 (SANFEED Doctoral program, with support by FCT and SPAROS Lda, Portugal). Sofia Engrola and Luísa Custódio were supported by FCT investigator grant IF/00482/2014/CP1217/CT0005 and by the FCT Scientific Employment Stimulus (CEEIND/00425/2017), respectively, both funded by the European Social Fund, the Operational Programme Human Potential and FCT.

Authors' contributions

MX conducted the experiment, performed all the analytical analyses, analysed all data, performed the statistical analysis, prepared the figures and wrote the manuscript. SE, LECC and LV designed the study and supervised the research. AR, RR performed the ELISA and spectrophotometry analyses. MM, CC contributed to the analyses of RT-qPCR. RC collaborate in experiment and sampling. LC contribute to the preparation of diet extracts and spectrophotometry analyses. LECC formulated the diets. All the authors contribute to writing of the manuscript.

Acknowledgements

The authors acknowledge the collaboration of the Aquagroup (CCMAR), SPAROS Lda, Lanuce (CIIMAR/ICBAS) and IFAPA teams.

References

1. El-Beshbishy HA, Mohamadin AM, Abdel-Naim AB. In Vitro Evaluation of the Antioxidant Activities of Grape Seed (*Vitis vinifera*) Extract, Blackseed (*Nigella sativa*) Extract and Curcumin. J Taibah Univ Medical Sci. 2009;4(1):23-35.
2. Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, et al. Techniques for extraction of bioactive compounds from plant materials: A review. J Food Eng. 2013;117(4):426-36.
3. Abbas M, Saeed F, Anjum FM, Afzaal M, Tufail T, Bashir MS, et al. Natural polyphenols: An overview. Int J Food Prop. 2016;20(8):1689-99.
4. Yahfoufi N, Alsadi N, Jambi M, Matar C. The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients. 2018;10(11).
5. Bouarab-Chibane L, Forquet V, Lantéri P, Clément Y, Léonard-Akkari L, Oulahal N, et al. Antibacterial properties of polyphenols: Characterization and QSAR (Quantitative Structure–Activity Relationship) models. Frontiers in Microbiology. 2019;10.
6. Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients. 2010;2(12):1231-46.
7. Kidd PM. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. Altern Med Rev. 2009;14 3:226-46.
8. Reverter M, Bontemps N, Lecchini D, Banaigs B, Sasal P. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. Aquaculture. 2014;433:50-61.
9. Rauf A, Imran M, Orhan IE, Bawazeer S. Health perspectives of a bioactive compound curcumin: A review. Trends in Food Sci Tech. 2018;74:33-45.
10. Giri SS, Sukumaran V, Park SC. Effects of bioactive substance from turmeric on growth, skin mucosal immunity and antioxidant factors in common carp, *Cyprinus carpio*. Fish Shellfish Immunol. 2019;92:612-20.
11. Xia S-l, Ge X-p, Liu B, Xie J, Miao L-h, Zhou Q-l, et al. Effects of supplemented dietary curcumin on growth and non-specific immune responses in juvenile wuchang bream (*Megalobrama amblycephala*). Isr J Aquacult-Bamid. 2015.
12. Midhun SJ, Arun D, Edatt L, Sruthi MV, Thushara VV, Oommen OV, et al. Modulation of digestive enzymes, GH, IGF-1 and IGF-2 genes in the teleost, Tilapia (*Oreochromis mossambicus*) by dietary curcumin. Aquaculture International. 2016;24(5):1277-86.
13. Cui H, Liu B, Ge X-p, XIE J, Xu P, Miao L-h, et al. Effects of dietary curcumin on growth performance, biochemical parameters, HSP70 gene expression and resistance to *Streptococcus iniae* of juvenile Gift Tilapia, *Oreochromis niloticus*. Isr J Aquac. 2013;66:986-96.
14. Akdemir F, Orhan C, Tuzcu M, Sahin N, Juturu V, Sahin K. The efficacy of dietary curcumin on growth performance, lipid peroxidation and hepatic transcription factors in rainbow trout *Oncorhynchus Mykiss* (Walbaum) reared under different stocking densities. Aquac Res. 2017;48(8):4012-21.
15. Mahmoud HK, Al-Sagheer AA, Reda FM, Mahgoub SA, Ayyat MS. Dietary curcumin supplement influence on growth, immunity, antioxidant status, and resistance to *Aeromonas hydrophila* in *Oreochromis niloticus*. Aquaculture. 2017;475:16-23.
16. Zhou J, Lin Y, Ji H, Yu H. The effect of green tea waste on growth and health of grass carp (*Ctenopharyngodon idellus*). Turk J Fish Aquat Sci. 2016;16:679-89.
17. Nootash S, Sheikhzadeh N, Baradaran B, Oushani AK, Maleki Moghadam MR, Nofouzi K, et al. Green tea (*Camellia sinensis*) administration induces expression of immune relevant genes and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 2013;35(6):1916-23.
18. Shi J, Yu J, Pohorly JE, Kakuda Y. Polyphenolics in grape seeds—biochemistry and functionality. J Medicinal Food. 2003;6(4):291-9.
19. Hamza AA, Heeba GH, Elwy HM, Murali C, El-Awady R, Amin A. Molecular characterization of the grape seeds extract's effect against chemically induced liver cancer: in vivo and in vitro analyses. Scientific reports. 2018;8(1):1-16.
20. Kesbiç OS, Yigit M. Structural and chemical changes of grape seed extract after thermal processing and its use in rainbow trout (*Oncorhynchus mykiss*) diets as an organic feed supplement. Aquaculture. 2019;503:275-81.
21. Xavier MJ, Engrola S, Conceição LEC, Manchado M, Carballo C, Gonçalves R, et al. Dietary antioxidant supplementation promotes growth in Senegalese sole postlarvae. Frontiers in Physiology. 2020;11(1472).
22. Croft KD. Dietary polyphenols: Antioxidants or not? Arch Biochem Biophys. 2016;595:120-4.
23. Husain H, Latief U, Ahmad R. Pomegranate action in curbing the incidence of liver injury triggered by Diethylnitrosamine by declining oxidative stress via Nrf2 and NFκB regulation. Scientific reports. 2018;8(1):1-17.

24. Halliwell B, Gutteridge JM. Free radicals in biology and medicine: Oxford University Press, USA; 2015.
25. Lau AT, Wang Y, Chiu JF. Reactive oxygen species: current knowledge and applications in cancer research and therapeutic. J Cell Biochem. 2008;104(2):657-67.
26. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 2006;141(2):312-22.
27. Wilhelm FD. Fish antioxidant defenses - A comparative approach. Braz Journal Med Biol Res. 1996;29(12):1735.
28. Martínez-Álvarez RM, Morales AE, Sanz A. Antioxidant Defenses in Fish: Biotic and Abiotic Factors. Reviews in Fish Biology and Fisheries. 2005;15(1-2):75-88.
29. Lushchak VI. Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol. 2011;101(1):13-30.
30. Engrola S, Conceição LE, Gavaia PJ, Cancela L, Dinis MT. Effect of pre-weaning feeding regime on weaning performance of Senegalese sole, *Solea senegalensis* (Kaup, 1858). Isr J Aquacult-Bamid. 2005;57:10-8.
31. Ricker WE. Handbook of computations for biological statistics of fish populations: Fisheries Research Board of Canada; 1958.
32. Rodrigues MJ, Soszynski A, Martins A, Rauter AP, Neng NR, Nogueira JMF, et al. Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*. Ind Crops and Prod. 2015;77:315-22.
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54.
34. Clairborne A. Catalase activity. In: Greenwald RA, editor. Handbook methods for oxygen radical research: CRC press, Boca Raton; 1985. p. 283-4.
35. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J Biol Chem. 1974;249(22):7130-9.
36. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal biochem. 1969;27(3):502-22.
37. Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem. 1990;190(2):360-5.
38. Rodrigues AC, Gravato C, Quintaneiro C, Bordalo MD, Golovko O, Žlábek V, et al. Exposure to chlorantraniliprole affects the energy metabolism of the caddisfly *Sericostoma vittatum*. Environ Toxicol Chem. 2017;36(6):1584-91.
39. Bird R, Draper H. Comparative studies on different methods of malonaldehyde determination. Methods in enzymology. 105: Elsevier; 1984. p. 299-305.
40. Mesquita CS, Oliveira R, Bento F, Geraldo D, Rodrigues JV, Marcos JC. Simplified 2, 4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Anal Biochem. 2014;458:69-71.
41. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem. 2004;37(4):277-85.
42. Rosa R, Trübenbach K, Pimentel MS, Boavida-Portugal J, Faleiro F, Baptista M, et al. Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). J Exp Biol. 2014;217(4):518-25.
43. Machado M, Salas-Leiton E, Infante C, Ponce M, Asensio E, Crespo A, et al. Molecular characterization, gene expression and transcriptional regulation of cytosolic HSP90 genes in the flatfish Senegalese sole (*Solea senegalensis* Kaup). Gene. 2008;416(1-2):77-84.
44. Firmino J, Carballo C, Armesto P, Campinho MA, Power DM, Machado M. Phylogeny, expression patterns and regulation of DNA Methyltransferases in early development of the flatfish, *Solea senegalensis*. BMC Dev Biol. 2017;17(1):11.
45. Infante C, Matsuoka MP, Asensio E, Cañavate JP, Reith M, Machado M. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. BMC Mol Biol. 2008;9(1):28.
46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. Methods. 2001;25(4):402-8.
47. Ho C-T, Chen Q, Shi H, Zhang K-Q, Rosen RT. Antioxidative effect of polyphenol extract prepared from various Chinese teas. Preve Med. 1992;21(4):520-5.

48. Minatel IO, Borges CV, Ferreira MI, Gomez HAG, Chen C-YO, Lima GPP. Phenolic compounds: Functional properties, impact of processing and bioavailability. 2017.
49. Jiang J, Wu X-Y, Zhou X-Q, Feng L, Liu Y, Jiang W-D, et al. Effects of dietary curcumin supplementation on growth performance, intestinal digestive enzyme activities and antioxidant capacity of crucian carp *Carassius auratus*. *Aquaculture*. 2016;463:174-80.
50. Arslan G, Sönmez AY, Yank T. Effects of grape *Vitis vinifera* seed oil supplementation on growth, survival, fatty acid profiles, antioxidant contents and blood parameters in rainbow trout *Oncorhynchus mykiss*. *Aquaculture Res*. 2018;49(6):2256-66.
51. Baldissera MD, Souza CF, Descovi SN, Verdi CM, Zeppenfeld CC, de Lima Silva L, et al. Effects of dietary grape pomace flour on the purinergic signaling and inflammatory response of grass carp experimentally infected with *Pseudomonas aeruginosa*. *Aquaculture*. 2019;503:217-24.
52. Thawonsuwan J, Kiron V, Satoh S, Panigrahi A, Verlhac V. Epigallocatechin-3-gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol Biochem*. 2010;36(3):687-97.
53. Cho SH, Lee S-M, Park BH, Ji S-C, Lee J, Bae J, et al. Effect of dietary inclusion of various sources of green tea on growth, body composition and blood chemistry of the juvenile olive flounder, *Paralichthys olivaceus*. *Fish Physiol Biochem*. 2006;33(1):49-57.
54. Abdel-Tawwab M, Ahmad MH, Seden ME, Sakr SF. Use of green tea, *Camellia sinensis* L., in practical diet for growth and protection of Nile tilapia, *Oreochromis niloticus* (L.), against *Aeromonas hydrophila* infection. *J World Aquacult Soc*. 2010;41:203-13.
55. Xu Y, Zhang M, Wu T, Dai S, Xu J, Zhou Z. The anti-obesity effect of green tea polysaccharides, polyphenols and caffeine in rats fed with a high-fat diet. *Food Funct*. 2015;6(1):296-303.
56. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biol Med*. 2001;30(11):1191-212.
57. Beaulieu M, Costantini D. Biomarkers of oxidative status: missing tools in conservation physiology. *Conserv Physiol*. 2014;2(1).
58. Reeg S, Jung T, Castro JP, Davies KJ, Henze A, Grune T. The molecular chaperone Hsp70 promotes the proteolytic removal of oxidatively damaged proteins by the proteasome. *Free Radical Biol Med*. 2016;99:153-66.
59. Abd El-Hakim YM, El-Houseiny W, El-Murr AE, Ebraheim LLM, Moustafa AA, Rahman Mohamed AA. Melamine and curcumin enriched diets modulate the haemato-immune response, growth performance, oxidative stress, disease resistance, and cytokine production in *Oreochromis niloticus*. *Aquat Toxicol*. 2020;220:105406.
60. Manju M, Akbarsha MA, Oommen OV. In vivo protective effect of dietary curcumin in fish *Anabas testudineus* (Bloch). *Fish Physiol Biochem*. 2012;38(2):309-18.
61. Yonar ME, Mise Yonar S, Ispir U, Ural MS. Effects of curcumin on haematological values, immunity, antioxidant status and resistance of rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas salmonicida* subsp. achromogenes. *Fish Shellfish Immunol*. 2019;89:83-90.
62. Li G, Zhou X, Jiang W, Wu P, Liu Y, Jiang J, et al. Dietary curcumin supplementation enhanced growth performance, intestinal digestion, and absorption and amino acid transportation abilities in on-growing grass carp (*Ctenopharyngodon idella*). *Aquaculture Res*. 2020;51(12):4863-73.
63. Galati G, O'brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biol Med*. 2004;37(3):287-303.
64. Chedea VS, Braicu C, Socaciu C. Antioxidant/prooxidant activity of a polyphenolic grape seed extract. *Food Chemistry*. 2010;121(1):132-9.
65. Mousavi S, Sheikhzadeh N, Tayefi-Nasrabadi H, Alizadeh-Salteh S, Khani Oushani A, Firouzmandi M, et al. Administration of grape (*Vitis vinifera*) seed extract to rainbow trout (*Oncorhynchus mykiss*) modulates growth performance, some biochemical parameters, and antioxidant-relevant gene expression. *Fish Physiol Biochem*. 2020;46(3):777-86.
66. Iftikar FI, Hickey AJ. Do mitochondria limit hot fish hearts? Understanding the role of mitochondrial function with heat stress in *Notolabrus celidotus*. *PLoS One*. 2013;8(5):e64120.
67. Lygren B, Hamre K, Waagbø R. Effects of dietary pro-and antioxidants on some protective mechanisms and health parameters in Atlantic salmon. *J Aquat Anim Health*. 1999;11(3):211-21.
68. Jia R, Li Y, Cao L, Du J, Zheng T, Qian H, et al. Antioxidative, anti-inflammatory and hepatoprotective effects of resveratrol on oxidative stress-induced liver damage in tilapia (*Oreochromis niloticus*). *Comp Biochem Physiol C Toxicol Pharmacol*.

2019;215:56-66.

69. Akbarian A, Michiels J, Degroote J, Majdeddin M, Golian A, De Smet S. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *J Anim Sci Biotechnol.* 2016;7(1):1-14.
70. Sayed AEH, Soliman HAM. Modulatory effects of green tea extract against the hepatotoxic effects of 4-nonylphenol in catfish (*Clarias gariepinus*). *Ecotoxicol Environ Saf.* 2018;149:159-65.
71. Hasanpour S, Salati AP, Falahatkar B, Azarm HM. Effects of dietary green tea (*Camellia sinensis* L.) supplementation on growth performance, lipid metabolism, and antioxidant status in a sturgeon hybrid of Sterlet (*Huso huso* × *Acipenser ruthenus*) fed oxidized fish oil. *Fish Physiol Biochem.* 2017;43(5):1315-23.

Tables

Table 1 Proximate composition of the three experimental diets (CTRL, CC, GT and GS)

Diets proximal composition	
Crude protein (% DM)	65.6
Crude fat (% DM)	19.4
Gross energy (MJ kg ⁻¹)	22.9

Table 2 Primers used in qPCR

Gene	Fwd sequence (5' → 3')	Rev sequence (5' → 3')	Acession nr (GenBank)	Size (bp)
<i>sod3</i>	AGTCGGAAGCAACACTGTTTGAAGGGATGT	GCCAGCATCTCCACCCAGTCCTAGGTCA	unigene29222	98
<i>cat</i>	CCAAGCCCCGACAAAATGCTTCAGGGT	CCACGCGGGTTCTGAATGGGCAGTTGAC	unigene326680	118
<i>gsr</i>	AGATGCTTACGTTGGCCGCCTGAACCAC	CCTCCACTGTCGGCTCAGGGTCATCTGT	unigene3049	116
<i>gpx1</i>	TGTGAACGGAGCAGATGCACACCCTT	AACTTTGGATCGGTCATGAGAGCCATGGTA	unigene65687	99
<i>gpx3</i>	GTTGTGGACCTTTGTGCCGCCGCTG	GCTGCACTGCCGAGTCAACGATGCCT	unigene29032	88
<i>hsp70</i>	GCTATACCAGGGAGGGATGGAAGGAGGG	CGACCTCCTCAATATTTGGGCCAGCA	AB513855	119
<i>hsp90aa</i>	GACCAAGCCTATCTGGACCCGCAAC	TTGACAGCCAGGTGGTCTCCAGT	Manchado et al., 2009	105
<i>hsp90ab</i>	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA	GCCAAGGGGCTCACCTGTGTGCG	Manchado et al., 2009	148

Gene name, sequences, accession numbers at GenBank or SoleaDB and amplicon sizes are indicated.

Table 3 Antioxidant properties (expressed as % of activity) and total phenolics (TPC; expressed as mg GAE/ g diet) and flavonoids contents (TFC; mg QE/g diet) of methanol extracts from the tested diets (CTRL, CC, GT and GS).

	Diets				1-way Anova
	CTRL	CC	GT	GS	P value
Assay/ parameter					
ABTS (% of activity)	19.4±2.4 ^b	18.5±0.9 ^b	17.3±1.4 ^b	28.6±1.3 ^a	<0.001
DPPH (% of activity)	8.9±3.2 ^c	50.2±9.9 ^a	34.1±2.0 ^b	39.9±4.1 ^b	<0.001
TPC (mg GAE/ g diet)	1.3±0.6 ^b	6.4±1.9 ^a	5.0±2.1 ^a	5.4±0.9 ^a	<0.001
TFC (mg QE/g diet)	0.7±0.3 ^c	2.9±0.7 ^{ab}	1.5±0.2 ^{bc}	4.4±3.7 ^a	<0.001

Values were expressed as mean ± SD. Different superscript letters indicate significant differences between the dietary treatments.

Table 4 Senegalese sole key performance indicators at the end of the growth trail (70 DAH) fed different diets.

	Initial	Treatments				1-way Anova
		CTRL	CC	GT	GS	P value
DW (mg)	12.3±5.7	163.8±40.2 ^c	192.2±39.4 ^a	175.7±43.2 ^{bc}	182.9±44.8 ^{ab}	<0.001
SL (mm)	17.7±3.1	35.0±2.9 ^c	39.1±2.7 ^a	38.0±2.3 ^{ab}	37.8±2.8 ^b	<0.001
K		1.8±0.3 ^a	1.6±0.2 ^{bc}	1.6±0.2 ^c	1.7±0.2 ^b	<0.001
RGR 45-70 DAH (% day ⁻¹)		10.9±0.8	11.6±0.2	11.2±0.4	11.4±0.4	0.364
Survival (%)		97.6±1.7	98.5±0.7	98.2±0.9	99.0±0.0	0.460

Values are presented means ± SD. Different superscript letters indicate significant differences between the dietary treatments.

Table 5 The response of oxidative stress-related biomarkers in 70 DAH Senegalese sole fed with different diets at the end of the growth trial (standard condition).

	Standard Condition				1-way Anova
	CTRL	CC	GT	GS	P value
Physiological indicators					
CAT ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	13.3 \pm 0.8	13.2 \pm 0.9	12.6 \pm 1.0	12.2 \pm 1.3	0.120
GST (nmol/min/mg protein)	21.5 \pm 2.5 ^a	18.1 \pm 1.6 ^b	17.9 \pm 2.7 ^b	16.0 \pm 3.1 ^b	0.001
GSH ($\mu\text{M}/\text{min}/\text{mg}$ protein)	102.9 \pm 11.9 ^a	97.9 \pm 4.7 ^a	39.6 \pm 6.9 ^b	36.6 \pm 4.4 ^b	<0.001
TAC (μM Trolox equivalents/mg protein)	1.3 \pm 0.2	1.3 \pm 0.4	1.5 \pm 0.4	1.1 \pm 0.1	0.126
HSP70 ($\mu\text{g}/\text{mg}$ protein)	1.3 \pm 0.1 ^a	0.9 \pm 0.3 ^b	1.6 \pm 0.4 ^a	0.6 \pm 0.1 ^b	<0.001
LPO (nmol TBARS/mg protein)	6.7 \pm 1.0 ^{ab}	6.2 \pm 0.9 ^b	8.0 \pm 1.7 ^a	6.6 \pm 0.7 ^{ab}	0.024
PC (nmol carbonyl/mg protein)	29.5 \pm 11.4 ^b	23.0 \pm 10.1 ^b	66.7 \pm 16.0 ^a	58.7 \pm 7.5 ^a	<0.001
Gene expression					
<i>sod3</i>	1.0 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.1	1.0 \pm 0.1	0.446
<i>cat</i>	1.0 \pm 0.1 ^{ab}	1.1 \pm 0.1 ^{ab}	0.9 \pm 0.1 ^b	1.4 \pm 0.1 ^a	0.046
<i>gsr</i>	1.0 \pm 0.1	1.3 \pm 0.2	1.1 \pm 0.0	1.4 \pm 0.2	0.266
<i>gpx1</i>	1.0 \pm 0.1 ^b	1.9 \pm 0.5 ^a	1.1 \pm 0.1 ^b	1.6 \pm 0.2 ^a	0.047
<i>gpx3</i>	1.0 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	1.1 \pm 0.2	0.431
<i>hsp70</i>	1.0 \pm 0.1	1.2 \pm 0.2	1.3 \pm 0.1	1.6 \pm 0.3	0.309
<i>hsp90aa</i>	1.0 \pm 0.1	3.3 \pm 0.6	1.5 \pm 0.5	1.8 \pm 0.4	0.051
<i>hsp90b</i>	1.0 \pm 0.1	1.0 \pm 0.1	0.8 \pm 0.0	1.0 \pm 0.1	0.453

Values are presented means \pm SD. Different superscript letters indicate significant differences between the dietary treatments.

Figures

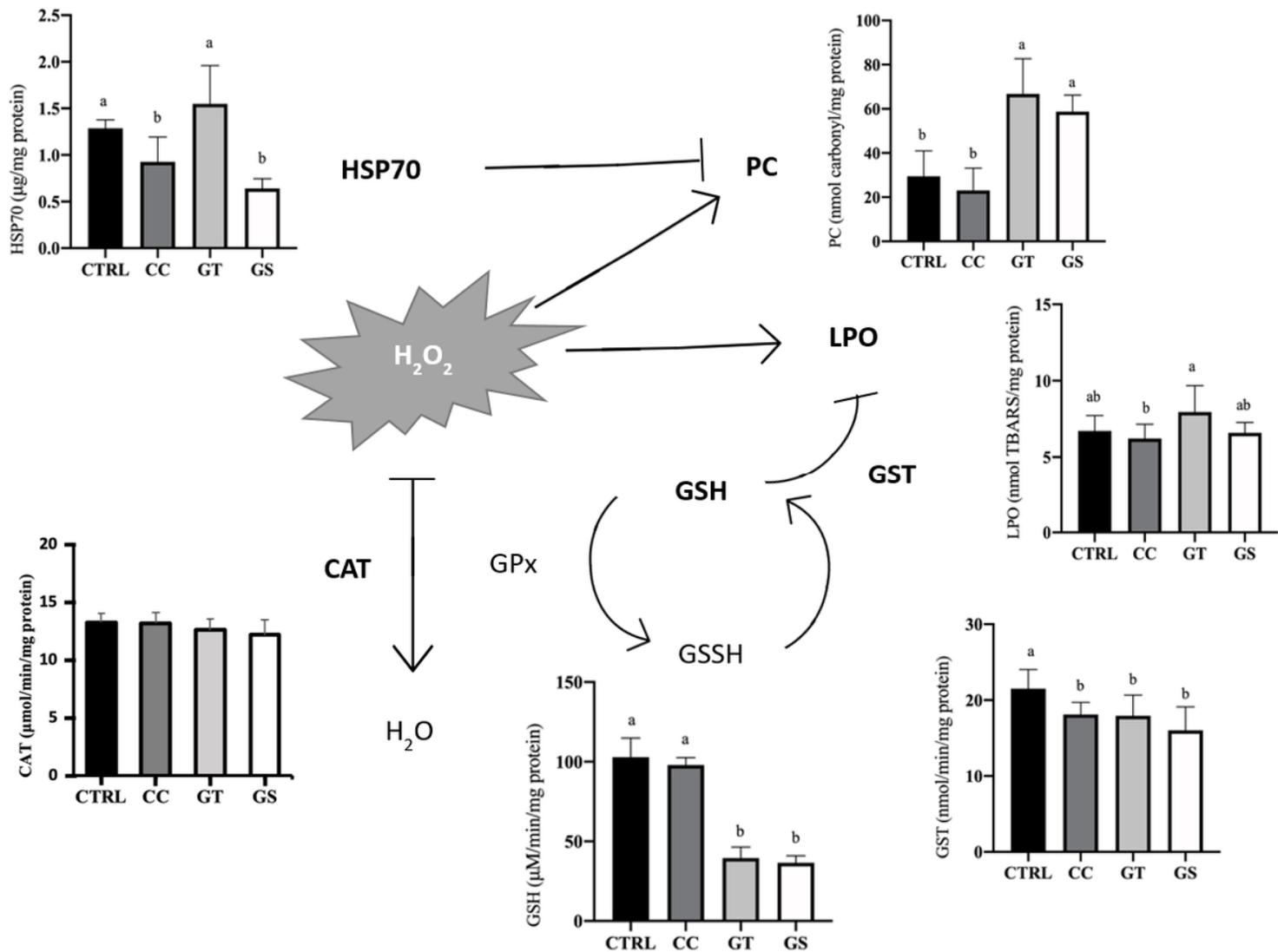


Figure 1

Schematic (simplistic) representation of the response and pathways of several oxidative stress related biomarkers of Senegalese sole fed with different diets at the end of the growth trail (70 DAH). Values are present as means ± SD. Different superscript letters indicate significant differences ($P < 0.05$, 1-way ANOVA) between the dietary treatments.

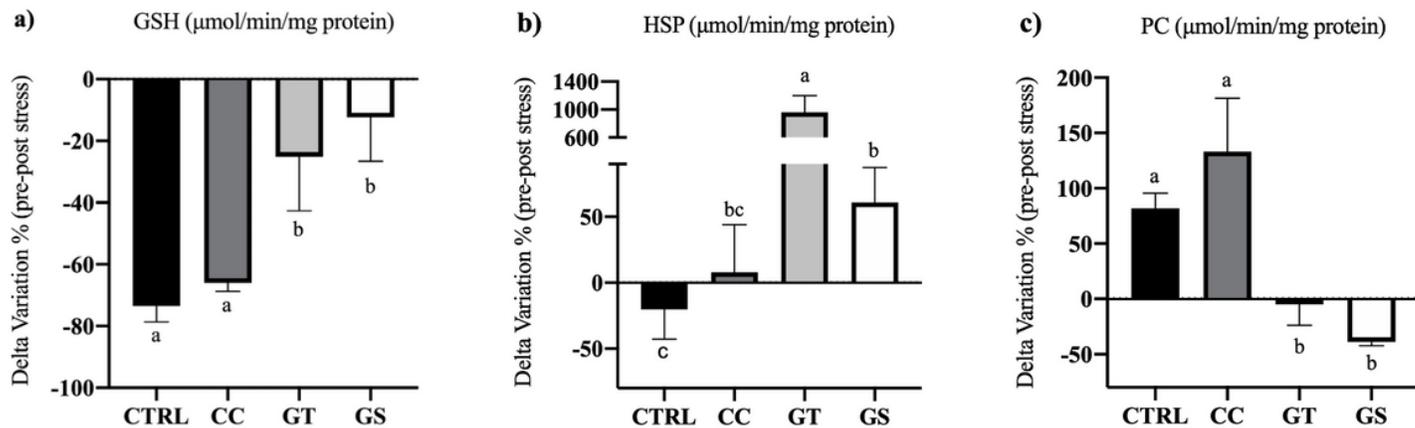


Figure 2

Delta variation (%) of the response of oxidative stress-related biomarkers from standard to acute stress conditions. Values are presented means \pm SD, n = 3. Different superscript letters indicate significant differences (P < 0.05, 1-way ANOVA) between the dietary treatments.

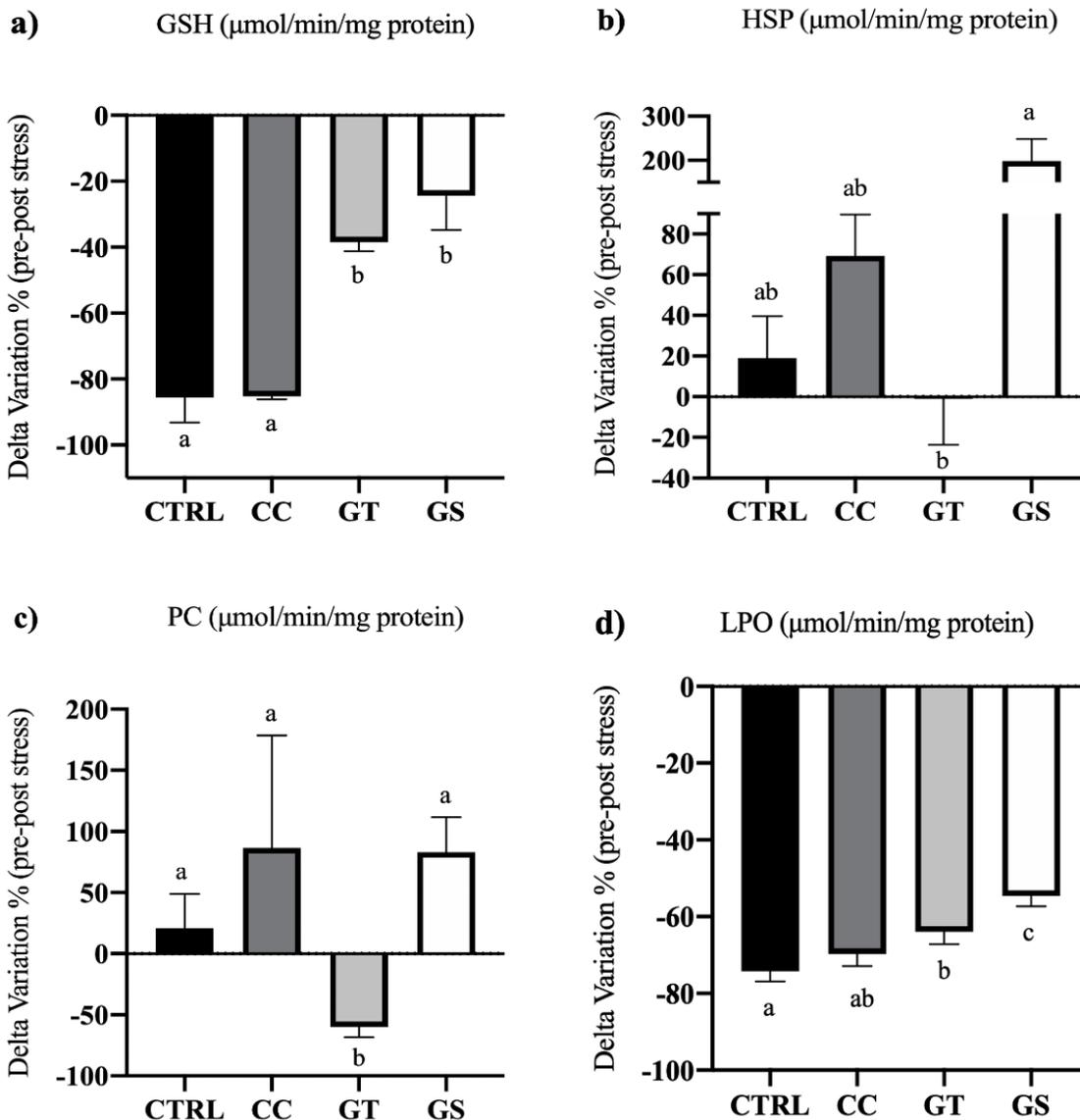


Figure 3

Delta variation (%) of the response of oxidative stress-related biomarkers from standard to chronic stress conditions. Values are presented means \pm SD, n = 3. Different superscript letters indicate significant differences (P < 0.05, 1-way ANOVA) between the dietary treatments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)
- [Additionalfile2.docx](#)