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Application of transcriptomic profiling to investigate the toxicity mechanisms caused by dietary exposure of nanoplastics in fish

Alberto Cuesta^a, Cristóbal Espinosa^a, María A. Esteban^a, Carmen González-Fernández^{b,*}

^a Immunobiology for Aquaculture Group, Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Murcia 30100, Spain

^b Laboratoire d'écotoxicologie, INRAE UR RiverLy, Villeurbanne F-69625, France

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ABSTRACT

Nowadays, nanoplastics (NPs) are one of the main concerns regarding plastic pollution. The increasing presence of plastic particles, fibers and fragments in the marine environment pose an additional risk to both, wild and cultured fish. Ingestion is the main mechanism by which particles are internalized. Thus, this study evaluated the impact of a diet containing NPs in one of the most cultivated species across the Mediterranean Sea, the European sea bass (*Dicentrarchus labrax*). Polystyrene NPs (50 nm) were supplied in the food for a period of 21 days and the transcriptomic changes were measured in the intestine through RNA-seq. Additionally, enzymatic and bactericidal activities were measured in the liver or serum, respectively of the same fish to evaluate the organism stress. No significant changes in the enzymatic activities were observed in the liver, whilst the seric bactericidal activity decreased by NPs dietary treatments. This suggests that ingestion of NPs at low dosages might have an impact on fish health. In addition, our data suggested that NPs impact some important biological pathways related to fish morphogenesis, organ development, membrane receptors, and fish immunity. These routes are extremely important for fish development and growth and can have long-term impact, since the early stages of fish are the most sensitive to this kind of pollution. This study provides information on the impact of the ingestion of NPs in sea bass and can serve as a basis for future investigations on the prevention and treatment of such pollutants in aquaculture.

1. Introduction

Plastic pollution is an emerging concern in both freshwater and marine environments. During the last decades, studies identifying the potential harmful effects of microplastics (MPs; 5 mm to 100 nm in size) and nanoplastics (NPs; < 100 nm in size) have flourished due to the improvement in equipment and techniques that allow for better detection and quantification them (Erni-Cassola et al., 2019). However, seawater conditions generate problems when quantifying small particles such as NPs, making it difficult to estimate the real concentration present in oceans (Lv et al., 2020). To date, there are few studies reporting the detection and analysis of NPs in real field samples (Cai et al., 2021; Ter Halle et al., 2017). Recent studies have evidenced the presence of small plastic particles (around 200 nm in size) in the Wadden Sea water column and remote surface waters with average concentrations of 4.2 and 563 µg/L, respectively (Materić et al., 2022a; 2022b). Ingestion of plastic debris has been recognized as one of the main entry sources in aquatic animals, easily going through the trophic chain (Nelms et al.,

2018). Once ingested, plastics can accumulate in the gastrointestinal tracts and stomachs of fish (Alomar et al., 2021) and produce physical intestinal injuries (Del Piano et al., 2023; Espinosa et al., 2019). In fact, *ex vivo* assays have demonstrated that 50 nm NPs are able to cross the European sea bass intestine barrier (Vagner et al., 2022). However, the effects caused by the ingestion of NPs in fish “*in vivo*” remain unclear. We were able to find some studies revealing that ingestion of NPs can impair fathead minnow’s (*Pimephales promelas*) innate immunity (Elizalde-Velázquez et al., 2020) as well as the survival rate, the growth and down-regulate the expression of lipolysis-related genes in the liver of large yellow croaker (*Larimichthys crocea*) juveniles (Lai et al., 2021). However, further information is required to identify the potential risks associated to these particles in natural environments.

According to the FAO, aquaculture has largely grown over the last decades as a food production system due to the increasing demand for seafood, mainly due to the population growth and the beneficial effects on human health related to fish consumption (FAO., 2020). The European sea bass (*Dicentrarchus labrax*) is one of the most exploited species

* Corresponding author.

E-mail address: carmen.gonzalez-fernandez@inrae.fr (C. González-Fernández).

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aquaculture in the Mediterranean areas (Grigorakis, 2007). This species is particularly sensitive to stressful conditions such as overcrowding and susceptible to pathogenic infections (Chi et al., 2016). The presence of NPs in the environment can be an additional stress factors leading to risks in production. Thus, the objective of the present study was to investigate whether a diet containing NPs can impact the intestinal integrity and functions of European sea bass, by means of the RNA sequencing (RNA-seq) technique. This technique has previously revealed that dietary-administered NPs alter important biological routes including immunity, oxidative stress, gene transcription and translation, protein degradation, lipid metabolism, oxygen demand, and reproduction in red swamp crayfish (*Procambarus clarkii*) (Capanni et al., 2021). We wanted to apply the same technology to identify specific pathways involved in the toxicity of NPs in the cultivated species European sea bass.

2. Material and methods

2.1. Fish maintenance

Healthy juvenile specimens of European sea bass (20.5 ± 0.7 g body weight) were purchased from a hatchery (Alevines Guardamar, Alicante, Spain). The fish were maintained in recirculatory laboratory aquaculture systems (RAS; 28 ‰ salinity, 22–26 °C and with a 12 h light: 12 h dark photoperiod) with suitable aeration and filtration devices and were fed with a commercial diet (Skretting) daily. The handling of the specimens was always performed in accordance with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committee of the University of Murcia (reference REGA ES-300305440012 and Permit Number A13210701).

2.2. NPs characterization and diet preparation

Commercial polystyrene (PS) nanoplastics (NPs) of 50 nm were purchased from Bangs Laboratories. NPs properties had been previously confirmed by dynamic light scattering (DLS) and transmission electron microscopy (TEM) (González-Fernández et al., 2021). All these techniques confirmed the composition, sizes below 100 nm and a PDI < 0.2 and no aggregation.

For diet preparation, commercial pellets (Skretting) were used to feed animals. Two plastic concentrations were used: 1 and 0.250 g plastic/kg food, forming the High and Low groups, respectively. These concentrations were chosen based on previous papers reporting similar doses, polymer type and time of exposure in teleost fish (Del Piano et al., 2023). NPs were resuspended in RNA-free water and sprayed onto diet pellets. The volume was adjusted to ensure that all pellets were fully covered by the solution without this entailing a structural loss of the feed. Control treatment was prepared identically but without containing NPs. The pellets were allowed to dry and then mixed with < 1 % of cod oil to avoid the dispersion of plastics once supplied in water (Espinosa et al., 2019).

2.3. Exposure and sample collection

For experimental procedure, 18 fish juveniles were randomly distributed in 6 tanks forming three groups. Fish groups received the control (without NPs), Low (0.25 g NPs / kg feed) or High (1 g NPs / kg feed) diets. Animals were fed daily for a period of 21 days with 1 % of fish biomass to ensure that all pellets were ingested. After 21 days, fish ($n = 6$ fish/treatment) were euthanized through an overdose of clove oil, and blood, liver and intestine tissues from each fish were sampled individually. Their whole intestine was conserved in TRIzol® Reagent (ThermoFisher Scientific) for RNA-seq and real-time PCR (qPCR) analysis and the liver was snap frozen in liquid nitrogen for enzymatic analysis. Blood was maintained at 4 °C for 2 h and then centrifugated at

5000 g for 20 min to obtain the serum for later immunological analyses. All the samples were individually obtained and analysed.

2.4. Enzymatic activities in liver

Liver samples from 6 fish/treatment were homogenized in a 50 mM potassium phosphate buffer (pH 7.0) for 30–60 s in ice, then vortexed and centrifuged (12,000 g, 10 min, 4 °C). The protein concentration in each liver homogenate was measured with Bradford protocol and was adjusted to 500 µg protein/mL (diluted in 50 mM potassium phosphate buffer, pH 7.0). Samples of liver homogenate were used to determine the superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidase (GPx) activities following established protocols (Espinosa-Ruiz et al., 2023). In all cases the enzymatic activities were expressed as units/mg protein. All analyses were performed in triplicates.

2.5. Bactericidal activity on serum

Bactericidal activity was measured in fish serum ($n = 6$ /treatment) which had been prepared as described in Section 2.3. The pathogenic marine bacteria *Vibrio harveyi* (strain Lg 16/100) was used to study bacterial inhibition by fish serum (Espinosa-Ruiz et al., 2023). Briefly, aliquots of 5 µL of bacterial culture were added to wells of a flat-bottomed 96-well plate (in duplicates) and incubated with the same volume of fish serum for 2 h at 25 °C. Then, 180 µL of tryptic soy agar (TSA, Sigma-Aldrich) was added to each well. The absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25 °C. Samples without bacteria were used as blanks (negative control). Samples without serum were used as positive controls (100 % growth or 0 % antibacterial activity). Bactericidal activity was expressed as % of bacterial growth inhibition.

2.6. RNA isolation for high-throughput transcriptome sequencing

Total RNA was isolated using the TRIzol® Reagent followed by purification with PureLink RNA Mini Kit columns according to the user's guide. Quality of RNA was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and showed an RNA Integrity Number (RIN) over 9.5. RNA from three independent individuals per condition (Control and Low groups) were subsequently delivered to Novogene company (UK Cambridge based) for independent library preparation and sequencing ($n = 3$ per group), using the Illumina technique.

2.7. RNA-seq data processing and differential expression analysis

The RNA-seq analysis was conducted to evaluate the transcriptome modulation in the intestine of fish fed with NPs. Based on the functional results and in potential future environmental scenarios, the Low NP concentration was selected and compared with the control group. RNA-seq pipeline is presented in Fig. 1. Files and first curation were performed by the company supplier Novogene. Two fastq files were obtained from each of the RNA samples analysed, which corresponded to the forward and reverse sequences. The raw read sequences were deposited in the Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra>) under BioProject accession number PRJNA945811. The sequences obtained were analyzed with the nf-core/rnaseq workflow (Batut et al., 2021) using Transcripts Alignment to a Reference (STAR) as aligner, and Salmon as quantifier, and based on the transcriptome available at ENSEMBL (dlabrax2021 “GCA_905,237,075.1”). The quantification results were processed and analyzed with the DESeq2 library of R, with which the differential expression genes (DEGs) was obtained. The expression $|\text{Log2Foldchange}| \geq 1.5$ (non-adjusted p -value < 0.05 in all cases) were

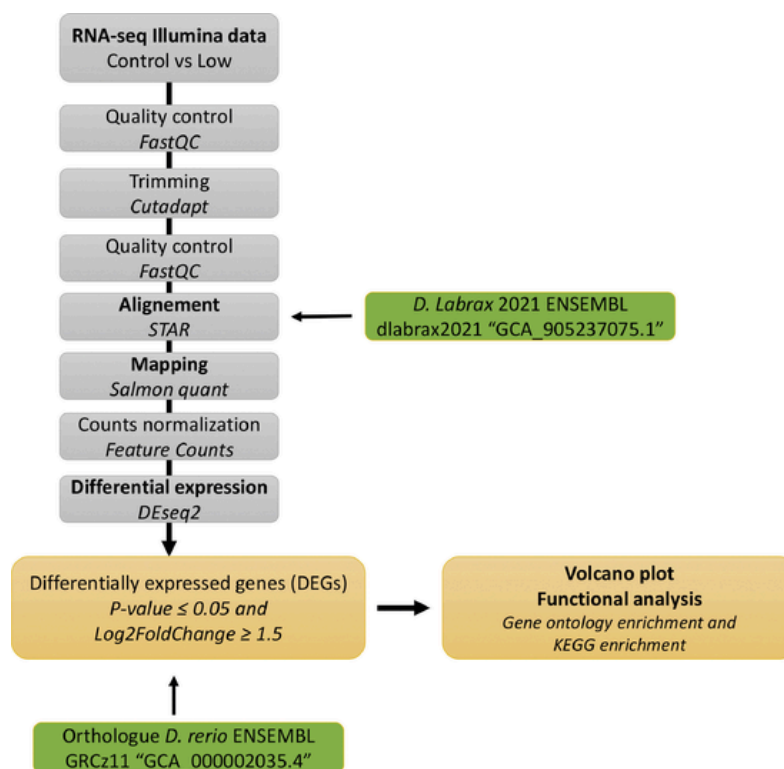


Fig. 1. Pipeline followed for RNA-seq analysis in this study.

used for selecting genes differentially expressed between Low NPs and Control treatments. To validate the up and down-regulated genes used for functional analysis, real time PCR (qPCR) was performed (Section 2.8). Enrichment analyses were executed using R software with a custom version of the *goana* (Gene Ontology enrichment). The Gene Ontology database (<http://www.geneontology.org/>) provides a controlled vocabulary keeping update to describe the properties of genes and gene products in organisms generally, classifies genes into three terms: “molecular function (MF),” “cellular component (CC),” and “biological process (BP)”. KEGG enrichment analysis (<http://www.genome.jp/kegg/>) of DEGs was performed with *ClusterProfiler* and *kegg* (KEGG pathway enrichment) libraries using *limma* package. All enrichment was performed with the *Danio rerio* species databases.

2.8. Validation of RNA-seq data by real-time (qPCR)

We evaluated the expression of selected genes using qPCR to validate some of the DEGs found in the RNA-seq data. To this end, individual total RNA from 6 samples (not pooled) was used to generate the cDNA. cDNA was produced with the SuperScript™ IV Reverse Transcriptase (ThermoFisher Scientific). Real-time PCR (qPCR) was performed using a 7500 Fast Real Time PCR System (Applied Biosystems) and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). Reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. Gene expression was normalized by the geometric mean of the housekeeping elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*rps18*) gene expression and expressed as $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). Primers are listed in supplementary Table S1. Negative controls with no sample were always included in the reactions.

2.9. Statistical analyses

The results of enzymatic activities were expressed as means \pm SEM. The normality of the variables was confirmed by the Shapiro–Wilk test

and homogeneity of variance by the Levene’s test. Statistical differences among the three groups were assessed by one-way ANOVA followed by the Tukey’ comparison of means when applicable. The significance level was 95 % in all cases (p -value < 0.05). Statistical analysis and graphics were performed with R software (version 4.3.1) using *factoextra*, *ggplot2* or *ggpubr* packages and SRPlot (<https://www.bioinformatics.com>). Visual representation of GO terms obtained from the enrichment was performed with *Revigo* (<http://revigo.irb.hr/>) by removing redundant GO terms.

3. Results

3.1. Physiological responses in the European sea bass

We analysed several enzymatic activities related to the oxidative stress including SOD, CAT, GR and GPx activities in sea bass liver (Fig. 2). After 21 days of NPs-diet supply, liver SOD and CAT activities were not changed whilst GR and GPx activities (Fig. 2C, D) decreased respect to the control group, although they failed to reach statistical significance.

Regarding the immune response, bactericidal activity was analysed in fish serum to evaluate the effects of dietary NPs. The inhibition of the bacterial growth was decreased in the serum of fish fed NPs (Fig. 2E), being significant in the Low group, compared to those from the control group.

3.2. DEGs in European sea bass intestine upon dietary exposure to NPs

The RNA-seq analysis resulted in an average of 1.13×10^8 reads in both control and Low NPs-diet treatment, with more than 98.97 % effective reads and an estimate error of 0.03 % (Table 1). These reads were aligned to the available transcriptome and resulted in a total of 20,482 genes.

Based on the differential expression analysis by DESeq2, we obtained the list of DEGs upon dietary intake of NPs and visualized in the

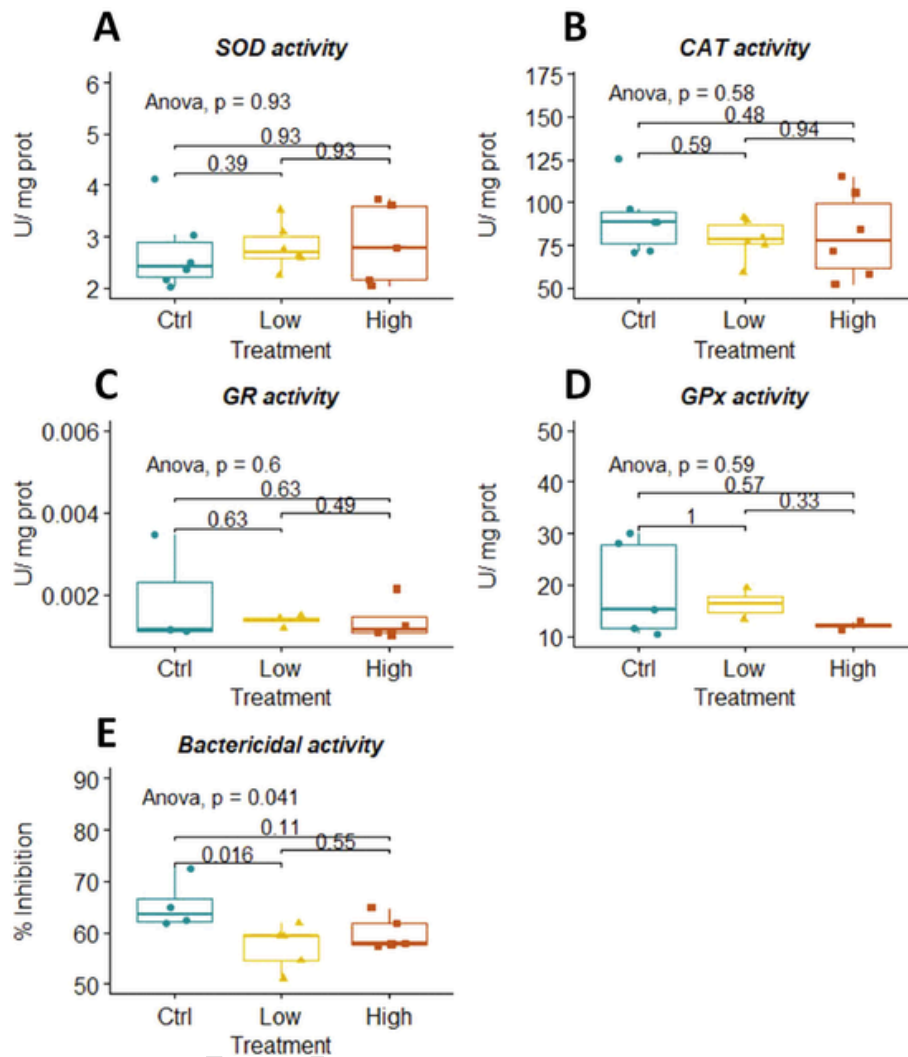


Fig. 2. Boxplot of the liver oxidative stress (A, B, C, D) and serum immune (E) activities analysed in European sea bass fed with commercial diet alone (control) or containing 0.25 (Low) or 1 (High) g NPs / kg feed for 21 days. Statistical differences by one-way ANOVA ($p < 0.05$) and Tukey' comparison of means are indicated.

Table 1

Summary of the effective sample reads and quality of data provided by Novogene.

Sample	Raw reads	Effective(%)	Error(%)	Q20(%)
Control 1	9.85E + 07	98.98	0.03	95.87
Control 2	1.15E + 08	98.91	0.03	97.39
Control 3	1.39E + 08	98.89	0.03	97.43
Low 1	1.03E + 08	99.01	0.03	96.1
Low 2	1.07E + 08	99.06	0.03	97.22
Low 3	1.17E + 08	98.95	0.03	95.78
Mean	1.13E + 08	98.97	0.03	96.63

volcano plot (Fig. 3A). From the total annotated genes, less than 0.5 % presented significant differences, 64 were up-regulated and 37 down-regulated, being most of the mapped genes not impacted (Fig. 3A). The heatmap carried out on the significant DEGs allowed us to visualize the similarities and differences between biological replicas (Fig. 3B). Although the clustering allowed us to separate Control and Low treatments, we observed that one Control replicate have higher expression of some up-regulated genes compared to the others. In this line, we can appreciate that Low treatment' replicates differ on the expression of up-regulated genes, which might explain the low percentage of significant DEGs observed. Among the DEGs, some of them are not annotated and

removed from the analysis. The remaining DEGs, either up- or down-regulated, are identified and presented in the Fig. 4.

To verify the reliability of the RNA-seq data, we selected DEGs with negative (*serhl*, *oscp1a*, *pole* and *neil3*) and positive (*serpinh1b*, *itgb1a*, *laynb* and *slc12a3*) values for qPCR analysis. Results demonstrated that expression values of these genes agreed with results obtained in the RNA sequencing analysis, which confirmed the reliability of the RNA-seq results (Fig. 5).

3.3. GO and KEGG enrichment analysis of DEGs

DEGs are commonly used to find functional or biological pathways of interest. GO enrichment analyses were carried out to explore the biological processes, molecular functions and biological compartments significantly impacted by a diet containing NPs. Due to technical limitations in identifying routes in European sea bass, annotated genes were translated to orthologues of the model zebrafish, with a total of 16,825 genes identified, to proceed with further analysis. For the enrichment analysis, up- (Tables S2) and down-regulated (Tables S3) DEGs were analysed separate. The results demonstrated that most of the up-regulated genes selected are related to the immune response and morphogenesis biological processes (Fig. 6A; Tables S2) whilst the principal molecular functions related to the endopeptidase activity were also im-

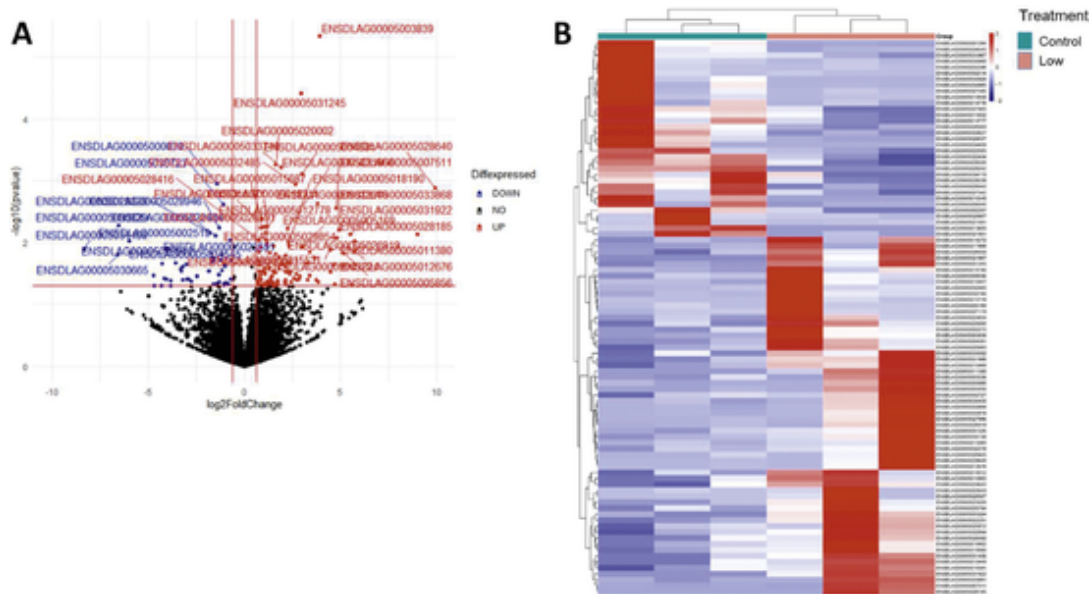


Fig. 3. A. Volcano plot of differentially expressed genes (DEGs) up-regulated genes: red; down-regulated genes: blue; and unaltered genes: black. B. Heatmap based on the significant DEGs in the intestine from European sea bass fed with 0.25 g NPs / kg feed (Low) for 21 days compared to Control fish.

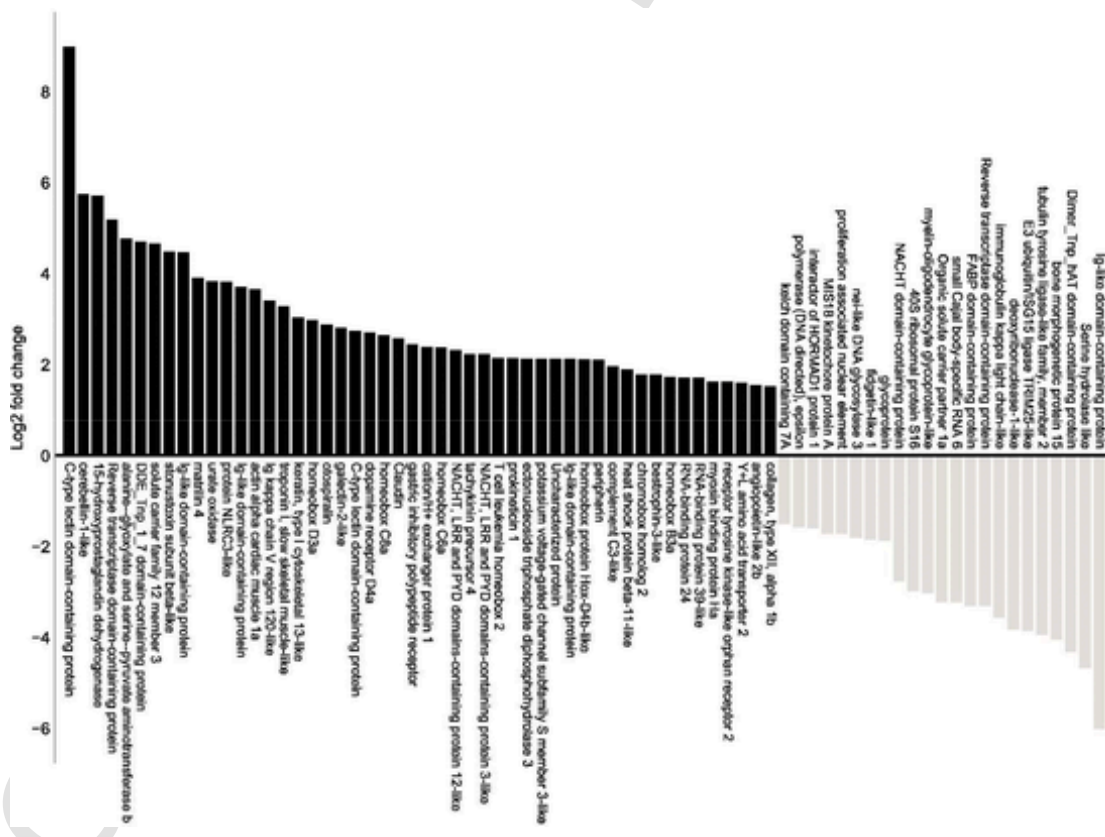


Fig. 4. Identified and annotated DEGs in the intestine of European sea bass fed with 0.25 g NPs / kg feed (Low) for 21 days.

ected by NPs exposure. A GO enrichment map on the biological processes allowed us to organize the enriched terms obtained with edges and connect the genes according to their biological functions (Fig. 7A). We observed a significant impact of NPs on complement activation and the immune response, which was also reflected by the *revigo* analysis, showing that the strongest cluster corresponded to that including complement activation and cellular responses to stimulus (Fig. 7A). The second stronger cluster was composed of biological processes re-

lated to cellular maintenance and membrane transport. Another two clusters were observed grouping morphogenesis pathways as well as those related to cell differentiation and signaling. Conversely, we observed a down-regulation expression upon NPs diet feeding in genes involved in the SMAD (suppressor of mothers against decapentaplegic) and BMP (bone morphogenetic proteins) protein signaling, critically important for regulating cell development, growth and morphogenesis (Fig. 6B; Tables S3). In addition, the down-regulation of enzymes par-

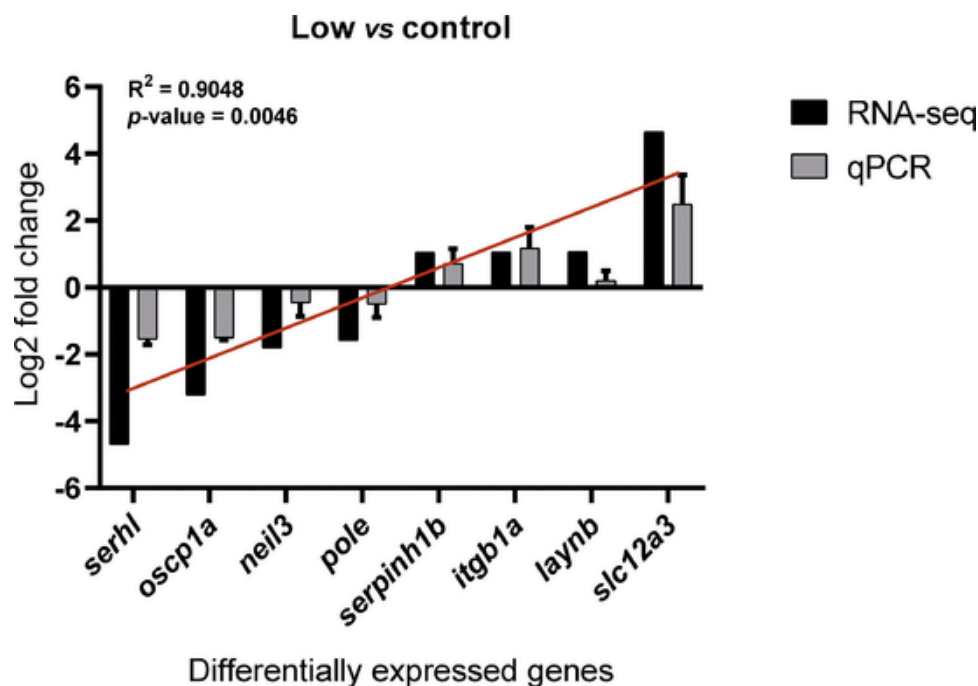


Fig. 5. Validation of the RNA-seq data by qPCR. Bar plot showing the fold change in the expression of selected genes in the intestine of European sea bass fed NPs (0.25 g NPs / kg feed) for 21 days compared to controls. qPCR data are presented as the mean \pm SEM ($n = 5$ replicates). The correlation between RNA-seq and qPCR samples was performed using a two-tail spearman correlation.

ticipating in the replication and repair of DNA was also observed (Figs. 6B, 7B), including the DNA polymerase epsilon catalytic subunit (*pole*) and the nei-like DNA glycosylase 3 (*nei13*).

Finally, we used the DEGs to perform a KEGG analysis to obtain a systematic analysis of gene functions, linking genomic information with higher order functional information. We could only identify 5 pathways corresponding to significantly up-regulated genes (phagosome, neuroactive ligand-receptor interaction, herpes simplex virus 1 infection, caffeine metabolism and purine metabolism) and 4 to down-regulated genes (base excision repair, DNA replication, Nucleotide excision repair and N-glycan biosynthesis) (Fig. 8).

4. Discussion

In the present study, we studied the impact that dietary NPs might have on fish's intestine for two main reasons: it is one of the key organs, together with the gills and kidney, involved in the first line of immunity and osmoregulation in fish, but mainly because the dietary exposure is one of the main sources of NPs entry into fish (Alomar et al., 2021), playing a major role for bioaccumulation through the trophic chain in fish. As result, we failed to observe significant changes in the enzymatic activities measured in the liver samples after 21 days of NPs dietary intake. Nevertheless, we observed a decrease in the seric bactericidal activity, which implies an impact on fish health. The RNA-seq analysis performed on the fish intestine allowed us to select a few DEGs between treatments. The functional analysis performed on this gene selection allowed us to identify the biological and metabolic pathways associated to these genes. We observed that the main pathways were related to immune response, cellular maintenance, signaling, differentiation and repair as well as pathways involved in protein function, morphogenesis and reproduction. Our results reveal that the dietary ingestion of NPs may impact some functional pathways and may lead to problems in fish growth having an impact on fish aquaculture.

4.1. NPs dietary exposure did not impact biochemical biomarkers in the liver but decreased serum bactericidal activity

Whatever the origin and route, the production of oxidative stress upon NPs exposure has been evidenced in fish (Bhagat et al., 2020; Brandts et al., 2022; Sarasamma et al., 2020). In vertebrates, the liver is a central metabolic organ that responds to adverse environmental conditions. For this, it regulates oxidative stress by the production of antioxidant enzymes such as SOD, CAT, GR and GPx, playing an important role in protecting against reactive oxygen species (ROS) damage (Regoli and Giuliani, 2014). As already demonstrated, NPs can the intestine barrier (Vagner et al., 2022), migrate to the bloodstream, pass through the hepatic portal vein and accumulate in the liver (Pitt et al., 2018). Therefore, NPs might affect the liver functions due to their presence and accumulation inside the organ or as a consequence of the injuries in other tissues, probably through blood communications. Although it is difficult to compare among studies due to different NPs properties, administration routes and dosages, fish species, etc., our data show a tendency to decrease liver GR and GPx activities, suggesting a potential accumulation of ROS, leading to liver damage (Lai et al., 2021) if the exposure is higher or chronic. Our results are consistent with previous studies where NPs exposure *in vivo* promotes a decrease of antioxidant enzymes in zebrafish liver (Cheng et al., 2022). Therefore, we may suggest that the exposure to NPs (21 days of feeding with NPs) promoted an incipient accumulation of ROS.

Among the important functions of the liver is its implication in immunity. The liver is an important source of many humoral immune factors including the complement system components, antimicrobial enzymes (lysozyme) and peptides (AMPs), proteases, or antiproteases amongst others, acting either locally in the liver or systematically by their release to the bloodstream. Regarding this, we studied the capacity of fish serum to inhibit bacterial growth, whatever the molecules and mechanism it uses, since this is a very easy and practical approximation. In a previous study, we have shown that dietary MPs can produce a little decrease in the mucus bactericidal activity, which was related to significant liver histopathological alterations, oxidative stress

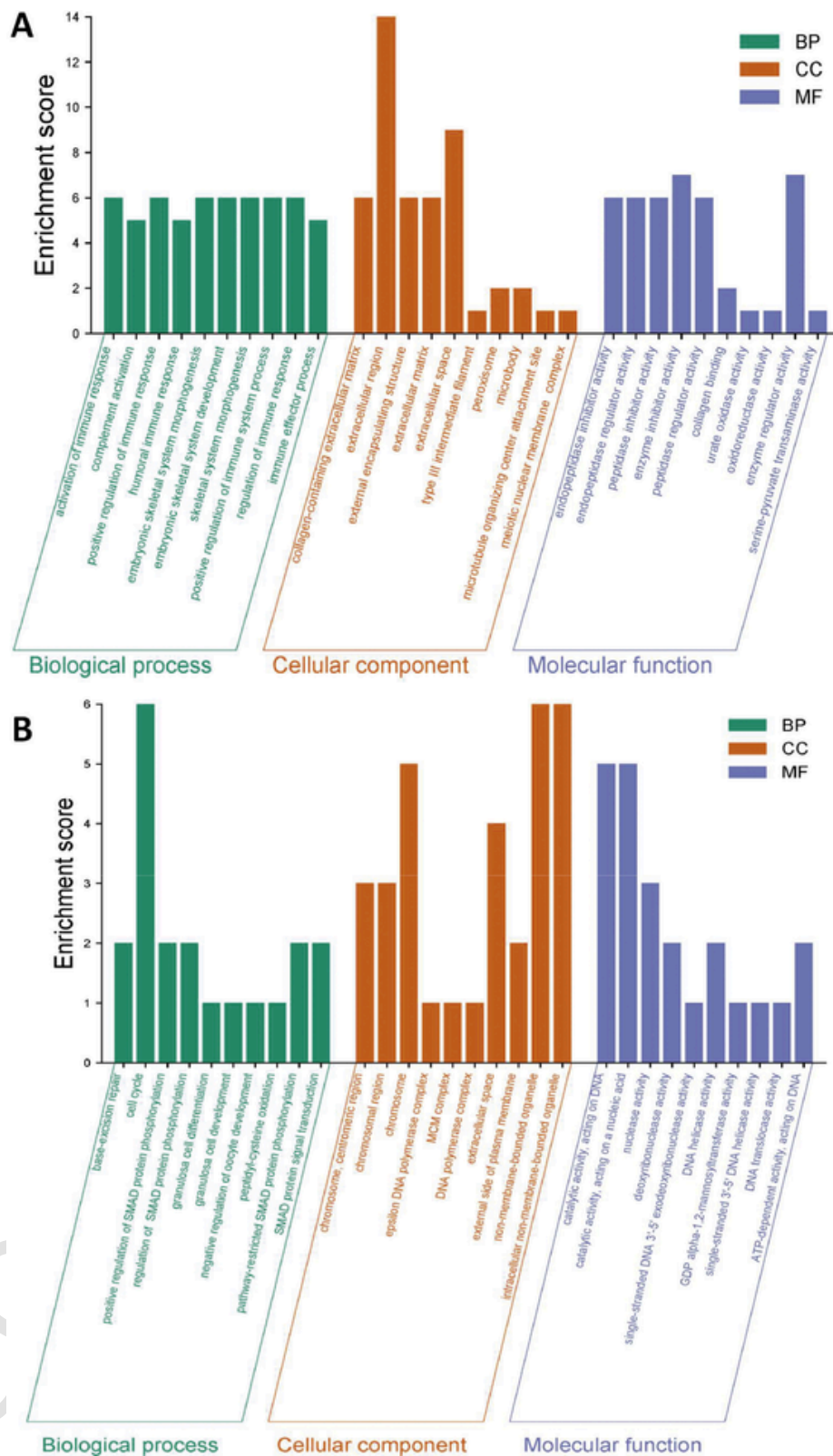


Fig. 6. Bar plots showing the 10 most significantly enriched GO terms based on the number of up-regulated (A) and down-regulated (B) DEGs impacted in the intestine of European sea bass fed NPs (0.25 g NPs / kg feed) for 21 days. Significant values are considered when $p < 0.05$. BP, biological process; CC, cellular component; and MF, molecular function.

and inflammation (Espinosa-Ruiz et al., 2023). Similarly, our results show decreased serum bactericidal activity upon dietary intake of NPs, which might be due to incipient oxidative stress and damage in the liver as previously reported for MPs. Therefore, these data suggest that fish

exposed to NPs would be more susceptible to infections, a fact that has been already documented (González-Fernández and Cuesta, 2022; Wang et al., 2021), which is a relevant problem for both wild and cultured species.

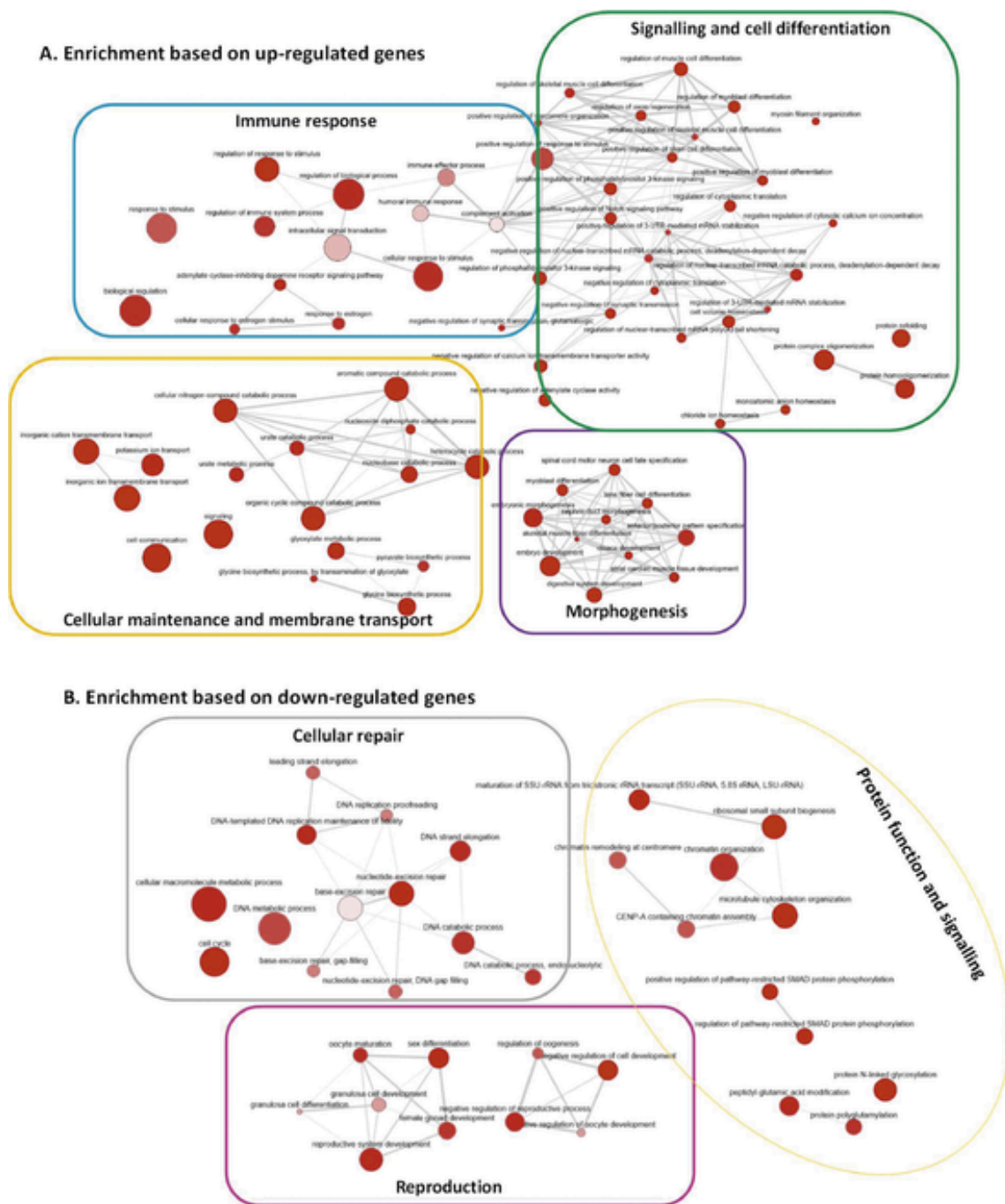


Fig. 7. Results from the Revigo analysis providing a cluster analysis on GO terms obtained by enrichment analysis for biological process GOSlim categories. The color of the bubble corresponds to the *p*-value provided (greater intensity color the more significant the *p*-value) and the size of the bubble corresponds to the LogSize value for the GO Term.

4.2. NPs dietary exposure produce little changes in intestine transcriptome of European sea bass

RNA-seq techniques have emerged as very useful tools to identify important biological pathways impacted by pollutants. However, they still have limitations when working with non-model species such as European sea bass. In the present study, we encountered limitations related to the process of gene annotation and the identification of biological pathways associated with these genes due to incomplete annotation and analysis of sea bass genome databases. As occurred in other studies, we had to search for orthologues in model species such as zebrafish to obtain a sufficient number of genes for bioinformatic analysis. Due to

the low NPs concentration used and the use of individual (not pooled) mRNA samples, we failed to find a high percentage of differentially expressed genes, reaching less than 0.5 % of the total genes identified. We decided to perform a functional analysis on some DEG genes to identify the biological pathways involved. We consider that these results provide useful information about the biological routes that may be impacted by dietary NPs.

European sea bass fed with NPs showed up-regulated genes related to immune function, morphogenesis and organ development in the intestine. The impact on these biological pathways have been particularly studied in zebrafish (Teng et al., 2022) due to this specie has a well described rapid growth and development. Depending on their functional-

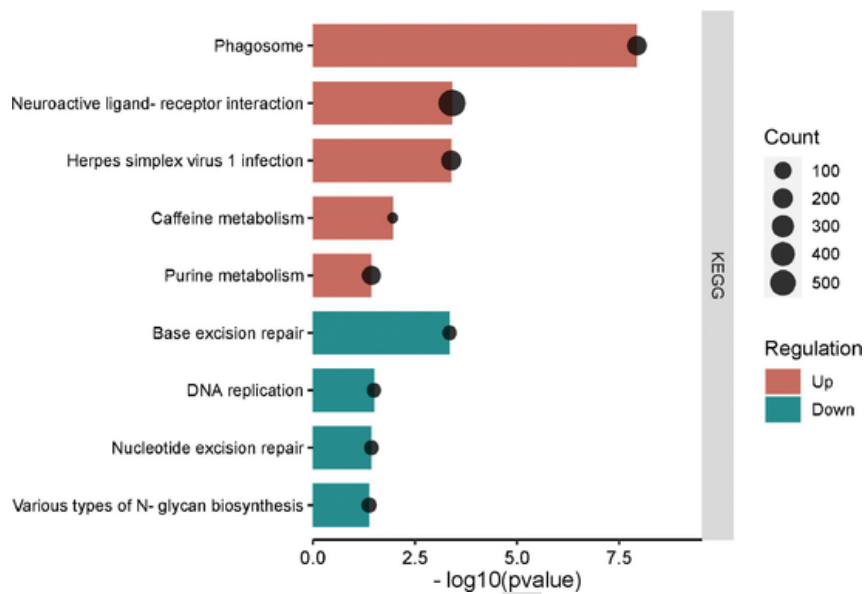


Fig. 8. Bar plots showing the KEGG enrichment analysis based on the number of differentially expressed genes (DEGs) in the intestine of European sea bass fed NPs (0.25 g NPs / kg feed) for 21 days.

ization and charge, PS-NPs have evidenced different effects at molecular and cellular levels. In zebrafish, previous studies revealed that positive PS-NH₂ NPs induced developmental toxicity (decreased spontaneous movement, heartbeat, hatching rate, and length) and cell apoptosis in the brain while negative PS-COOH NPs impacted metabolism inducing the abnormal behavior of zebrafish larvae (Teng et al., 2022). One of the biological processes suggested to be modified by NPs exposure was immunity, probably due to the importance of the intestine as a first line of defense by the gut-associated lymphoid tissue (GALT), where an increase in the humoral immune response and the complement activation was highlighted. In particular, some genes involved in the complement activation of the immune response such as *complement c3-like* and *Ig-like domain-containing proteins* were evidenced. The complement system participates in the humoral and cellular responses against pathogens and inflammatory processes. In particular, the complement component 3 (*c3*) is an essential protein for the activation of the complement system and responsible for enhancing the ability of phagocytic cells to clear antigens. Changes in the *c3* expression may suggest that NPs have the ability to initiate the complement pathway and promote inflammation. Previous studies have revealed an up-regulation of *c3* genes in fish liver upon NPs ingestion (Elizalde-Velázquez et al., 2020) supporting our results. To further investigate the routes impacted by NPs, we performed a KEGG analysis. It revealed that a diet containing NPs may alter immunological processes involved in the phagosome, neuroactive ligand-receptor interaction, and purine metabolism. Activation of phagosomes is a logical process occurring after NPs internalization by micropinocytosis by enterocytes or to clear NPs-containing cells or even single or aggregated NPs by phagocytic cells, accompanied by low but maintained ROS production (Aguilar-Guzmán et al., 2022). We observed an up-regulation of genes such as *hsp11beta* and *NLRC3-like*. These genes participate as molecular chaperones, probably maintaining denatured proteins in a folding-competent state, and as inhibitors of inflammation, which supports our hypothesis. In addition, our data is in accordance with results obtained in tilapia exposed to NPs, where the alteration of genes associated with CAMs and interactions between neuroactive ligands and receptors was documented (Pang et al., 2021; Wei et al., 2020). Neuroactive ligands influence neuronal function by binding to intracellular receptors, which can bind transcription factors and regulate gene expressions. Disruption of the genes involved in neuroactive ligand-receptor interactions have been observed also in zebrafish exposed to silica NPs having an impact

on memory function (Wei et al., 2020). Our results showed that the dopamine receptor d4 was up-regulated by NPs. Dopaminergic receptors can modulate extracellular matrix remodeling, resulting in malformation of the neural extracellular matrix (Wei et al., 2020). Other studies carried out with zebrafish larvae have also evidenced that concentrations of 1 mg L⁻¹ of NPs impacted the morphology of the nervous system, promoting neuromuscular diseases (Pedersen et al., 2020). The purine metabolism is important in the regulation of the innate immune response, to stimulate resolution of inflammation and self-limiting the immune cell activity (Antonioni et al., 2014). As herein, this pathway was previously enhanced by NPs exposure in seabream (Brandts et al., 2021) and zebrafish (Cheng et al., 2022). The up-regulation of genes involved in purine metabolism might be a consequence of the ROS attack and this would be supported by the liver biochemical markers that suggest an incipient increase of ROS. By contrast, it should be noted that, in both analyses, GO and KEGG enrichments, we observed a down-regulation of genes such as *nei-like DNA glycosylase 3 (nei3)* and *polymerase DNA epsilon (pole)*, both involved in DNA repair pathways. The fact that NPs can impair the DNA repair machinery has been already highlighted in grass carp (*Ctenopharyngodon idella*) exposed to water NPs, which resulted in DNA damage and micronuclei formation (Guimarães et al., 2021). Similar results were observed in goldfish (*Carassius auratus*) and zebrafish embryos exposed to NPs (Brandts et al., 2022; Sökmen et al., 2020). At the gene level, studies in zebrafish have observed alteration of genes implicated in DNA repair, caused by the increase of ROS accumulation in cells, promoting apoptosis and inflammation (Martin-Folgar et al., 2023). We have previously observed an increase in genes related to oxidative stress, apoptosis and inflammation upon *in vitro* and *in vivo* exposure to NPs (González-Fernández et al., 2021; González-Fernández and Cuesta, 2022) supporting these results. Taken together, our results indicate that NPs ingestion may cause functional and immunological changes in European sea bass and that further studies are needed to identify its interaction with other factors.

5. Conclusions

Our results evidenced that ingestion of NPs tends to reduce the antioxidant status, which might drive ROS accumulation, reduces the bactericidal activity as well as induce transcriptomic changes in fish intestine. The main routes affected by NPs exposure were those related to fish immunity, morphogenesis, organ development, membrane recep-

tors and DNA repair. The specific routes impacted need to be studied in detail to unravel their relevance since they are extremely important for fish development and growth and might have an impact at long term. Thus, the presence of NPs in the environment possesses and additional risk to fish aquaculture, which is already, affected by physical stress and natural pathogen outbreaks.

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CRedit authorship contribution statement

Alberto Cuesta : Writing – review & editing, Supervision, Project administration, Funding acquisition. **Cristóbal Espinosa** : Writing – review & editing. **María A. Esteban** : Writing – review & editing, Funding acquisition. **Carmen González-Fernández** : Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

The raw read sequences were deposited in the Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra>) under BioProject accession number PRJNA945811.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.aquatox.2023.106712](https://doi.org/10.1016/j.aquatox.2023.106712).

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