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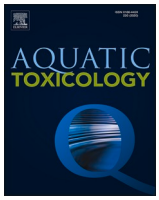

# The effect of post-biotics-enriched diet on the antioxidant capacity in *Mytilus galloprovincialis* exposed to $Cu_2O$

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ABSTRACT

This study explores the effects of copper (I) oxide ($Cu_2O$) nanoparticle exposure on *Mytilus galloprovincialis* gills and the modulation of oxidative stress by a diet enriched with a postbiotic derived from kefir microorganisms. Postbiotics, which include components such as proteins, lipopolysaccharides, and short-chain fatty acids, have shown biological benefits, including enhancing gut health and modulating immunity. They are known for their antioxidant properties, which can counteract oxidative stress by scavenging free radicals, making them useful in both human and animal health. Mussels were exposed to 2 mg $L^{-1}$ of $Cu_2O$ nanoparticles for three days. Nanoparticle characterization showed rapid agglomeration in seawater, with hydrodynamic diameters increasing from 76 ± 11 nm to 529 ± 75 nm within 24–48 h. Biochemical analyses revealed a significant increase in catalase (CAT) activity in mussels exposed to $Cu_2O$ nanoparticles ($G1^+$ group; $p < 0.0001$), which was not observed in mussels exposed to $Cu_2O$ nanoparticles after feeding with postbiotics ($G2^+$ group); indicating the postbiotic's protective role. Glutathione reductase (GR) activity was significantly reduced in the $G1^+$ group ($p < 0.01$), but maintained at control levels in the $G2^+$ group. While glutathione S-transferase (GST) activity decreased in both exposed groups, the reduction was notably less pronounced in the $G2^+$ group compared to $G1^+$, suggesting mitigation of the pollutant's adverse effects on the GST-mediated detoxification pathway. A strong negative correlation was observed between CAT and GST (and to a lesser extent GR), indicating that $Cu_2O$ exposure at the tested concentrations can overburden glutathione defense pathways despite robust CAT activity. These results highlight the potential of postbiotics from kefir microorganisms as effective feed additives in mussel aquaculture to enhance resilience against environmental pollutants and mitigate oxidative stress.

## 1. Introduction

Human activities continuously introduce various pollutants into marine environments, such as use of pesticides, metals, pharmaceuticals and other chemicals that reach wastewater streams, rivers and estuaries and eventually coastal waters, hence presenting a threat to biota. Indeed, pollution in the marine environment is believed to have affected around 600 species, such as marine mammals, fish, seabirds, turtles, and invertebrates, including plankton and algae (Mandil et al., 2020).

Heavy metals (HMs) that may be present in air, water and soil are significant pollutants due to their non-biodegradable and bio-accumulative characteristics (Chen et al., 2019). Their accumulation in exposed organisms can vary at different trophic levels, leading to numerous toxic effects such as oxidative stress, inflammation, histological damage, and genetic mutations, along with alterations in gut microbiota (Amoatey and Baawain, 2019) . Toxic effects arise when HMs are taken up by the body through permeable membranes like gills, interfering with metabolic processes by replacing the native metal ions in metabolic enzymes, disrupting protein structures, and causing DNA cross-links that can disrupt the cell cycle (Pramita Garai, 2021). Despite being known for their potential to accumulate environmental toxins, seafood demand is rising, and consuming aquatic organisms that contain HMs can result in similar adverse effects in humans, including neuronal, hepatic, renal and reproductive issues, as well as cardiovascular and

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peripheral vascular diseases (Engwa et al., 2019). Since HM transfer through the food chain is inevitable, effective strategies for mitigating the effects of HMs and eventually supporting their removal from aquatic organisms before human consumption are crucial.

Copper (Cu), a naturally occurring metal that serves as a vital co-factor in the structure of enzymes and proteins in living organisms (Liess et al., 2020), is also one of the most common pollutants. Various forms of Cu, such as sulfate, hydroxide, and carbonate, are frequently used in agriculture as pesticides, fungicides, herbicides and bactericides. Sources of anthropogenic Cu in marine environments include agricultural runoff, sewage, industrial discharges, mining waste and anti-fouling paints, and is often found in high concentrations in seawater and sediments of coastal and estuarine systems. Aquatic organisms are particularly susceptible to Cu contamination, as soft tissues, such as skin and gills, can easily absorb dissolved copper. Cu mimics essential ions like sodium (Na) and calcium (Ca) to cross epithelial membranes, after which it undergoes redox cycling, generating ROS that cause DNA damage and lipid peroxidation (Grosell, 2011). Moreover, excess Cu can lead to oxidative stress, acid-base imbalances, enzyme inhibition, immune suppression and reduced growth in marine organisms (Cao et al., 2019). Copper in ionic form has been previously used in ship hull antifouling paints, however the release of copper ions from those coatings is relatively rapid which may give rise to unintended non-target organism toxicity as well as requiring the ship to be more regularly re-painted. A recent advance on this is to encapsulate nanoparticulate copper (Cu and CuO) that releases copper ions more slowly, which has positive implications in terms of duration of the antifouling activity of the coating. However, relatively little attention has been given to $Cu_2O$ nanoparticles, even though these latter nanoparticles have increasingly become an important constituent of marine antifouling paints, for example in Sea Hawk's Cukote Biocide {Adeleye, 2016 #43}. Recent studies have confirmed that $Cu_2O$ present in aquatic environments eventually accumulates in tissues and generates oxidative stress which, combined with an inadequate antioxidant defense, leads to damage to biomolecular structures including lipids, proteins, and DNA and histological changes (Haverroth et al., 2015). Marine organisms require a balance between oxidative stress and antioxidant defenses. They serve as crucial bioindicators in pollution studies, reflecting the accumulation of pollutants and associated health impacts in aquatic ecosystems. Depending on the concentration and duration of exposure, Cu can adversely affect fish reproduction, growth, development, and survival, ultimately influencing population dynamics and ecosystem health (Birnie-Gauvin et al., 2017).

According to the latest SOFIA report (FAO, 2016), global food production must increase significantly to support a projected population of 9.7 billion by 2050. Marine products are vital to the human diet, as they constitute a primary source of animal protein, with per capita consumption doubling since 1960.

Currently, over half of marine products come from aquaculture, a figure that is expected to exceed 65 % by 2030 due to overfishing in traditional fisheries. The aquaculture sector is crucial for meeting rising food demand, but intensifying fish farming practices brings challenges, including disease outbreaks and environmental issues (Boyd et al., 2020). This necessitates effective strategies to enhance health and productivity (Zorriehzahra et al., 2016).

Bivalves, particularly oysters, mussels, clams and scallops, are vital to aquaculture, with global production approaching 14 million tonnes. Bivalves are frequently used in toxicological studies due to their sessile nature, filter-feeding behaviour, and capacity to accumulate both soluble and insoluble pollutants from the water column (Impellizzeri et al., 2023). Accumulated toxicants can distribute throughout their tissues, causing harmful effects (Aguirre-Rubí et al., 2019; Auguste et al., 2021; De Felice and Parolini, 2020), including damage to hemocytes which play a crucial role in homeostasis, supporting digestion, nutrient transport, shell repair and immune responses (Curpan et al., 2022; de la Ballina et al., 2022; Donaghy et al., 2012; Song et al., 2010). Mussels, such as the Mediterranean mussel *Mytilus galloprovincialis*, are important in coastal food webs and are commonly used in aquaculture. As sessile filter feeders, they produce strong byssal threads to attach to hard substrates, and their feeding behavior leads to the bioaccumulation of environmental contaminants, making them valuable for monitoring water quality and providing location-specific insights (Melwani et al., 2011). Indeed, their efficient filtering ability (3–9 L/h) allows them to rapidly accumulate various pollutants, making them valuable biological indicators for monitoring anthropogenic pollution trends in coastal waters.

In the past two decades, there has been significant growth in the quantity and quality of clinical studies examining the health benefits of probiotics. These beneficial microorganisms offer a promising solution for various health issues. Research indicates that probiotic supplementation can enhance fish growth, optimize feed efficiency, boost immune responses, and improve stress resistance (Abdul Hakim et al., 2023).

Probiotics and prebiotics are widely used in fish and shrimp aquaculture to improve growth performance, enhance immunity, produce pathogen-inhibiting compounds, suppress virulence genes and reduce pro-inflammatory cytokines like interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Kumar et al., 2016). In addition, probiotics are used in aquaculture to reduce reliance on antibiotics (Akhter et al., 2015), and have garnered significant attention due to their greater effectiveness in mitigating the negative impact of heavy metals (HMs). Their application in aquaculture as feed and water additives for pathogen inhibition and disease prevention has also been documented (Butt et al., 2021; Kumar et al., 2016; Sayes et al., 2017). Little is known about post-biotic substances. Postbiotics are based on the observation that microbiota's beneficial effects are mediated by the release of different metabolites. The exact definition of it is still being debated. The term postbiotics is used by Tsilingiri at al. to describe any substance that microorganisms release or produce that has a beneficial effect on the host, either directly or indirectly. Studies have demonstrated that postbiotics can directly scavenge free radicals or indirectly modulate endogenous antioxidant pathways such as the Nrf2-Keap1 signaling axis, promoting the expression of detoxifying enzymes like GST and SOD. For instance, postbiotics derived from *Lactobacillus* spp. have shown protective effects against oxidative damage in fish and invertebrate models, improving redox homeostasis under chemical or thermal stress {Wang, 2023 #41}{Yu, 2023 #42} However, the effects of HMs and postbiotics on mussels as regard the possibility to develop a protective strategy against the toxic effects of HMs is still underexplored (Tsilingiri and Rescigno, 2013; Żółkiewicz et al., 2020).

Previous studies carried out in our laboratory show the probiotic capacities of three microorganisms isolated from a homemade Kefir, a yeast *Pichia kudriavzevii* (Y1) and two bacteria *Lactococcus lactis* subsp. *hordniae* (LAB1) and *Lactococcus lactis* subsp. *lactis* (LAB2). We also tested the postbiotic capacity of their cell-free supernatant (CFS) against both planktonic cells and pathogenic microorganisms biofilms and its cytotoxicity on Caco-2 cells (Maione et al., 2024). As the protective effect of postbiotics in mussels exposed to HMs is still unexplored, we hypothesized that dietary postbiotics from our consortium could potentially modulate antioxidant activity in mussels exposed to $Cu_2O$ nanoparticles and mitigate the negative effects of this pollutant.

Therefore, our goal in this research was to determine whether a diet enriched with postbiotic substances could protect *M. galloprovincialis* from negative impacts on growth performance and on oxidative stress based on assessment of a range of antioxidant markers and biomolecular markers in gills of bivalves exposed to $Cu_2O$. To the best of our knowledge, there are currently few available studies that have investigated biomarker responses to these contaminants in the mussel species *M. galloprovincialis* fed by a diet enriched with a postbiotics.

## 2. Materials and methods

### 2.1. Reagents

EDTA, Bradford reagent, hydrogen peroxide, trypan blue, NADPH, ethanol, salts for sodium and potassium phosphate buffers were purchased from Sigma Aldrich, St. Louis, MO, USA.

### 2.2. Nanoparticle characterisation

In this study $Cu_2O$ nanoparticles, purchased from Nanografi, Ankara, Türkiye, were used without further modification. Particle size distributions for nanoparticles in ultrapure water and artificial seawater were determined by dynamic light scattering (DLS) using a Malvern Panalytical Zetasizer Nano ZS (Malvern, UK) instrument using a 532 nm green laser with scattered light measured at 173° Samples were measured 10 times at 25 °C and the hydrodynamic diameters were calculated as the average of the peak maximum of the size volume distribution. Data were processed on proprietary Zetasizer software.

The size and shape of the nanoparticles, as well as the influence of salinity on aggregation, were investigated using transmission electron microscopy (TEM). A drop of nanoparticle dispersion in artificial seawater (salinity S·38), previously dispersed using a sonicator, was placed on a carbon-coated copper grid, air-dried and imaged using a Tecnai F20 instrument at an acceleration voltage of 200 kV. The elemental composition of the nanoparticles was confirmed as comprising of copper using a Sapphire Si (Li) detector for energy-dispersive X-ray spectroscopy (EDX).

The kinetics of $Cu_2O$ nanoparticle dissolution in ultrapure water and in filtered seawater was determined by preparing nanoparticle dispersions (1 g $L^{-1}$) in the respective media after which an aliquot was withdrawn every 12 h (over a period of 96 h) and was filtered through an Amicon Ultra 3 kDa MWCO centrifugal filter to remove particulate matter (Merck KGaA, Darmstadt, Germany). The pH of the filtrate was reduced to pH 5 to promote the oxidation of any cuprous ions, and the metal ion concentration was then measured using a cupric ion selective electrode (Mettler Toledo, Columbus, OH, USA).

### 2.3. Microorganisms and growth conditions

*P. kudriavzevii* (Y1), *L. lactis* subsp. *Hordniae* (LAB1) and *L. lactis* subsp. *lactis* (LAB2) isolated from a homemade kefir and previously molecularly characterized (Maione et al., 2024) were used. For the cultures, yeast extract peptone dextrose broth (YPD, Oxoid Ltd.) (1 % yeast extract (Sigma Aldrich, Co., St. Louis, MO, USA), 2 % Bacto Peptone (Gibco,Life Technologies Corporation, Detroit, MI, USA), 2 % glucose (SERVA Electrophoresis GmbH, Heidelberg, Germany) for Y1 and De Man, Rogosa, and Sharpe broth (MRS), (Oxoid Ltd., Basingstoke, UK) for LAB1 and LAB 2, were used at 37 °C with shaking overnight.

### 2.4. Cell-free supernatant (CFS) production

The preparation of the cell-free supernatant was carried out as described elsewhere (Maione et al., 2024). Briefly, after growing a co-culture of the three microorganisms (*ratio* = 1:1:1, *v/v/v*) for 48 h at 37 °C under agitation, the supernatant was obtained by centrifugation at 4000 g for 30 min at 4 °C. After removal of all cells, the solution was then sterilized using a 0.22 μm pore filter. To ensure the sterility of CFS, 100 μL were plated in TSA (Scharlab S.L., Spain) and grown to 37 °C for 24 h. The solution was then lyophilized and stored at −80 °C.

### 2.5. Total antioxidant activity of postbiotics

CFS postbiotics produced from LAB1, LAB2 and Y1 were quantified for total antioxidant activity of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay according to previous studies (Brand-Williams et al., 1995; *Re* et al., 1999). To evaluate DPPH free radical scavenging activity, 900 μL of DPPH methanolic solution (0.005 % (w/v)) was mixed with 100 μL of CFS and incubated in darkness at room temperature for 30 min. The determination of DPPH radical scavenging ability was detected at 517 nm absorbance using a spectrophotometer (Varioskan LUX Multimode Microplate Reader Thermofisher Scientific USA**)**. The assay was conducted in triplicates. Ascorbic acid was used as a reference antioxidant. The scavenging activity of DPPH was calculated as below:

DPPH radical scavenging activity ( %) = [(Ac − As)/Ac × 100] and Ac is the absorbance of the control and As is the absorbance of the sample.

The ABTS+ radical scavenging assay was conducted on postbiotics preparation, at first, the ABTS reagent by reacting the reaction mixture 7 mM ABTS solution and 140 mM potassium persulphate, incubating in the dark at room temperature for 12 h. The ABTS+ solution was diluted to reach absorbance of 0.70 ± 0.02 at 732 nm and the diluted sample was mixed with aliquots of CFS in dark for 10 min at room temperature. The absorbance was measured at 734 nm with Varioskan LUX Multimode Microplate. Ascorbic acid was used as a reference antioxidant. The scavenging activity of ABTS+ was calculated as below:

ABTS+ radical scavenging activity ( %) = [(Ac − As)/Ac × 100] and Ac is the absorbance of the control and As is the absorbance of the sample

### 2.6. M. galloprovincialis acquisition and acclimation

*M. galloprovincialis* (ca. 6–7 cm), were obtained from a shellfish farm in Lim Bay in the northern Adriatic Sea, Croatia. A total number of 180 mussels were separated from a pergola and from each other by cutting the byssus threads, scraped clean of fouling organisms and subsequently maintained in open-circuit seawater tanks at a temperature of 18 °C and salinity of S 36.7 for three days before use.

### 2.7. Experimental design and setup

Following the acclimatation period, mussels were divided into two experimental groups G1 and G2. For a period of 15 days, the G1 group was fed daily only with algae (*Dunaliella salina*) at a concentration of about 1 × $10^6$ cells, while the G2 group daily received a diet of algae supplemented with the postbiotic substance (CFS) at a concentration of 25 mg $L^{-1}$. After the 15-day feeding period, the experiment progressed to evaluate the effect of $Cu_2O$ nanoparticles (2 mg-Cu $L^{-1}$) on mussels fed with algae compared to those fed with algae supplemented with CFS. To achieve this, each experimental group (G1 and G2) was divided into two subgroups, resulting in a total of four experimental aquaria: $G1^-$ (not exposed to $Cu_2O$), $G1^+$ (exposed to $Cu_2O$), $G2^-$ (not exposed to $Cu_2O$), and $G2^+$ (exposed to $Cu_2O$), where groups $G1^-$ and $G2^-$ served as controls. Additionally, a fifth aquarium was set up as a positive control in which copper sulfate ($CuSO_4$) was added to a final concentration of 2 mg-Cu $L^{-1}$, and the mussels were not fed during the exposure period. Each aquarium contained 36 mussels, maintained at a volume of 0.75 L per individual, for a total aquarium volume of 27 L. Throughout the entire experimental period, the water in each aquarium was aerated and replaced daily to ensure optimal water quality and maintain experimental conditions. Survival of *M. galloprovincialis,* temperature, pH and salinity of water were recorded each day, and the latter three parameters did not change over the duration of the experiment.

After three days of exposure, the condition index (CI) was determined, and the gills were harvested and kept at −80 °C until chemical, biochemical and molecular analyses were performed. Chemical analysis of the gills was carried out by digesting tissue in 65 % nitric acid and determining copper concentrations by atomic absorption spectroscopy (AAS) using a Perkin Elmer AAnalyst 200. Standards of 2.5, 4 and 5 mg $L^{-1}$ were prepared and used to generate the calibration curve. The measurements were carried out at the characteristic absorption maximum

for copper at 324.8 nm. For biochemical analysis, samples were first homogenized in ice-cold potassium phosphate-EDTA homogenization buffer in a potter-elvehjem tissue grinder by raising and lowering the Teflon pestle 10 times. Following homogenization, the samples were centrifuged at 10,000 g and + 4 °C for 30 min to obtain the supernatants.

### 2.8. Biometric parameters and condition index (CI)

The measurement of mussel total weight (TW)without water and wet meat weight (WMW) was carried out using a digital scales with 0.01 g precision. The measurement of shell length, width, and height was carried out using digital calipers with 0.1 mm accuracy.

The condition index (CI) was assessed in mussels to determine their physiological status in the different groups and was calculated by determining the ratio between the soft tissue and the total mass of the five mussels taken from each aquarium. The CI was determined by the following formula (Pavičić-Hamer et al., 2022)

$$CI\ (\%) = WMW\ (g) \times 100/TW\ (g)$$

### 2.9. Total haemocyte count

Hemolymph was taken from the anterior adductor muscle using a 1 mL syringe with a G2325 mm needle. All samples were stored in ice to avoid the clustering of haemocytes. The number of cells present in the suspensions was determined by light microscopy. Haemocytes were counted in triplicate for each mussel and the results were expressed in cell $mL^{-1}$. To determine the viability of the haemocytes, aliquots of haemolymph from each mussel were stained with trypan blue. The unstained cells represented the viable cells in the sample. Cell viability was calculated as following formula:

$$\text{Cell Viability}\ (\%) = \text{viable haemocytes} \times 100/\ \text{total haemocytes}.$$

### 2.10. Biochemical analyses

A biomarker battery was used to evaluate tissue-specific responses to the $Cu_2O$ nanoparticles, with the goal of determining antioxidant capacity (catalase (CAT)) and as well as activation of the glutathione detoxification pathway (glutathione reductase (GR) and glutathione S-transferase (GST)). Each biomarker analysis was carried out on thirty individual mussels for each treatment group and absorbance measurements were read by using a UV/Vis spectrophotometer (Shimadzu UV1800). The Bradford method (1976) was used to determine total protein concentrations in gill tissue homogenates (mg protein $g^{-1}$ tissue) by mixing 5 µL homogenate with 250 µL Bradford Coomassie reagent and measuring absorbance at 595 nm in a Tecan Infinite 200 Pro (Männedorf, Switzerland) microplate reader. Bovine serum albumin (BSA) used as a standard, and enzyme activity values in gill homogenates were finally expressed per mg of protein..

### 2.11. Catalase activity (CAT)

Catalase activity was determined according to a modified protocol described by Aebi. (Aebi, 1984). After adding potassium phosphate reaction buffer, sample and hydrogen peroxide solution to a quartz cuvette, they were mixed quickly and measurement of absorbance was started immediately. The decrease in absorbance caused by hydrogen peroxide's catalytic breakdown was measured for 30 s at a wavelength of 240 nm. The enzyme activity was is calculated by the formula:

$$A_{CAT} = (\Delta A/\text{time}) \cdot V_{\text{total vol}} \cdot 1000 / \varepsilon \cdot d \cdot V_{\text{sample}}$$

Catalase activity was finally calculated as specific activity in nmol $min^{-1}$ $mg^{-1}_{prot.}$.

### 2.12. Activity of glutathione reductase (GR)

GR activity was measured using the Paglia and Valentine method (Paglia and Valentine, 1967). The activity level of GR was determined by measuring the GR-catalyzed NADPH-dependent reduction of GSSG to two molecules of GSH. Specifically, oxidized glutathione was added to the supernatants of gill homogenates with NADPH and the reduction in absorbance at 340 nm was measured in a multiwell plate for 10 min. The enzyme activity was calculated by the formula:

$$A_{GR} = (\Delta A/\text{time}) \cdot V_{\text{total vol}} \cdot 1000 / \varepsilon \cdot d \cdot V_{\text{sample}}$$

Glutathione reductase activity was finally calculated as specific activity in nmol $min^{-1}$ $mg^{-1}_{prot.}$.

### 2.13. Activity of glutathione S–transferase (GST)

Using the adapted method of Habig et al. (Habig et al., 1974), the activity of the enzyme GST in the gills of 30 mussels was evaluated. The method involved conjugation of 200 mM reduced glutathione (GSH) with 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) sodium phosphate reaction buffer (100 mM pH 6.5) in multiwell plates, incubated at room temperature for 1.5 min followed by measurement of the increase of absorbance at 340 nm every 30 s for 5 min.

The enzyme activity as is calculated by the formula:

$$A_{GST} = (\Delta A/\text{time}) \cdot V_{\text{total vol}} \cdot 1000 / \varepsilon \cdot d \cdot V_{\text{sample}}$$

Glutathione-S-transferase activity was finally calculated as specific activity in nmol $min^{-1}$ $mg^{-1}_{prot.}$.

### 2.14. RNA extraction and gene expression analysis

Total RNA was extracted from mussels' gills using the RNeasy mini-kit (Qiagen, USA), following the manufacturer's protocol. The concentration of purified RNA was analyzed spectrophotometrically with Nanodrop2000 (Thermo Scientific Inc., Waltham, MA, USA). RNA was reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen, USA) according to the manufacturer's instructions. We focused on five gene markers: Catalase, Superoxide dismutase, Glutathione S-transferases, and Heat shock Proteins (HSP 70 and HSP 90). All primers used for real-time PCR (RT-PCR) studies are shown in Table S1, Actin was used as housekeeping gene. PCRs were performed in a final volume of 20 µL, with 10 µL of Invitrogen™ Platinum™ Hot Start PCR Master Mix (2X), 1 µL pf 10 µM of each primer and 50 ng of cDNA template under the following conditions: a denaturing step at 94 °C for 2 min; 35 cycles at 94 °C for 15 s, at 60 °C 15 s and at 68 °C for 15 s; a final extension step of at 68 °C for 5 min. To confirm the identity of the fragments, PCR products were sequenced in both directions (Eurofins Genomics GmbH, Ebersberg, Germany). Electropherograms were assembled to examine any base pair ambiguities, and the new sequences were aligned using the software Geneious version 14 (Biomatters, http://www.geneious.com/). Sequences were analyzed with GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool) to confirm gene identity. Subsequently, RT-PCR was performed using the Mic qPCR Cycler (Bio molecular systems, USA) in a final volume of 20µ L, with 50 ng of cDNA, 1 mM of each primer, and 10µ L of qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems, USA). The RT-PCR cycling profile consisted of a step at 95 °C for 2 min, 40 step cycles at 95 °C for 5 s, at 60 °C for 30 s, and the melting curve analysis. Quantitative RT -PCR analysis was conducted using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). At the end of each test, a melting curve analysis was done (plate read every 0.5 °C/s from 50 °C to 95 °C) to determine the formation of the specific products. Each sample was tested and run in triplicate. We compared and analyzed results using a T-test and a *p*-value

$< 0.05$ was considered statistically significant.

### 2.15. Statistical analysis

All statistical analyses and graphical representations were performed using GraphPad Prism software. The data were analysed for normality and homoscedasticity, and one-way or two-way ANOVA, followed by Tukey's post-hoc test, was used to analyze the data for all experimental results, which are expressed as mean ± standard deviation (SD). Pearson correlation analysis was performed to evaluate the relationships between different parameters. For molecular analyses, a *t*-test was applied to compare groups. Statistical significance was set at $p < 0.05$ for all tests.

## 3. Results

### 3.1. Nanoparticle characterisation

DLS analysis indicated a hydrodynamic diameter of 76 ± 11 nm for $Cu_2O$ nanoparticles dispersed in ultrapure water, while addition of nanoparticles to artificial seawater resulted in immediate agglomeration and aggregation, with the hydrodynamic diameter immediately increasing to 286 ± 53 nm and eventually reaching a value of 529 ± 75 nm over 24–48 h.

Transmission electron micrographs indicated a primary particle size of about 80 nm as well as the presence of a range of large agglomerates/aggregates that arose primarily due to loss of colloidal stability in artificial seawater (but also likely contributed to by the drying process of the nanoparticle dispersion on the TEM grid).Figs. 1, 2

While $Cu_2O$ is relatively insoluble in seawater, any cuprous ions ($Cu^{+}$) released may be oxidised to cupric ions ($Cu^{2+}$), hence the concentration of cupric ($Cu^{2+}$) ions in ultrapure water and in filtered seawater was used as a means for estimating the kinetics of metal ion release from the nanoparticles as they underwent oxidative dissolution over 96 h $Cu^{2+}$ ion concentration was found to remain relatively constant over time, with about 1 mg $L^{-1}$ and 0.01 mg $L^{-1}$ copper measured in ultrapure water and in filtered seawater, respectively, indicating that about 0.1 % and 0.001 % of nanoparticulate copper respectively was present in ionic form. Thus, for the experimental exposure concentration

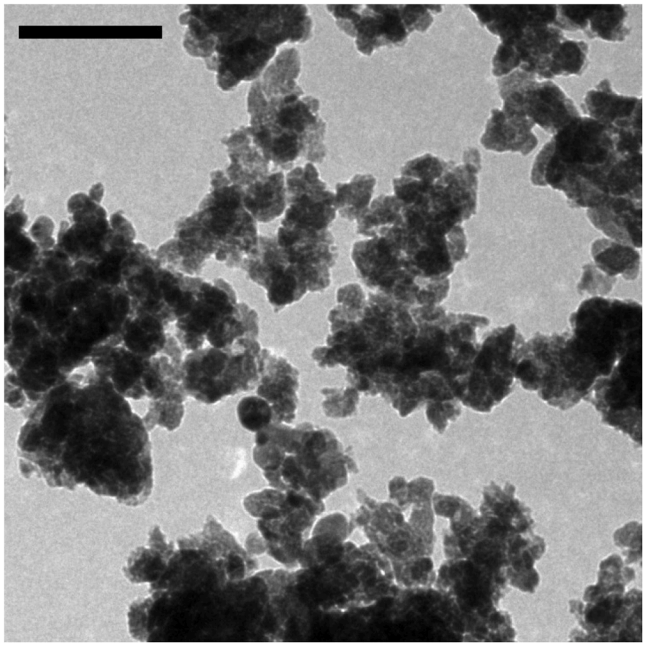

**Fig. 1.** Transmission electron micrograph of $Cu_2O$ nanoparticles dispersed in artificial seawater. The scale bar indicated 200 nm.

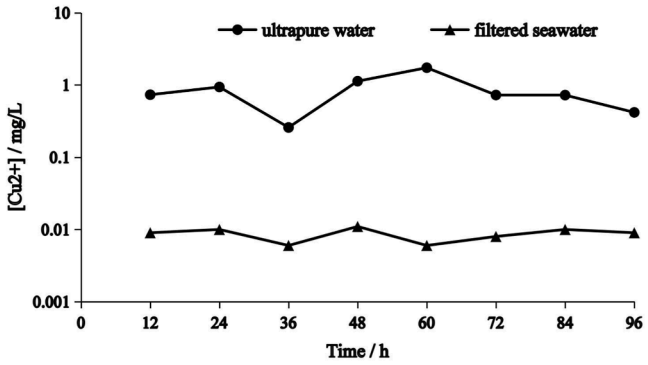


**Fig. 2.** Temporal change in cupric ion concentration as a function of salinity for $Cu_2O$ nanoparticle dispersions.

of 2 mg-Cu $L^{-1}$, it may be expected that the concentration of ionic copper present in seawater in the experimental tanks would be in the order of 0.02 μg $L^{-1}$ (far below the level at which mortality would occur in M. galloprovincialis over 4 days), hence most of the copper remained in particulate form.

### 3.2. Antioxidant activity of postbiotics

The results reported in Table 1 show the DPPH and $ABTS^{\bullet}$ radical scavenging activity expressed as a percentage for CFS at the concentration of 25 mg $L^{-1}$, compared with ascorbic acid (AA) as a positive control. CFS demonstrated a lower scavenging activity than AA, with a radical-neutralizing capacity of 34.5 % for DPPH and 52.2 % for $ABTS^{\bullet}$, showing a moderate antioxidant activity at the concentration used.

### 3.3. Biometric parameters and condition index (CI)

After 18 days of experimental trial, measurement of biometric parameters was carried out in G1 and G2 groups. As shown in Fig. 3, A, B, C, there are no significant differences in the different experimental groups in length, width and height of shell.

To evaluate the physiological conditions of the mussels *M. galloprovincialis* under different experimental conditions, the conditional index (CI) was calculated. As shown in Fig. 3, D the group that was fed with CFS supplement ($G2^{-}$) showed a significant increase in CI ( %) compared to $G1^{-}$ group, fed only with algae ($p < 0.05$). Exposure to $Cu_2O$ at a concentration of 2 mg $L^{-1}$ led to a significant reduction in the percentage of CI in $G1^{+}$ group ($p < 0.05$), showing a toxic effect of the pollutant. Furthermore, the $G2^{+}$ group did not show significant differences compared to $G1^{-}$ and $G2^{-}$ groups, suggesting a protective effect of the postbiotic.

### 3.4. Total haemocyte count and cell viability

The total haemocyte count (THC) and cell viability in exposed ($G1^{+}$ and $G2^{+}$) and non-exposed ($G1^{-}$ and $G2^{-}$) groups after $Cu_2O$ exposure at 24, 48, and 72 h are presented in Fig. 4 (A, B).

In the unexposed groups that were fed a normal diet and a CFS-supplemented diet ($G1^{-}$ and $G2^{-}$, respectively), THC remained stable over the 72 h period, with an average value of approximately $1.7 \times 10^{6}$ cells $mL^{-1}$. In contrast, the $G1^{+}$ group exposed to $Cu_2O$ nanoparticles

**Table 1**
DPPH radical scavenging activity ( %) and ABTS+ radical scavenging activity ( %) of CFS at concentration of 25 mg $L^{-1}$.

| | AA | CFS |
|---|---|---|
| DPPH | 90.4 ± 0.03 | 34.5 ± 0.6 |
| ABTS+ | 97.3 ± 0.07 | 52.2 ± 0.08 |

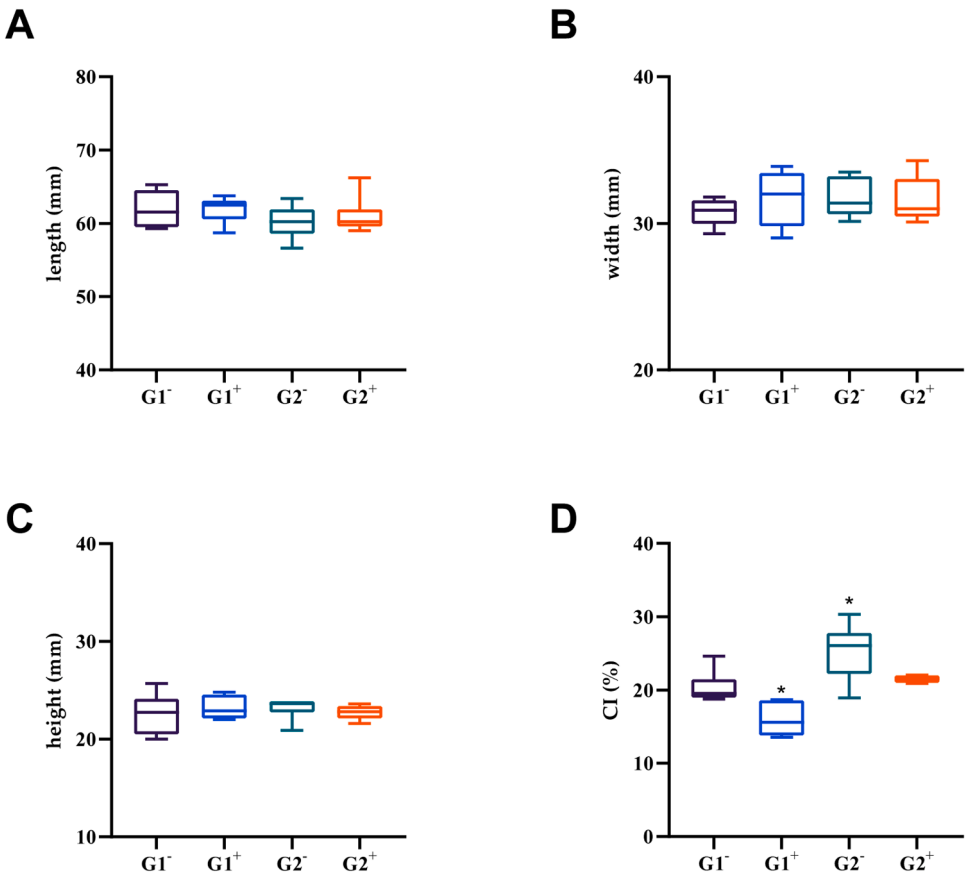


**Fig. 3.** Biometric parameters measurement of *M. galloprovincialis* exposed ($G1^+$ and $G2^+$ groups) and not exposed ($G1^-$ ang $G2^-$ groups) to $Cu_2O$ nanoparticles; (A) shell length (mn), (B) shell width (mn); (C) shell height (mn); (D) Conditional Index ( %). *= $p < 0.05$.

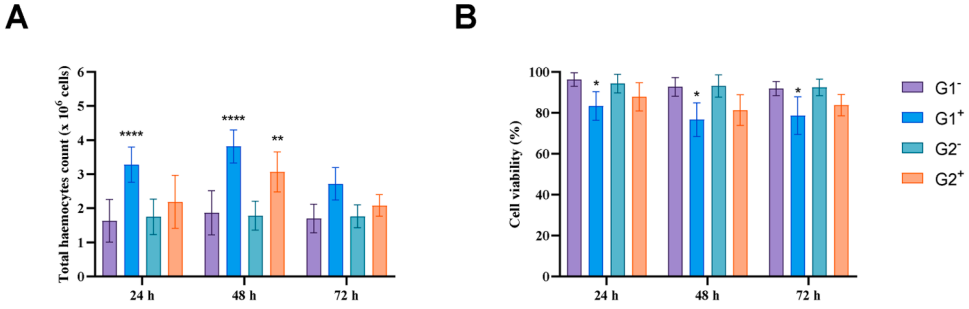


**Fig. 4.** Total haemocytes count (A) and cell viability (B) in mussel groups exposed ($G1^+$ and $G2^+$ groups) and not exposed ($G1^-$ ang $G2^-$ groups) to $Cu_2O$. Asterisks represent significant differences vs $G1^-$ group (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$).

showed a significant increase in THC at 24 and 48 h compared to the unexposed groups ($p < 0.0001$). Interestingly, in the $G2^+$ group exposed to nanoparticles, THC increased only at 48 h, after which it returned to baseline levels.

Cell viability was also assessed at 24, 48, and 72 h in all experimental groups (Fig. 4B). The unexposed groups ($G1^-$ and $G2^-$) maintained a constant viability of approximately 95 % throughout the study. In the exposed groups, cell viability decreased to 79 % in $G1^+$ and 84 % in $G2^+$, with a more pronounced reduction observed in the group not supplemented with CFS.

### 3.5. Copper bioaccumulation

The bioaccumulation of copper in gill tissue was assessed by atomic absorption spectroscopy, and it was noted that copper concentrations in non-treated controls ($G1^-$) and mussels fed with postbiotics ($G2^-$) showed very low levels (0.27–0.36 mg $g^{-1}$) which likely derives from background copper in seawater (Fig. 5). A large and significant increase in copper concentration in gill tissue was noted for mussels treated with $Cu_2O$ nanoparticles ($G1^+$, 20.32 mg $g^{-1}$), with the mussels additionally fed postbiotics showing lower, though still statistically significant, accumulation of copper ($G2^+$, 8.42 mg $g^{-1}$).

### 3.5. Biochemical analyses

To assess the effect of a CFS-enriched diet on the antioxidant system of mussels exposed to $Cu_2O$, the enzymatic activities of catalase (CAT), glutathione reductase (GR), and glutathione S-transferase (GST) were analyzed.

As shown in Fig. 6 (A, B, C), the non-exposed groups that were fed a

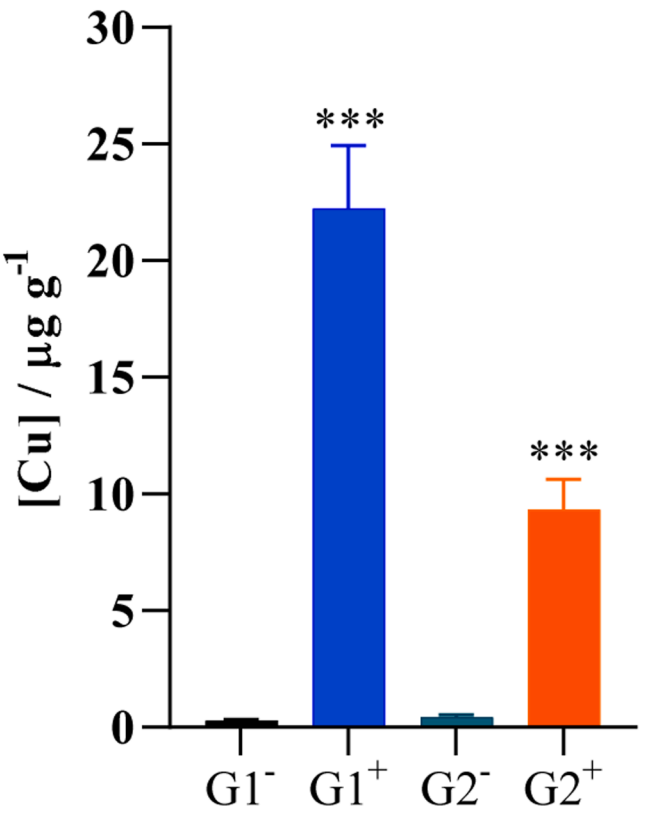


**Fig. 5.** Copper concentrations in gill tissue for non-treated mussels and mussels treated with postbiotics and/or $Cu_2O$ nanoparticles. Values are given as the mean ± SD. Asterisks represent significant differences vs $G1^-$ group (***$p < 0.001$).

normal diet and a CFS-enriched diet ($G1^-$ and $G2^-$, respectively) exhibited no significant differences in enzymatic activity for all three enzymes, indicating a comparable baseline condition.

Regarding CAT activity (Fig. 6A), $Cu_2O$ exposure in the $G1^+$ group led to a significant increase compared to the control group $G1^-$ ($p < 0.0001$), suggesting a response to oxidative stress. Interestingly, this effect was not observed in the $G2^+$ group, which received the CFS-enriched diet, indicating a potential protective role of the diet.

It was noted that GR activity (Fig. 6B) was significantly reduced in the $G1^+$ group ($p < 0.01$), highlighting a possible burdening of the antioxidant pathway and hence toxic effect of $Cu_2O$. In contrast, the $G2^+$ group displayed GR activity levels comparable to those of the $G1^-$ and $G2^-$ groups, further supporting the antioxidant protective effect of the CFS-enriched diet.

Finally, GST activity (Fig. 6C) was found to decrease in both $Cu_2O$-exposed groups ($G1^+$ and $G2^+$). However, the reduction was less pronounced in the $G2^+$ group compared to $G1^+$, suggesting that the enriched diet mitigated the pollutant's adverse effects resulting in less inhibition of the GST-mediated detoxification pathway.

The correlation coefficients among the examined biomarkers are presented in Table 2 showing Pearson's correlation. A correlation coefficient exceeding 0.5 was deemed significant at $p < 0.05$. In particular, it was noted that the condition index and total haemocyte counts were inversely related indicating that for a weakening of the overall physiological status of the mussels, the immune system may have been activated in response to the presence of the $Cu_2O$ nanoparticles. In addition, the strong correlation between the GR and GST enzyme activities clearly indicates how the functioning of the glutathione antioxidant defence system may be affected at all levels when exposed to $Cu_2O$ nanoparticles xenobiotics. Moreover, the strong negative correlation between CAT and GST (and to a slightly lesser extent GR) in these experiments may indicate that high turnover rate CAT can continue to function more robustly than glutathione defence pathways which become overburdened upon exposure to $Cu_2O$ nanoparticles at the tested concentrations. Analysis indicates that there is little correlation between activation of the immune system (THC) and increased enzymatic activity related to anti-oxidative stress pathways (CAT, GR, GST), although this is likely due to temporal mismatch between the two defense systems and our endpoints. The number of hemocytes increases over the first 48 h of the experiment indicating a relatively rapid activation of the immune response while hemocyte count returns to near baseline levels by 72 h from the start of the exposure. However, enzyme activity was measured only after 72 h by which time hemocyte count had reduced. It is possible that increased measurement frequency of enzyme activities would also show greater activation at the 24 h and 48 h timepoints, consistent with increased the total hemocyte count, giving rise to a higher correlation coefficient.

### 3.6. Gene expression analysis

RT-PCR analysis revealed that Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione S-transferases (GST) gene expression levels were up-regulated in the $Cu_2O$ treatment compared to $G1^-$ group, reflecting an enhanced antioxidant response (Fig.7). In the postbiotic treatment ($G2^-$), CAT expression was moderately elevated compared to $G1^-$, while SOD and GST expression was downregulated. For $G2^-$ and $G2^+$ groups, all gene expression levels were slightly down-regulated relative to $G1^+$.

HSPs gene expression showed strong upregulation in the $G1^+$group, increasing values by about 3- and 18-fold for HSP70 and HPS90, respectively. Interestingly, the postbiotic-only treatment ($G2^-$) does not affect gene HSP expression, but when it was used in combination with the $Cu_2O$ exposure ($G2^+$), the HSP expression showed a significant and intense downregulation compared to mussels exposed to $Cu_2O$ and fed only a normal diet. This effect was much greater forHSP90 than HSP70.

## 4. Discussion

Oxidative stress is a common response to chemical stress in many organisms, making oxidative stress markers useful tools in ecotoxicological assessments. However, the stress response is complex and may be modulated by various factors, ranging from the nature and duration of exposure to antagonistic or synergistic effects deriving from other compounds co-present. Therefore, this study was designed to examine the effects of $Cu_2O$ exposure on mussel gills, and to determine if a diet enriched with a postbiotic, derived from a consortium of microorganisms isolated from homemade kefir, could modulate any stress response arising from the $Cu_2O$.

Postbiotics are substances that include components of microbial cell walls, such as proteins and lipopolysaccharides, extracellular polysaccharides, and microbial metabolites produced during carbohydrate fermentation or protein breakdown, such as short-chain fatty acids (SCFAs). Numerous studies have shown that postbiotics can provide beneficial biological effects for the host. It has been demonstrated that postbiotics have great potential in maintaining intestinal microbiota homeostasis and improving gut health by inhibiting the growth and activity of harmful bacteria while stimulating the growth of beneficial bacteria. Additionally, postbiotics modulate the host's immunity by enhancing gastrointestinal barrier function and preventing pathogen translocation. This is achieved by increasing tight junction proteins, which improve the intestinal epithelial barrier function. Postbiotics can influence both the innate and adaptive immune systems by interacting with various cell types along the mucosa, such as B cells, T cells, monocytes, macrophages, NK cells, and dendritic cells (DCs). Furthermore, postbiotics may impact the immune response by modifying immune signaling pathways and modulating inflammatory cytokines. These findings suggest that the immunomodulatory activity of postbiotics primarily depends on their ability to differentially regulate the

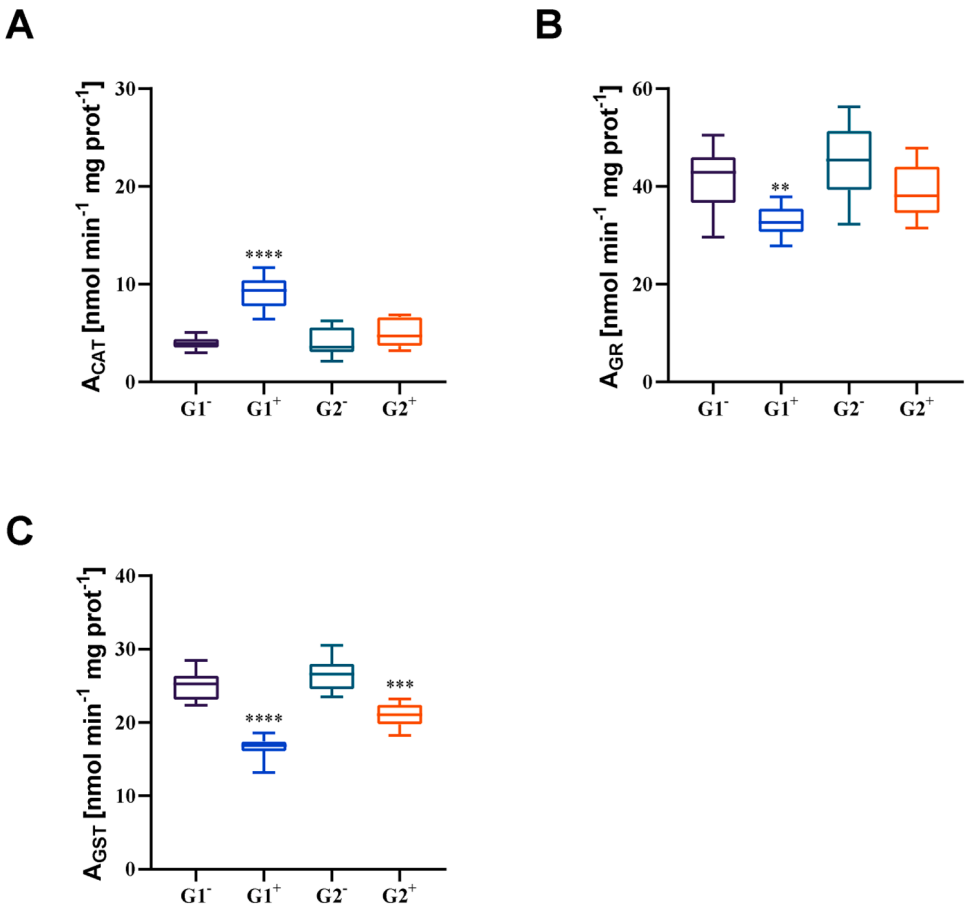


**Fig. 6.** Biochemical analyses of *M. galloprovincialis* exposed (G1$^+$ and G2$^+$ groups) and not exposed (G1$^-$ ang G2$^-$ groups) to $Cu_2O$. (A) Enzymatic activities of catalase (CAT), (B) enzymatic activities of glutathione reductase (GR) and (C) enzymatic activities of glutathione S-transferase (GST). Asterisks represent significant differences vs G1$^-$ group (**= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$).

**Table 2**
Pearson's correlation coefficients (r).

| | CAT | GR | GST | THC | CI | *SOD* | *CAT* | *GSTs* | *hsp_70* | *hsp_90* |
|---|---|---|---|---|---|---|---|---|---|---|
| CAT | 1 | | | | | | | | | |
| GR | **−0441,346** | 1 | | | | | | | | |
| GST | **−0719,182** | **0,5535,081** | 1 | | | | | | | |
| THC | −0001,084 | −0,08,298 | 0,0735,664 | 1 | | | | | | |
| CI | −0224,429 | 0,0234,883 | 0,0015,735 | **−0631,052** | 1 | | | | | |
| *SOD* | 0,1428,965 | −0045,154 | 0,1030,011 | −0245,895 | 0,2286,359 | 1 | | | | |
| *CAT* | 0,0632,394 | −0018,282 | 0,1704,822 | **−0429,363** | **0,3732,385** | 0,0910,595 | 1 | | | |
| *GSTs* | 0,1655,054 | 0,0300,744 | −0127,463 | 0,2544,551 | −0127,594 | −0151,254 | −0032,317 | 1 | | |
| *hsp_70* | −0274,683 | 0,0951,739 | −0237,418 | −0045,131 | 0,1308,591 | −0203,299 | 0,1111,668 | **0,5345,052** | 1 | |
| *hsp_90* | −0135,842 | −0,0261 | 0,0441,204 | **−0705,984** | **0,5374,785** | 0324,708 | **0,4930,862** | −0008,596 | 0160,812 | 1 |

production of anti-inflammatory and pro-inflammatory cytokines, as well as the balance between types 1 and 2 T helper cells (Th1 and Th2, respectively). They also effectively regulate gene expression in immune cells and influence transcription factors, thereby driving the differentiation of the immune system (Izuddin et al., 2020). In addition, postbiotics are rich in organic acids, mainly lactic acid and acetic acid, which are excellent electron donors due to the presence of hydroxyl groups that enhance free radical scavenging activity, showing in vitro antioxidant activity. Thus, the antioxidant properties of postbiotics have increased the potential for their integration as feed additives, even in animal farming diets (Wang et al., 2017).

However, there is scarce information on the antioxidant activity of postbiotics, and their potentially antagonistic mitigation of toxicity caused by heavy metal pollutants, in marine species such as commercially important bivalve mussels.

In our study, we first evaluated growth and immunological parameters in all four groups. The biometric parameters after 18 days of a diet supplemented with the post-biotic showed no differences between the two groups G1- and G2-. When we evaluated the CI (Pavičić-Hamer et al., 2022), which provides a general means to assess the physiological activity of organisms and is an important ecophysiological indicator of the health status of mussels, it was noted to have increased in the supplemented diet group G2-. In contrast, a noticeable decrease was observed in the unsupplemented group (G1+) exposed to the pollutant, while the fed and exposed group (G2+) showed no change. It is clear that exposure to these nanoparticles is negatively affecting the overall health of the mussels, with a decrease in the proportion of meat in the shellfish likely reflecting disruption to normal feeding patterns and the organism beginning to weaken. However, it should be borne in mind that the nanoparticles are too small and low in concentration to cause

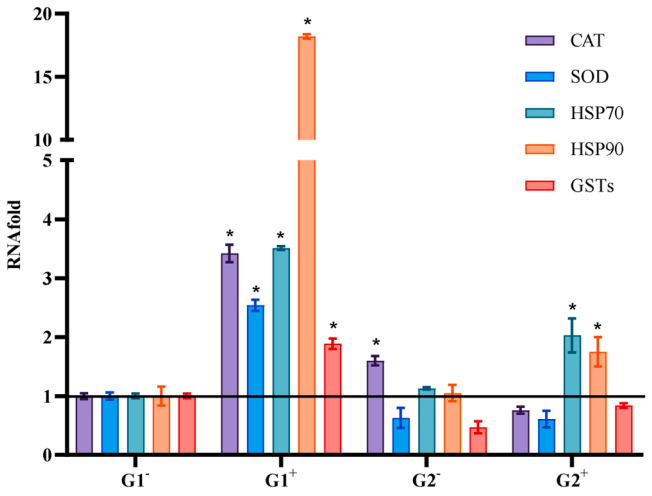


**Fig. 7.** Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S-transferases (GSTs), and Heat Shock Proteins (HSP 70 and HSP 90) gene expression levels in *Mytilus galloprovincialis* gills after treatments ($G1^-$, $G1^+$, $G2^-$ and $G2^+$). Asterisks represent significant differences vs $G1^-$ group (*= $p < 0.05$).

physical blockage of the digestive tract of the mussels, indicating rather that broader impacts on cellular processes are likely the source of any diminished feeding. In parallel to deleterious effects at the whole organism level, it would be expected that circulating haemocytes in the haemolymph would also display corresponding changes. Haemocytes, which are a type of macrophage, play a crucial role in the immune system by defending the organism against parasites, pathogens, and pollutants. They also serve as a useful tool for evaluating the stressors affecting the immune system. Our study shows that exposure $Cu_2O$ led to a notable rise in haemocyte production in the first 24/48 h in G1+, and this increase is directly related to the exposure period with a decrease at 72 h. The same trend was observed in G2+ group even though the values reached were lower. Similar values were found in G1- and G2- group confirming the potent ability of the postbiotic in modulating immune responses. Cell viability, used to assess the response and health of cells in the mussels after treatment with the $Cu_2O$ nanoparticles, was also monitored. It revealed a significant decrease in haemocyte viability at all time points only in the G1+ group. Overall, this indicates both a clear activation of the mussel immune system and degradation of that system due to toxic burden from the nanoparticles. Moreover, this provides the initial and most rapid indication that postbiotics can provide a key boost to organisms' defense mechanisms, enabling them to function more robustly in the presence of xenobiotics.

It has been reported (Wang et al., 2017) that postbiotics show antioxidant activity and our postbiotic supplement CFS confirmed a high scavenging activity against hydroxyl superoxide and DPPH free radicals. As a consequence, in defending against oxidative stress, in both exposed groups (G1+ and G2+) we observed changes in the activity of intracellular antioxidant enzymes like CAT, GR and GST compared to non-exposed groups (G1- and G2-).

Aquatic organisms, especially marine bivalves, exhibit a variety of changes in enzymatic antioxidant defenses after exposure to pollutants (Regoli and Giuliani, 2014; Regoli et al., 2002; Regoli and Principato, 1995). Maintaining redox homeostasis is crucial for mitigating oxidative stress and promoting cellular survival (Zhang et al., 2022a). The enzymatic pathway consists of protective ROS-scavenging enzymes, such as SOD, CAT and GST (Atli et al., 2006; Feder and Hofmann, 1999) that play vital roles in reducing the harmful effects of ROS (Lu et al., 2022; Zhang et al., 2022b). Prominent among this antioxidant defense system are superoxide dismutase (SOD), converting the superoxide anion into hydrogen peroxide, catalase (CAT), converting $H_2O_2$ into the water, and the glutathione S-transferase (GST) phase II detoxifying enzyme that plays a crucial role in eliminating lipid peroxidation byproducts, thereby reducing cellular damage (Yin et al., 2024). Antioxidant defense enzymes are induced by various environmental pollutants under pro-oxidant conditions, initially their concentrations increase to counteract oxidative stress but prolonged exposure causes their depletion, leading to oxidative damage to basic biological molecules, such as protein and DNA (Bebianno et al., 2005). Furthermore, elevated oxidative stress levels are consistently associated with apoptosis and are considered a potential mechanism driving this cellular process (Meng et al., 2020).

Bivalve mollusks, such as mussels and oysters are filter-feeding sedentary species prone to the accumulation of pollutants (Coppola et al., 2017; Leite et al., 2020; Lopes et al., 2022). They have been used extensively in recent years as sentinel organisms and sensitive bioindicators for pollutants associated with ROS generation and oxidative damage (Frenzilli et al., 2004). Mussels, such as *M. galloprovincialis* are used to evaluate contaminated coastal environments (Lionetto et al., 2001) in programmes such as the international "Mussel Watch". The bivalve *M. galloprovincialis* was selected as a model organism due to its extensive use in ecotoxicological studies and high sensitivity to chemical stressors (Capolupo et al., 2018; Curpan et al., 2022; Leite et al., 2020, 2023). Like other organisms, mussels can control the increasing ROS levels in their tissues during exposure to heavy metals, activating the cellular antioxidative defense systems composed of both enzymatic and non-enzymatic components. Antioxidant enzymes are actively involved in response to heavy metals such as copper (Cu) in aquatic organisms (Canesi et al., 1999; El-Gendy et al., 2009) and their induction have been considered as reliable molecular biomarkers for oxidative stress caused by various environmental contaminants.

As reported in other studies (Pintueles-Tamayo et al., 2025) the activity of antioxidant enzymes can vary across different tissues, with different degrees of inhibition in groups exposed to xenobiotics. In the present study, we found that the exposure to $Cu_2O$ for 3 days enhanced the activity of CAT enzyme in group G1+, suggesting its role in counteracting ROS production compared to G2+ confirming its activation after acute exposure to a pollutant and the protective role of CFS. In our experiment, we observed a general decrease in antioxidant GR activity in G1+, indicating a weakened antioxidant capacity in mussels exposed to $Cu_2O$ and fed with a normal diet while activity similar to the control was observed in the other three groups (G2+, G1-, G2-). Notably, GST, a multifunctional enzyme essential for detoxifying both exogenous and endogenous toxic compounds, was significantly decreased in both G1+ and G2+ exposed mussels, likely due to the overwhelming stress imposed by $Cu_2O$ exposure and suggesting that GST activity might serve as a useful biomarker for $Cu_2O$ exposure.

The ability of CFS postbiotics to alleviate oxidative stress has been confirmed by the mRNA expression of genes related to oxidative stress including CAT, SOD, GST and heat shock proteins (HSP 70 and HSP 90).

Toxic chemicals and environmental parameters may affect the levels of heat shock proteins. Environmental stress was reported to significantly modify the mRNA expression of various heat shock protein genes (Banni et al., 2014; Negri et al., 2013). The folding proteins (HSP70, HSP90) play a crucial role in the restoration of the structure of partially denatured proteins as well as in protein folding and translocation. HSP70 and HSP90 mitigate cellular stress caused by heavy metals and pollutants. HSP70 is a widely recognized biomarker for environmental stress in marine invertebrates, with studies demonstrating its significant upregulation in *M. galloprovincialis* exposed to heavy metals like copper (Cu) and cadmium (Cd). This response reflects the organism's defense mechanism to counteract oxidative damage and maintain cellular functionality (Franzellitti and Fabbri, 2005). HSP90 is expressed to stabilize and repair proteins, preserving cellular homeostasis under stress conditions. Studies have previously highlighted its upregulation in mussels exposed to sub-lethal concentrations of antibiotics, such as oxytetracycline (OTC). This indicates its involvement in responses to both chemical and thermal stress (Banni et al., 2015). Interestingly, heat

shock proteins were previously suggested to act as redox sensors activating some antioxidant genes (Madeira et al., 2017).

In our experiments CAT, SOD and GST expressions were notably altered in G1+ group exposed to $Cu_2O$ and fed with a usual diet, indicating that they could serve as early indicators of stress. Specifically, SOD expression was upregulated by G1+ (3.33-fold) and downregulated when in the presence of CSF (G2- and G2+, 0.63 and 0.61-folds respectively). This result aligns with an initial response to oxidative damage caused by $Cu_2O$ exposure, a compound known to generate ROS (Negri, 2016) which activated the antioxidant system. On the contrary, in G2- and G2+, the presence of the dietary supplement may have reduced the need for an antioxidant response possibly through a potential protective mechanism mediated by CSF. Similarly, CAT expression was significantly upregulated in both G1+ (3.42-fold) and G2- (1.6-fold). In contrast, no changes were observed in G2+, indicating that CSF, when combined with a pollutant, may counteract its oxidant effects. Regarding GST expression, it was significantly upregulated in G1+ (1.87 fold) and downregulated in G2- mussels (0.47 fold). This dual response suggests GST may have acted in two distinct ways: in G1+, its expression could have been enhanced to compensate for the reduced activity observed in our biochemical assays, while in G2-, its expression might not have been necessary due to the protective effect of CSF, where no differences in GST activity were observed. These findings suggest that $Cu_2O$ treatment may impair the detoxification capacity of mussels, whereas CSF could enhance it. Regarding heat shock proteins, HSP70 and HSP90, their expression was significantly upregulated by the presence of $Cu_2O$ both alone and in combination with CSF (G1+ and G2+), even if in the presence of CSF the upregulation was mitigated. HSP70 expression increased 3.51-fold in G1+ and 2.03-fold in G2+ treatment. Similarly, HSP90 was upregulated 18.21-fold by $Cu_2O$ alone (G1+) and 1.75-fold when combined with CSF (G2+). No differences were observed in G2- treatment compared to the control, suggesting that the altered expression levels in G1+ and G2+ were driven by the presence of the pollutant, likely as a response to protein damage.

Metals, such as Cd, Ni, Cr, Pb and Hg are well known to be toxic in aquatic organisms mainly because of the oxidative potential whereas other metals, such as Fe, Zn, Cu, Se and Mn are essential for their metabolism but become toxic when their concentrations exceed certain levels (Chang et al., 1996). However, nanoparticulate metal and metal oxides such as those based on copper are known to undergo rapid aggregation in high strength electrolytes such as seawater, and the corresponding reduction in surface area compared to colloids that are well-dispersed as well as copper's sparing solubility in seawater, lead to the expectation that nanoparticulate copper would not be harmful at the tested concentrations. The observation that $Cu_2O$ nanoparticles can elicit a clear response in mussels in a short period, even though little ionic copper is expected to be released from the nanoparticles, may be related to copper's $+I$ oxidation state that can play a role in cellular redox chemistry as it may be either reduced or oxidized depending on the biochemical pathway involved (Mortezaee et al., 2019). Certainly, further studies are warranted on probing the nature of redox active nanoparticles' interaction with biota and how they may be a greater driver of oxidative stress, for example compared to copper in the 0 or +II oxidation states. In addition, significant accumulation of copper in the gill tissue was noted for organisms exposed to $Cu_2O$ nanoparticles, with much greater copper concentrations in mussels that did not have postbiotics supplementation. The reason for postbiotics disfavoring copper accumulation may be related to activation of detoxification pathways, for example though increased GST activity in the postbiotics-fed mussels. Thus, less accumulation of copper (I) results in reduced exposure to metal-based redox activity which contributes to lower oxidative stress. In conclusion, postbiotics, particularly CFS derived from a consortium of microorganisms isolated from homemade kefir, exhibited strong antioxidant capacity. This dietary supplementation, following exposure to heavy metals, was found beneficial in enhancing antioxidant capacity and reducing oxidative stress in mussels. This points to dietary postbiotics, as feed additives, having significant potential for use in mussel aquaculture for reducing oxidative stress and increasing yield.

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## CRediT authorship contribution statement

**Angela Maione:** Visualization, Methodology, Data curation. **Marianna Imparato:** Methodology. **Antonietta Siciliano:** Funding acquisition. **Valeria Maselli:** Writing – original draft, Supervision. **Tania Russo:** Investigation. **Mariangela Norcia:** Formal analysis. **Marco Guida:** Resources, Project administration. **Daniel Mark Lyons:** Writing – original draft, Supervision. **Emilia Galdiero:** Writing – review & editing, Conceptualization.

## Declaration of competing interest

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

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107514.

## Data availability

Data will be made available on request.

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