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Are bioplastics safe? Hazardous effects of polylactic acid (PLA) nanoplastics in *Drosophila*

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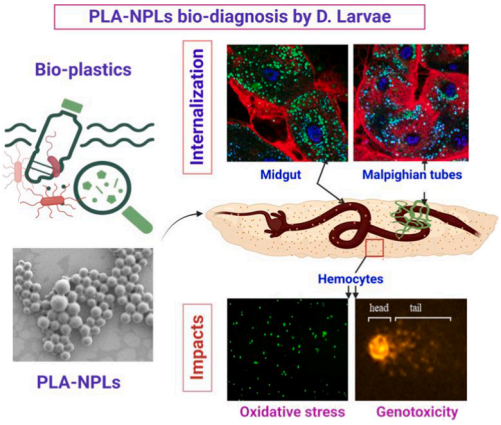
HIGHLIGHTS

- PLA nanoplastics were in-house obtained from PLA pellets.
- A physicochemical characterization was carried out.
- The potentially harmful effects were determined using Drosophila as *in vivo* model.
- The journey and fate after ingestion were determined in exposed larvae.
- PLA-NPLs triggered molecular responses associated with oxidative stress and genotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The expanded uses of bioplastics require understanding the potential health risks associated with their exposure. To address this issue, *Drosophila melanogaster* as a versatile terrestrial *in vivo* model was employed, and polylactic acid nanoplastics (PLA-NPLs), as a proxy for bioplastics, were tested as a material model. Effects were determined in larvae exposed for 4 days to different concentrations (25, 100, and 400 μg/mL) of 463.9 ± 129.4 nm PLA-NPLs. Transmission electron microscopy (TEM) and scanning electron microscope (SEM) approaches permitted the detection of PLA-NPLs in the midgut lumen of *Drosophila* larvae, interacting with symbiotic bacteria. Enzymatic vacuoles were observed as carriers, collecting PLA-NPLs and enabling the crossing of the peritrophic membrane, finally internalizing into enterocytes. Although no toxic effects were observed in egg-to-adult survival, cell uptake of PLA-NPLs causes cytological disturbances and the formation of large vacuoles. The translocation across the intestinal barrier was demonstrated by their presence in the hemolymph. PLA-NPL exposure triggered intestinal damage, oxidative stress, DNA damage, and inflammation responses, as evaluated *via* a wide set of marker genes. Collectively, these structural and molecular interferences caused by PLA-

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NPLs generated high levels of oxidative stress and DNA damage in the hemocytes of *Drosophila* larvae. The observed effects point out the need for further studies aiming to deepen the health risks of bioplastics before adopting their uses as a safe plastic alternative.

1. Introduction

Due to their wide presence in all types of environmental compartments, micro and nanoplastics (MNPLs) resulting from the degradation of plastic goods are considered emergent environmental pollutants. Despite the increasing number of recent papers dealing with the potential risks of MNPLs (Malafaia and Barceló, 2023; Kutralam-Muniasamy et al., 2023), there is still a lack of trusted information on their potential health effects on humans (Courtene-Jones et al., 2022). Despite that, a growing concern about their potential risks is spreading rapidly.

Due to the bad press of fossil fuel-based plastics, an increasing tendency to use what are denominated "bioplastics" is emerging. These plastics are wholly or partially made from biological materials and their use is quickly expanding, mainly for disposable plastic goods (Chen, 2022). A remarkable characteristic of bioplastics is their degradation rate which is faster than in conventional plastics (Kumar et al., 2023). Considering that one of the most relevant risks of fossil fuel-based plastics is associated with the production of MNPLs during their degradation, it could be assumed that the rate of producing MNPLs resulting from the degradation of biodegradable plastics should be higher (Fojt et al., 2023). As recently reported, when teabags made from PLA are used, they can release about one million PLA-NPLs during the normal tea preparation procedure (Banaei et al., 2023). Thus, despite the pioneering study of Roes and Patel (2007) pointing out that the conventional risks of biotechnologically produced chemicals are lower than those of fossil-fuel-derived ones, no relevant approaches have delved into this topic (Ribba et al., 2022). Consequently, determining the potentially harmful effects of the MNPLs resulting from the degradation of bioplastics is a hot topic that needs to be addressed.

Polylactic acid (PLA) is likely the most popular bio-based polymer, being a biodegradable aliphatic semicrystalline polyester synthesized *via* the polycondensation of lactic acid (LA) monomers, or through ring-opening polymerization of its cyclic dimer (lactide) (Castro-Aguirre et al., 2016). The great success of PLA is mainly due to its mechanical properties, which are like those of conventional plastics, such as polystyrene and polyethylene terephthalate (Malafaia et al., 2021). Consequently, the production of PLA is steadily growing reaching 20.7 % of bioplastics in 2022, which is expected to reach 37.9 % in 2027 (European Bioplastics, 2023).

Despite the increasing use of PLA, there is an alarming lack of studies aiming to determine the potential risks associated with MNPLs resulting from its degradation (Wang et al., 2023). Of the few existing studies, some of them have used *in vivo* models. Thus, using zebrafish, the accumulation of PLA in larvae was demonstrated, associated with altered locomotor and exploration activities, and inhibition of the acetylcholinesterase function (de Oliveira et al., 2021). Further, PLA-MPLs resulting from the photodegradation with UV light showed a high ability to trigger mitochondrial structural damage and apoptosis in infantile zebrafish (Zhang et al., 2021). Interestingly, PLA-MPLs were ingested much more efficiently than petroleum-based MPLs, inducing gastrointestinal damage in zebrafish and affecting the diversity of their intestinal microbiota (Duan et al., 2022). However, PLA-MPL internalization, as well as its associated impacts (including molecular response, oxidative stress, and genotoxicity), have not been well addressed, comparatively to traditional plastics (Zhang et al., 2023). We which to highlight the previously detected gaps in the identification of the potentially harmful effects of PLA-NPLs and, accordingly, we aim to get sound data covering such lack of information, focusing on genotoxicity as a very relevant biomarker of harmful effects.

In this context, and as a working hypothesis, we assume that PLA-NPLs can result as harmful as those NPLs resulting from petroleum-based plastics. Consequently, this study aims to expand our knowledge of the effects of PLA-MNPLs in an *in vivo* model. To this end, we have chosen *Drosophila* as an experimental model due to its multiple advantages. Among them, it should be highlighted that its genome contains genes sharing homology with 75 % of the genes involved in human pathologies (Yamaguchi et al., 2021) and, consequently, *Drosophila melanogaster* has been proposed as a good model to understand the etiology of human pathologies (Bellen et al., 2019). Accordingly, it is assumed that *Drosophila* represents a good model to extrapolate the obtained findings to humans, in terms of mechanisms of action (Alaraby et al., 2016a, 2016b).

2. Material and methods

2.1. PLA-MNPLs obtention

MNPL production requires a methodology allowing to get particles with a narrow size distribution, control of the morphology, and to be reproducible and robust. To obtain MNPLs with chemical characteristics like the particles that can be found in the environment, the top-bottom strategy was chosen starting from commercial-grade polymers in the form of pellets. In this way, we started with an additive polymer ready to obtain final plastic products. The following products were used, Pluronic® F-127 BioReagent and polyvinyl alcohol (PVA) (from Sigma-Aldrich). Dichloromethane (DCM), dried (max. 0.005 % H_2O) ExpertQ® provided by Scharlab. Commercial grade of polylactic acid in pellet form. The 5(6)-FAM (5-(and-6)-carboxyfluorescein), SE (succinimidyl ester), and mixed isomers (fluorophore compound) were purchased from ThermoFisher Scientific. Solvent evaporation, combined with the miniemulsion technique was the methodology used to obtain the PLA reference material. The procedure was like the one reported by other groups (Feng et al., 2018; Chen et al., 2020). A pre-mini emulsion was prepared by adding the aqueous phase consisting of dissolved pluronic and PVA (as co-stabilizer) in 120 g water (0.25 % wt and 2 % wt, respectively) to the organic phase composed of 1 g of PLA dissolved in 30 g DCM and stirring magnetically for 60 min. Furthermore, ultrasonication under ice cooling was applied for 120 s at 80 % amplitude, using a Bandelin Electronic UW2200 sonicator. The obtained miniemulsion was transferred to a round bottom flask to evaporate the organic solvent, under pressure.

The labeled PLA-NPLs were obtained in the same way as the unlabeled PLA-NPLs explained above. Previously, the functionalization of PLA was necessary using a reactive extrusion process without the use of solvents. Once PLA was functionalized with amino groups, it was labeled by reacting with the fluorophore compound. For this purpose, 1 g of amino-functionalized PLA was placed in a round-bottom flask provided with a magnetic stirring bar, an N_2 inlet, and a reflux condenser. 12 mL of tetrahydrofuran (THF) was added under an inert atmosphere, and when the material was completely dissolved, 25 mg (2.5 % w/w) of 5(6)-FAM, SE was added. The reaction mixture was stirred at room temperature and protected from light. After 1 h, water was added to precipitate the labeled PLA, which was filtered off, washed with water, and dried. At this point, the fluorophore compound is chemically anchored to the functionalized PLA, and ready to be used.

2.2. PLA-NPLs physicochemical characterization

Before use in *Drosophila*, PLA-NPLs were widely characterized using

a set of approaches. Shape, morphology, and degree of aggregation of PLA-NPLs were determined using both scanning electron microscopy (SEM) (Zeiss Merlin, Zeiss, Oberkochen, Germany) and a TEM instrument (JEOL JEM 1400, JEOL LTD, Tokyo, Japan). The transparency nature of PLA obstructed TEM investigation since the particles showed a low contrast. This was overcome by staining particles with lead acetate. To stain PLA-NPLs, 20 μL of a PLA-NPLs dispersion (2 mg/mL) was loaded upon a copper grid covered with carbon film, and the dried samples were stained with 20 μL of lead acetate. For SEM investigation, the PLA-NPLs suspension was loaded on the bright surface of cleaned silicon chips. Silicon chips were cleaned with drops of Milli-Q water and kept in a clean area. The average diameter of PLA-NPLs was determined by measuring 100 random particles of SEM images using the ImageJ software. The functional groups of PLA particles were detected with Fourier transform infrared spectroscopy (FTIR). A Zetasizer® Ultra device from Malvern Analytical (Cambridge, United Kingdom) was used to evaluate the hydrodynamic size (dynamic light scattering, DLS) as well as the total surface charge of PLA-NPLs (Zeta potential) in suspension. FTIR, DLS, and zeta potential were measured at the Molecular Spectroscopy and Optical Microscopy unit of the Institut Català de Nanociència i Nanotecnologia (ICN2) at the UAB campus.

2.3. *Drosophila melanogaster (in vivo study)*

2.3.1. *Toxicity studies*

To determine the potentially harmful effects associated with exposure to PLA-NPLs, the Canton-S strain of *Drosophila melanogaster* was employed in all the experimental approaches involved in the current study. The survival rate of flies was evaluated after treatment of their early stage with different concentrations of PLA-NPLs. To proceed, 4 g of instant medium (Carolina Biological Supply Co., Burlington, NC) was wetted with 10 mL of steadily increasing concentrations of PLA (25, 100, and 400 μg/mL) equivalent to (62.5, 250, and 1000 μg/g food). Canton-S eggs were directly transferred into treated media and nontreated media, which was used as a control. The experiment was carried out in five replicates, each vial containing 50 eggs, incubated in conditions of room temperature (24 ± 0.5 °C) and adequate relative humidity (65 ± 5 %), with a 12 h light/dark cycle. Emerged adults were counted to reveal the relative survival percentage, in comparison with untreated flies. To detect any developmental morphological malformation induced by PLA-NPL exposure, the emerged adults were carefully investigated under a stereomicroscope.

2.3.2. *PLA-NPLs internalization following ingestion*

The internalization journey of PLA-NPLs, from oral ingestion to deposition in deep tissues and hemolymph, was followed in detail using TEM and confocal microscopy as follows:

(i) **TEM.**

The presence of PLA-NPLs in the different body compartments of *Drosophila* larvae was detected with TEM, following our previous protocol (Alaraby et al., 2015a). Samples of four days treated larvae (3rd instar) with 1000 μg/g food were collected, dissected in buffer (PB; 0.1 M, pH 7.4), and the intestinal tissues were immediately fixed in 0.15 M phosphate buffer containing 4 % paraformaldehyde and 1 % glutaraldehyde, pH 7.4. In a cold temperature (4 °C) environment, tissues were post-fixed and stained with a mixture of 1 % (w/v) osmium tetroxide and 0.8 % (w/v) potassium hexacyanoferrate for 2 h. The tissues were further washed with deionized water and dehydrated with a graded series of acetone. The dehydrated samples were then embedded in Eponate 12TM resin (Ted Pella Inc., Redding, CA) and polymerized at 60 °C for 48 h. To get ultrathin sections, midgut tissues were cut with a diamond knife (450, Diatome, Biel, Switzerland). Finally, the sections were mounted upon non-coated copper grids and contrasted with uranyl acetate (30 min) and Reynolds lead citrate (5 min) solutions, respectively to be ready for TEM investigation. TEM (JEOL 1400, 120 kV) was applied to examine PLA-NPLs' existence at the different compartments of the gut including the lumen, the symbiotic bacteria of the gut lumen, and inside the enterocytes. To detect PLA-NPLs in larval hemolymph, larvae were dissected, and the hemolymph was mounted upon carbon girds, dried, and investigated with TEM, as well as with SEM.

(ii) **Confocal microscopy.**

Larvae treated for 4 days with labeled PLA-NPLs (400 μg/g food), were dissected in 1 % PBS, and the midgut tissues and hemolymph were immediately manipulated for confocal investigation. The manipulation includes staining with Hoechst 33342 (excitation of 405 nm and emission collected at 415–503) and Cellmark (excitation of 633 nm and emission collected at 645–786) to visualize nuclei and cellular membranes, respectively. A Leica TCS SP5 confocal microscope, with an excitation wavelength of 561 nm, and emission between 580 and 700 nm was used to investigate the stained tissues for PLA-NPLs incorporation.

2.3.3. *Molecular response (Real-Time PCR)*

Many molecular pathways are involved in the maintenance of cellular homeostasis, and they are extremely sensitive to mild stress changes induced by exposures to exogenous agents. Accordingly, they became sensitive indicators of stressor agents. Consequently, changes in the expression levels of genes representative of i) general stress (*Hsp70Aa* and *Mtna*), ii) oxidative stress (*PHGPX*, *Cat*, *Sod2*, and *Cu/Zn SOD*), iii) genotoxicity and DNA repair (*p53* and *Ogg1*), iv) physical stress-intestinal damage (*Muc68D*, *Duox*, and *Peri*), and v) immunity (*Cyp18a1*, *Toll*, *Imd*, *Def*, *Keap 1*, *Egr*, and *Spz*) were investigated after exposure to PLA-NPLs. The housekeeping *actine 5C* (*Act5C*), as a reference gene, was used for gene normalization. The list of forward and reverse primers (Supplementary Data, Table S1) was designed based on the National Centre for Biotechnology Information NCBI, as well as in the work of Frat et al. (2023). The gene expression was detected according to our previous protocol (Alaraby et al., 2015b). In brief, total RNA extracted from homogenized larvae in TRIzol® Reagent (Invitrogen, Carlsbad, CA) was quantified with a NanoDrop 1000 spectrophotometer and manipulated with RNase-free DNase Kits to discard DNA contamination. RNA samples were reversely transcribed to cDNA (100 ng/μL) that were later polymerized by Quantitative PCR using a LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). The cycling conditions were 95 °C for 1 min, followed by 55 cycles of 95 °C for 1 min, and 65 °C for 30 s. Cycle threshold (Ct) values were calculated with the LightCycler software.

2.3.4. *Oxidative stress induction*

The levels of reactive oxygen species (ROS) in the hemocytes of exposed *Drosophila* larvae were used as indicators of PLA-NPL's ability to induce oxidative stress. The hemolymph of 4 days larvae (~50 larvae are pooled from triplicate per each concentration of PLA-NPLs) was collected and incubated in 5 μM 6-carboxy-2,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) (Alaraby et al., 2020). Using a fluorescent microscope with an excitation of 485 nm and an emission of 530 nm (green filter), various fluorescent images were obtained. The values of ROS were obtained by quantitative analysis of the fluorescent images using the ImageJ program (da Costa Araújo et al., 2020). The mean fluorescent intensity of 10 random field images for each concentration was determined. ROS data from exposed larvae were compared with those of unexposed ones. As a positive control, the hemocytes of untreated larvae were incubated for 30 min with 0.5 mM H_2O_2.

2.3.5. *Genotoxic damage induction*

The percentage of DNA moving from the nucleus to the tail in hemocytes was used as an indicator of the genotoxic potential of PLA-NPLs,

by using the comet assay. Our previous protocol (Alaraby et al., 2019) was followed. In brief, 3-day-old *Drosophila* larvae were transferred, for 24 h, into an instant medium containing 62.5, 250, or 1000 μg/g food of PLA-NPLs. For negative and positive control, Milli-Q water and 4 mM EMS were used, respectively. The hemolymph of about 100 pooled larvae of each PLA-NPL concentration (triplicates) were collected and mixed with 0.75 % low-melting agarose (1:9) at 37 °C. Immediately, drops of hemolymph/gel mixture were distributed upon the hydrophilic surface of Gelbond film (GBF) and lysed for 1 h at 4 °C in a dark chamber. To unwind DNA, GBFs were incubated for 25 min in a cold electrophoresis solution (0.001 M EDTA, 0.3 M NaOH, pH 13.2) before performing electrophoresis for 20 min at 20 V and 300 mA. GBFs were immersed twice in 1%PBS (for 5 min each) and for 1 min in distilled water, to reduce alkalinity. DNA samples were dehydrated with ethanol, stained with SYBERGold, and investigated by using a Komet 5.5 Image-Analysis System (Kinetic Imaging Ltd., Liverpool, UK).

2.4. Statistical analysis

The obtained data were analyzed with the IBM SPSS Statistics 21 package. The normality distribution of data and homogeneity of variance were checked using the Shapiro-Wilk test and Levene's test, respectively. The parametric one-way ANOVA was applied for normally distributed data with equal variance, while Mann-Whitney *U* test and Kruskal-Wallis were applied for data with skewed distribution and unequal variance. The parametric data was represented as mean ± standard deviation, while the median and interquartile range were represented the nonparametric data. Correlation and linear regression analyses were performed for oxidative and genotoxicity data to assess the concentration dependence domain. $P \leq 0.05$ is indicated as the minimum threshold of significant differences.

3. Results and discussion

3.1. PLA-NPLs characterization

Bioplastics have emerged to be an effective and safe alternative to persistent petroleum-based plastics. Nevertheless, their potential detrimental impacts, mainly attributed to their high tendency to produce MNPLs, are not well studied (Zhang et al., 2021). It is important to point out the recent work of Wang et al. (2023) showing that PLA microplastics submitted to *in vitro* digestion, such as that which occurs in the gastrointestinal tract, were degraded into nanoplastics and nanoparticle oligomers, which once administered to mice bioaccumulated in the intestine, liver, and brain, resulting in acute inflammation and intestinal damage. In such context, our study proposes to determine the potential harmful impacts resulting from exposure to PLA-NPLs.

As a first step, in-house generated PLA-NPLs were extensively characterized according to size, morphology, and degree of agglomeration using SEM and TEM (Figs. 1a, b). Both techniques showed PLA particle agglomerations ranging from two or three particles to relatively large patches; the rounded shape, smooth surface, and homogeneity are predominant properties of PLA-NPLs. Based on morphometric analyses of various SEM images processed with Image J software (Fig. 1c), the mean size of PLA particles was determined as 463.9 ± 129.4 nm. The large standard error is due to the occurrence of a few large particles among smaller size-homogenous particles, as appeared in Fig. 1b (indicated by an arrow). This could explain the relatively low hydrodynamic size (Fig. 1d) of PLA-NPLs (425.7 ± 10.22 nm) after measuring thousands of particles in a Zetasizer device. According to the Zeta-potential measurements (Fig. 1d), PLA-NPLs bear a negative moderate charge (−16.6 ± 0.8). Considering the inverse relationship between Zeta-potential and particle aggregation (Liu et al., 2023), the relatively low value of PLA-NPLs Zeta-potential agrees with SEM images, showing aggregation. PLA contains main groups like C═O, —CH_3 asymmetric, —CH_3 symmetric, and C—O (Chieng et al., 2013). Thus, FTIR analysis (Fig. 1e) showed strong IR bands at 2924 and 2884 cm^{-1} that are assigned to —CH_3 asymmetric and —CH_3 symmetric, straight strong beak at 1751

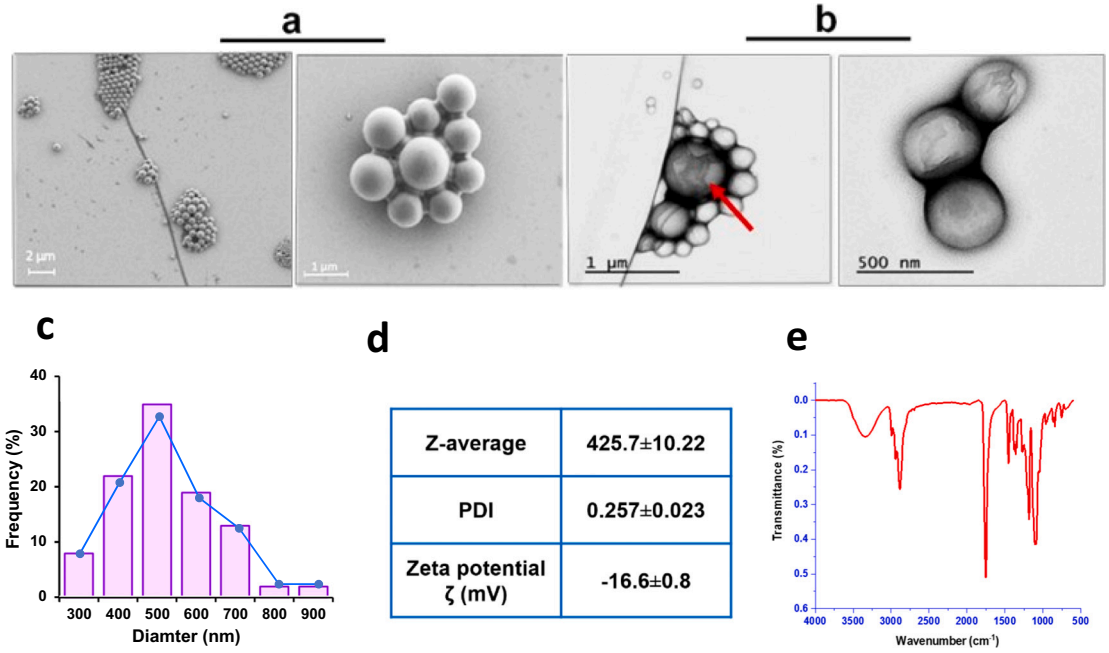

Fig. 1. PLA nanoplastics characterization. a) Two representative SEM images and b) two representative TEM images of PLA-NPLs showing the shape and degree of aggregation of PLA-NPLs. The images indicate some large PLA particles located among smaller ones, indicated by a red arrow. c) The size frequency of PLA measured with image J program with an average of 463.9 ± 129.4 nm. d) The hydrodynamic size and Z-potential of PLA. e) FTIR of PLA indicated the functional groups of its chemical composition.

cm^{-1} for C═O. The short beak appeared at 1452 cm^{-1} and 1380 cm^{-1} attributed to CH_3 and CH, respectively, while those observed at 1104 and 1091 cm^{-1} indicated C—O—C (Vargas-Villagran et al., 2014). Accordingly, FTIR results confirm the chemical nature of PLA-NPLs, belonging to lactic acid.

The categorization of MNPLs between MPLs and NPLs is a conflictive issue (Hartmann et al., 2019). Although the approach used for engineered nanoparticles has been proposed (Bleeker et al., 2013), such a scenario is far away from the characteristics of the secondary MNPLs present in the environment with variability in size and shape. Accordingly, we categorize MNPLs according to the conventional units of size: nano (1–1000 nm) and micro (1–1000 μm) (Villacorta et al., 2023). Consequently, the obtained PLA particles are considered PLA-NPLs.

3.2. Toxicity of PLA-NPLs

To the best of our knowledge, there is only one study (Legaz et al., 2016) that has evaluated the harmful impact of PLA-NPLs in *Drosophila*. Although most of that study used *Drosophila* cells in culture, larval viability was determined after 4 days of feeding. In such a preliminary study, the ingestion of PLA-NPLs (147 nm) was able to reduce larval viability at the highest tested concentration (500 μg/mL). In our study, egg-to-adult viability was accounted as an indicator of survival. Results indicated in supplementary Fig. S1 show that none of the three tested concentrations of PLA-NPLs was able to significantly reduce egg-to-adult viability. Although the emerging adults were carefully investigated to detect possible morphological abnormalities, no developmental effects were observed after PLA-NPL exposure.

Regarding the potentially toxic effects of PLA-NPLs in other *in vivo* models, the survival of *Daphnia magna* exposed to the breakdown products of different PLA goods was determined in an acute toxicity assay. Results showed an extension in the survival rate when the breakdown products of PLA plastic cups were used (Kelpsiene et al., 2023). The authors owed this elevated survival to the potential growth of symbiotic bacterial communities that benefited from the degradation of PLA nanoplastics. Although the survival rate is an important approach to detecting toxicity, often could not reflect other toxicological issues related to the health circumstances of exposed individuals that could appear with aging. In this sense, long-term exposure to PLA-MPs affected *Caenorhabditis elegans'* gonad development and reproduction process (Shao et al., 2023). Thus, PLA-NPLs internalization and their associated impacts are highlighted in detail in the present study.

3.3. Digestion and internalization

TEM was used to follow up the journey of the ingested PLA-NPLs in the intestinal tract. Firstly, PLA-NPLs were observed in the lumen of the four days exposed larvae (Fig. 2a, b), as well as attached and internalized in the symbiotic bacteria of the midgut (Fig. 2c–g). Despite the differences between human and fly intestines, they have unified similarities in terms of main structures, anatomy, and physiological function (Pitsouli et al., 2009). In addition, the chitinous peritrophic matrix of the *Drosophila* gut plays the same role as human gut mucus protecting enterocytes (Apidianakis and Rahme, 2011). Thus, the *Drosophila* intestine is an effective alternative model to the human intestine to explore the interaction and internalization of nano-scaled materials including MNPLs (Shen et al., 2021). Considering, the strong symbiotic relationship between midgut bacteria and their host health, reasonable questions regarding bacteria-MNPLs interaction arise. Herein, in the current study, PLA-NPLs were observed attached to the midgut bacteria (Fig. 2c) and internalized into midgut bacteria mainly through an invagination mechanism (Fig. 2d). Bacterial internalization, contributing to their bioaccumulation, was observed in the form of one particle (Fig. 2e), two particles (Fig. 2f), or in larger numbers (Fig. 2g). It is important to emphasize that the bioaccumulation of PLA-NPLs inside gut bacteria was not observed with other metal and plastic nanoparticles, despite their attachment to their outer membrane (Alaraby et al., 2018; Alaraby et al., 2021; Alaraby et al., 2022b). This could indicate the bacterial tendency to ingest this type of plastic.

It has been indicated that the lactic acid generated by the degradation of PLA might act as a carbon source for intestinal microbiota (Romera-Castillo et al., 2018; Chen et al., 2020). In this sense, it was observed that zebrafish prefer to ingest PLA-MPLs rather than petroleum-based PET-MPLs (Duan et al., 2022), which might be due to

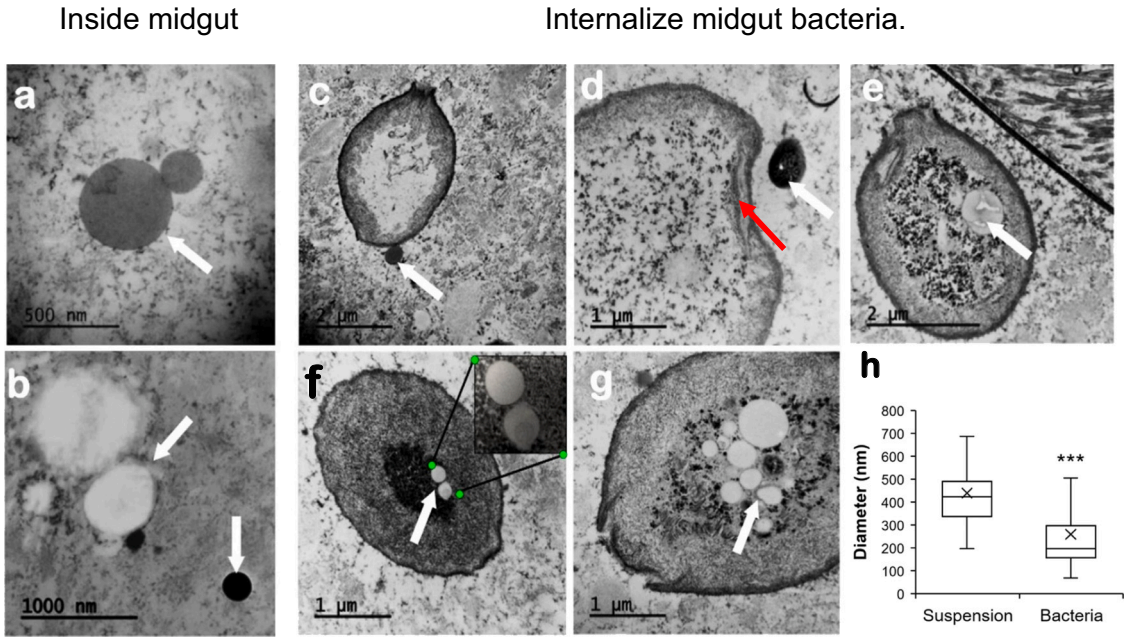

Fig. 2. PLA-NPLs in the midgut after oral administration. a) and b) PLA-NPLs are distributed inside the midgut lumen, where they interact with the midgut bacteria of *Drosophila* larvae (c–h). c) attach to bacteria membrane. d) bacteria amoeboid engulf PLA-NPLs (red arrow indicates the invagination furrow). PLA-NPLs inside bacteria cytoplasm whether a few particles (e and f) or huge particles (g). The median and interquartile range (IQR) of PLA diameter in suspension (424 and 156) and inside midgut bacteria (196 and 145), respectively. ***$p < 0.001$. (Mann-Whitney *U* test).

the specific nature of PLA as a simple organic chemical with a palatable scent (Peng et al., 2021). Thus, bacterial growth was found to be increased by 1.72 times, and their biomass acquisition by 2.29 times, with plastic leachate from plastic bags, due to the accessibility of added carbon natural organic matter (Sheridan et al., 2022). This hypothesis is strongly supported by our findings since the size of the PLA-NPLs observed inside the midgut bacteria was significantly decreased (259.8 ± 166.2 nm) regarding their initial size before internalization (Fig. 2h). This would agree with the results reported in earthworms where PLA microplastics depolymerization in their guts was higher than in the soil (Meng et al., 2023). Although the degradation and surface morphology changes of PLA-MPLs could not alter the homeostasis of the microbial community (Jiménez-Arroyo et al., 2023), they caused specific changes in the intestinal microbiota diversity and cellular disorders, promoting fish diseases (Duan et al., 2022).

As previously indicated, the *in vitro* digestion of PLA-MPLs generated nanoparticulated oligomers *via* enzymatic hydrolysis, which were bioaccumulated in the liver, intestine, and brain of exposed mice (Wang et al., 2023). Accordingly, the erosion of bioplastics in the gastrointestinal tract and their interaction with symbiotic bacteria open new venues about their hazardous impacts on the health of the exposed hosts.

Further, we have explored the potential mechanisms of PLA-NPL internalization into the midgut enterocytes. Fig. 3 describes the different stages of the uptake mechanism. Initially, the particles of PLA distributed in the midgut lumen are surrounded by semi-vacuole membranes (Fig. 3a, b), which drive them to be placed close to the peritrophic membrane (Fig. 3c–e), where they are finally surrounded by intestinal vacuoles (Fig. 3f) to be ready to be internalized by enterocytes. This mechanism has already been observed with TiO_2-NPs (Alaraby et al., 2021), and with polystyrene nanoplastics (Alaraby et al., 2022a). To elucidate PLA-NPLs fate inside enterocytes, several ultrathin sections were investigated. Fig. 4 shows the distribution of PLA-NPLs inside the enterocyte's cytoplasm, which appears in transparent or black dots, associated with many cytoplasmic large vacuoles. Although we treated to reduce the low contrast/density of PLA-NPLs by staining particles with lead acetate, differences in density appear, which are attributed to the section plain, which can be in the top or the bottom of the spheric shape of PLA-NPLs.

The high abundance of PLA-MNPLs inside enterocytes has been associated with the induction of damage in the intestinal epithelial tract of zebrafish (Duan et al., 2022). Finally, the ability of PLA-NPLs to translocate the intestinal barrier was confirmed by its presence in the hemolymph (Fig. 4g, h), as homologous to mammalian blood. Consequently, once PLA-NPLs are translocated into the circulatory system they can be distributed to any tissues and organs.

To confirm the journey described by using TEM/SEM methodologies, the use of fluorescence PLA-NPLs counterpart, associated with the use of confocal microscopy, is of great relevance. Thus, Fig. 5 demonstrates the presence of PLA-NPLs in different compartments throughout the entire intestine (Fig. 5a), the Malpighian tubes (Fig. 5b), as in hemolymph (Fig. 5c).

The demonstrated high internalization ability of PLA-NPLs, together with its easy depolymerization, could generate health detrimental impacts higher than those induced by petroleum-based nanoplastics (Duan et al., 2022).

3.4. The potential impacts of PLA-NPL exposure

Changes observed at the molecular scale are considered early and very sensitive indicators of the detrimental impacts resulting from exposure to exogenous agents, which may appear over the long term and with aging. To understand in depth the molecular response to PLA-NPL exposure, the expressions of several genes representing various mechanisms involved in maintaining cell and organism homeostasis were evaluated. Thus, Fig. 6 shows the expression levels of the 18 selected genes once larvae were exposed to PLA-NPLs. As an indicator of general stress status, it is remarkable the significant changes observed in the expression of the heat shock protein *Hsp70* gene. *Hsp70* is expressed in all cells, both prokaryotic and eukaryotic, to offer protection against any harmful insult (Kiang and Tsokos, 1998). Similar increased effects have

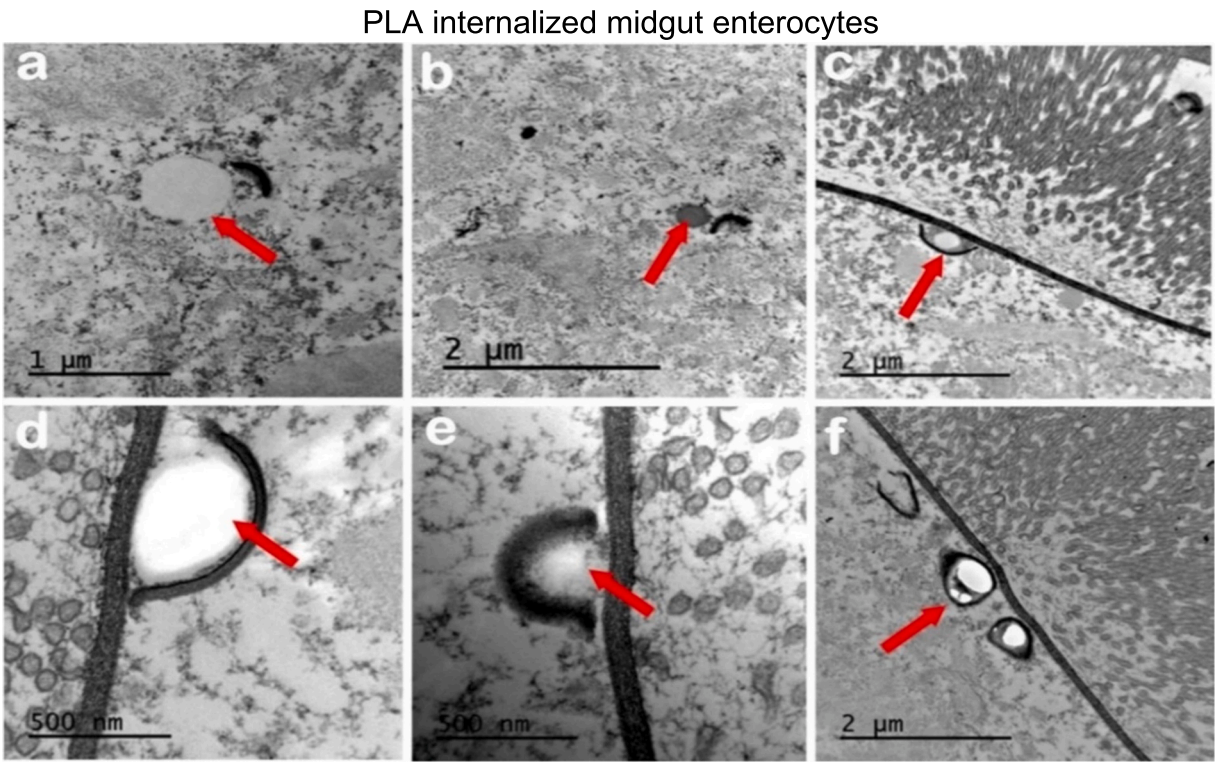

Fig. 3. PLA-NPLs internalization mechanism. a) and b) PLA-NPLs are semi-surrounded by midgut vacuoles. The semi-surrounded PLA-NPLs are pushed close to the peritrophic membrane (c–e). f) PLA-NPLs are surrounded by midgut vacuoles.

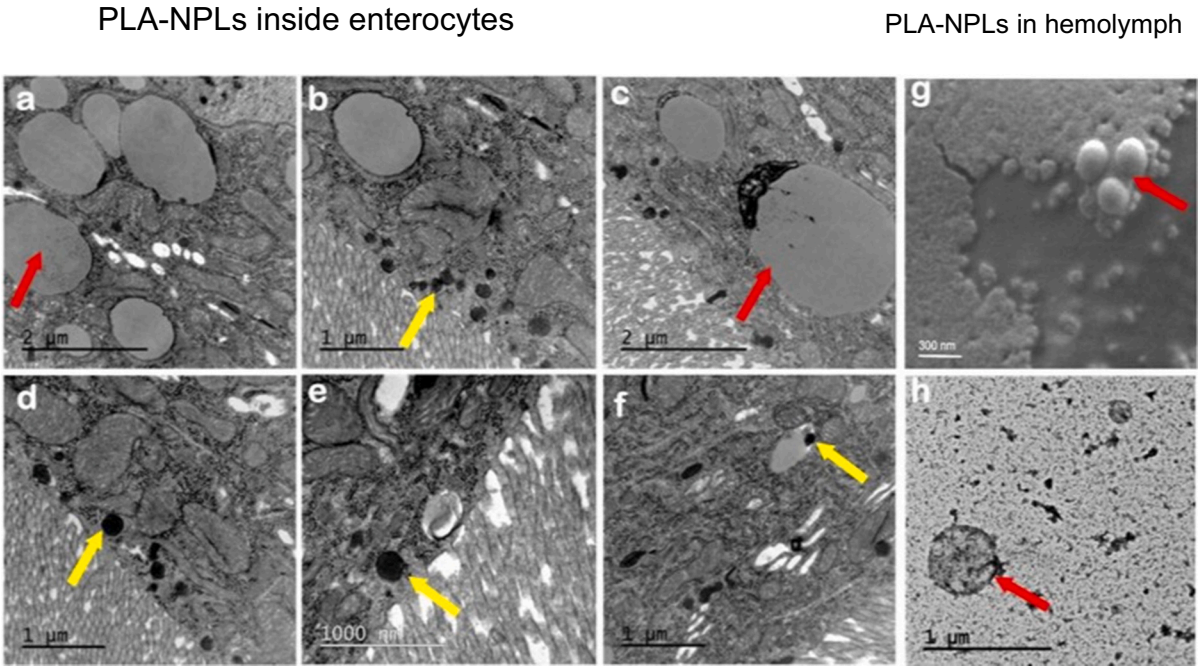

Fig. 4. Distribution of PLA-PNLs in enterocytes (a–f) and hemolymph (h). Midgut enterocytes contain large vacuoles (red arrow) and dense dark smaller rounded vacuoles containing PLA-NPLs (yellow arrow). PLA-NPLs distributed in hemolymph (red arrow) were detected with SEM (g) and TEM (h).

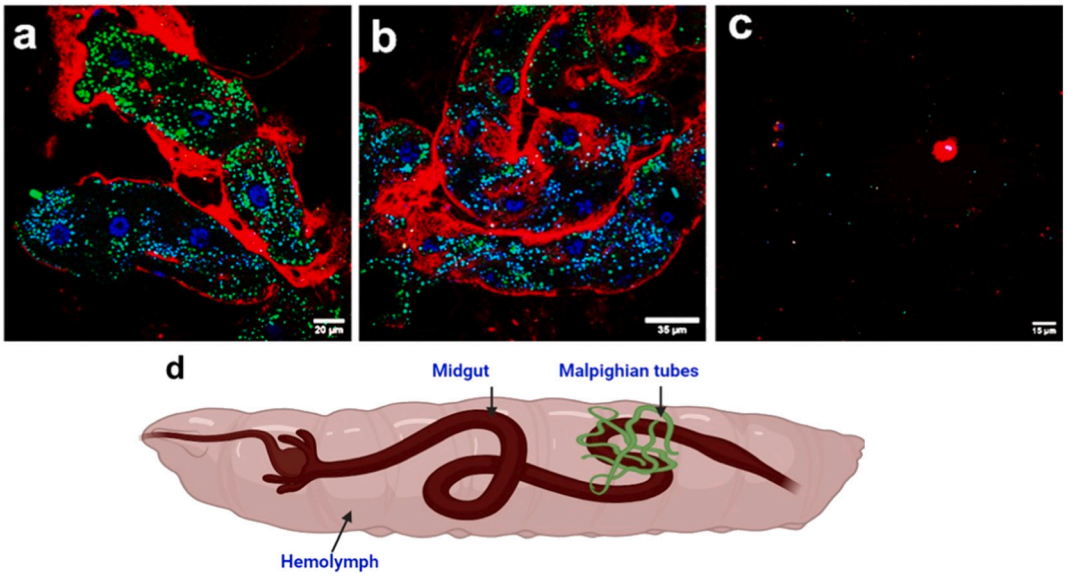

Fig. 5. Confocal fluorescent images of PLA-NPLs in different body organs; a) intestine, b) Malpighian tubes, and c) hemolymph. Schematic drawing of Drosophila larvae showing the major organs.

been reported in earthworms, where the expression levels of the *Hsp70* gene were significantly increased after exposure to PLA-MPLs (Baihetiyaer et al., 2023).

The antioxidant capacity is one of the most important defense mechanisms, considering its direct relation with the maintenance of the redox balance and mitigation of oxidative stress (Matés et al., 1999). Exposure to PLA-NPLs affects the activity of all studied antioxidant genes since the expressions of glutathione peroxidase (*PHGPX*), catalase (*Cat*), superoxide dismutase 2 (*Mn Sod2*), and superoxide dismutase 1 (*Cu/Zn Sod*) were significantly up-regulated with almost all the tested concentrations. This set of antioxidant genes constitutes important cellular biomarkers against xenobiotic-mediated oxidative stress, by reducing the high to toxic O_2^- into the less toxic H_2O_2, and finally into safe molecules (O_2 and H_2O) (Kurutas, 2015; Ighodaro and Akinloye, 2018).

Like in our findings, exposure to PLA-BioMPLs also mediated increases in the levels of SOD, catalase, and glutathione in tadpoles (Malafaia et al., 2021). It is important to point out the strong relationship between MNPL accumulation with the immune response, energy metabolism, and oxidative stress (Kim et al., 2021). Hence, the intestinal damage resulting from MNPL accumulation has been indicated as one potential scenario responsible for oxidative stress induction (Lei et al., 2018). In this sense, our results showed the ability of PLA-NPLs to alter the expression of genes responsible for keeping the integrity of the intestinal barrier, as is the case of *Duox* which is highly expressed at all PLA-NPLs concentrations. Duox is a central factor in the regulation of

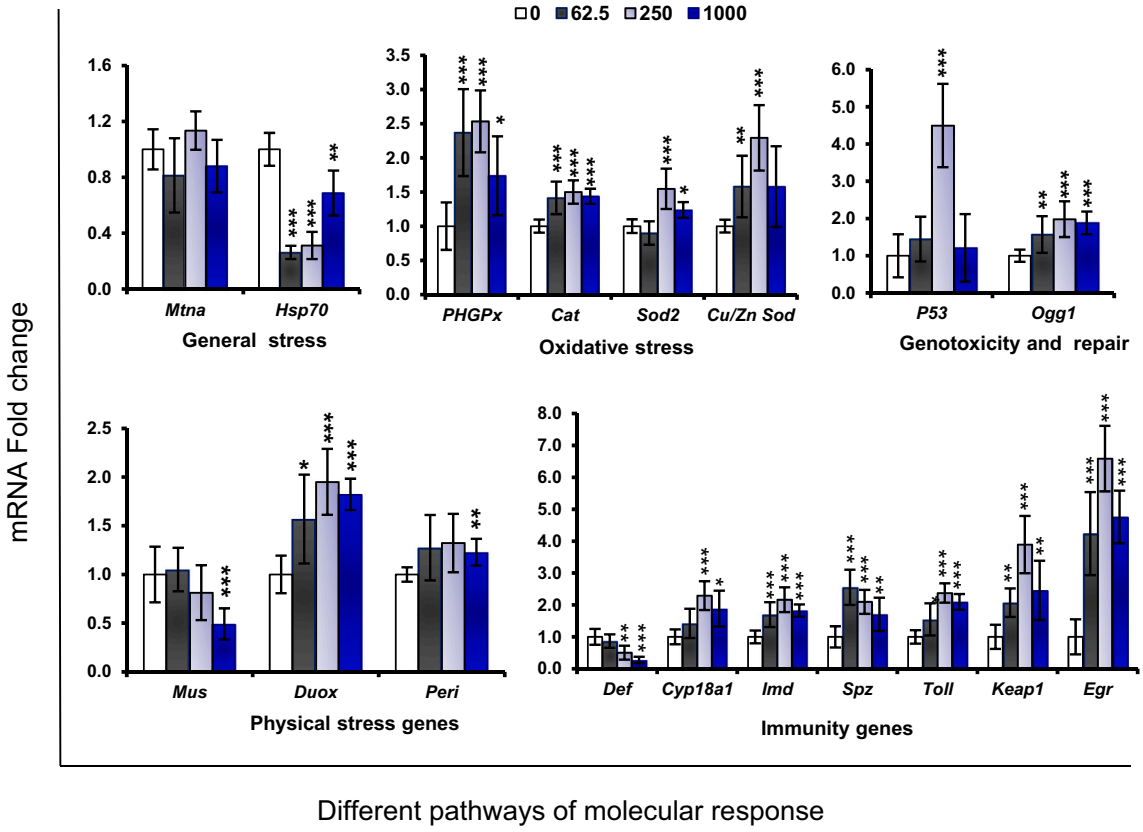

Fig. 6. The molecular response to PLA-NPL exposure. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (One-Way ANOVA test for parametric data and Mann-Whitney *U* test for non-parametric data, six independent replicates).

gut defense, as well as in gut permeability and wound healing (Kim and Lee, 2014), and it is found widely expressed after the impacts of nano-sized particles (Alaraby et al., 2016b).

Immunity is a robust mechanism that is mainly activated to attack the presence of xenobiotics or toxicants, including particulate nanosized materials (Jin et al., 2018). If the inflammation is not balanced or controlled, destructive disorders and dangerous diseases can be generated (Grivennikov et al., 2010). Considering that the immune system intrinsically recognizes micro- and nano-plastic particles as xenobiotics, variable inflammatory response endpoints of petroleum-based MNPLs have been detected, such as serious histopathological lesions and immune dysfunction (Ahrendt et al., 2020; Kang et al., 2021). However, information on the inflammation impacts of biodegradable MNPLs is very scary, particularly for NPLs. In a recent study, PLA-MPLs induced inflammation and infiltration of the small intestine, colon, and liver of exposed rats (Wang et al., 2023). The authors indicated the gradual leachate of oligomeric nanoparticles from PLA-MPLs, which permeate the intestinal tract and are associated with adverse inflammation effects In the present study, a set of inflammation biomarkers was employed to discover the immune response associated with PLA-NPL exposure. Our findings show that exposure to PLA-NPLs induces a wide upregulation in the battery of inflammation and immunity response genes including cytochrome P450 (*Cyp18a1*), Toll (*Toll*), immune deficiency (*Imd*), Defensine (*Def*), Keap 1 (*Keap 1*), Eiger (*Egr*), and Spatzle (*Spz*). This wide inflammation pattern could correlate with the wide distribution of PLA-NPLs in the body tissues of *Drosophila* larvae, especially in the intestine. Hence, it has been reported that PLA-BioMPL accumulation mediated various physiological changes/adaptations (Chagas et al., 2021). In addition, exposure to PLA-MPLs enhanced germline apoptosis through increased expressions of *egl-1*, *ced-3*, and *ced-4*, while decreasing the expression of *ced-9* in *Caenorhabditis elegans* (Shao et al., 2023).

DNA damage is one of the most severe toxic effects of environmental agents. To address whether PLA-NPLs can trigger disturbances in genes related to genotoxicity and repair mechanisms, *p53* and *Ogg1* expression levels were evaluated. Our results show that, although the nanoparticles of PLA have a low impact on the expression of the *p53* tumor suppressor gene, they stimulated the expression of the repair gene *Ogg1* (8-oxo-guanine DNA glycosylase) regardless of PLA concentrations. Ogg1 is a complex glycosylase included in the BER pathway that is extensively expressed against DNA oxidative damage (Radak and Boldogh, 2010), where it recognizes and detaches damaged bases from DNA (Klungland and Bjelland, 2007). The deregulation of *Ogg1* has been associated with exposure to conventional MNPLs (Feng et al., 2022; Alaraby et al., 2022a), but no data exist associated with bioplastic exposures. Therefore, our findings highlight the relevance of the BER pathway activation to defend against the oxidative DNA damage induced by PLA-NPLs exposure.

3.5. *Oxidative stress and genotoxicity*

Oxidative stress and genotoxicity are inevitable consequences of disturbing molecular homeostasis and continuous inflammations (Khanna et al., 2015). Several pieces of literature have reported the strong relationship between conventional MNPL exposure and oxidative stress induction, never mind the variation in polymer type, surface characteristics, sizes, concentrations, or exposure times (Hu and Palić, 2020).

Despite the very little data linking oxidative stress induction and bioplastics exposure, this could follow the same pattern that conventional petroleum-based ones, as micro/nano-scaled particles. Nevertheless, higher toxicological consequences have been proposed for bioplastics, according to their high ability to disintegrate into MNPLs (Ali et al., 2023) and their bio-preference (Duan et al., 2022).

However, the precise mechanisms leading to oxidative stress triggered by exposure to MNPLs are not easy to determine. Among the potential scenarios, homeostasis disturbance (Zheng et al., 2019), physical and chemical toxicity related to bioaccumulation (Solomando et al., 2020), and mitochondrial structural damage/depolarization (Zhang et al., 2021) have been proposed. Interestingly, our results revealed that PLA-NPL exposure raised the level of oxidative stress in the hemocytes of *Drosophila* larvae (Fig. 7), and the effects correlate significantly ($p < 0.001$, $r = 0.641$) with concentration increases (Fig. S2). This high oxidative stress mediated by PLA-NPLs might be associated with the high expression of antioxidant enzymes and the high immunity response previously reported. Hence, oxidative stress generation is linked with the antioxidant incapacity to neutralize ROS production, generating a redox imbalance (Malafaia et al., 2021; Chagas et al., 2021). On the other hand, oxidative stress could also be produced in association with the immune response that is generated to struggle with the exitance of MPNLs as foreign particles (Hu and Palić, 2020). Biodegradation of MNPLs (specifically PLA-MNPLs) is another possibility of oxidative stress induction. Hence, biodegraded particles have more surface-to-mass ratio, and their small size permitted their easier translocation through cellular organelles as well as interaction with lipid membranes that facilitated the generation, absorption, and translocation of free radicals (Hu and Palić, 2020). This has been recently reported in greater wax moths (*Galleria mellonella*) where feeding with PLA caused some metabolic stress, mediated oxidative stress, disturbances in metabolism, and inflammation (Shah et al., 2023).

Among the different biomarkers of effect, genotoxicity stands out. Thus, due to the relevance of DNA driving cell functionality, any damage to cellular DNA can drastically compromise human health (Carbone et al., 2020). Accordingly, genotoxicity must be determined when testing the potential health effects of emergent pollutants, as is the case of bioplastics. Possibly because the interest in exploring the potential impacts of MNPLs originating from bioplastics is very recent, the data about their genotoxic impact is almost lacking. This highlights the relevance of our results. As indicated in Fig. 8, exposure to PLA-NPLs was able to induce significant levels of DNA damage in the hemocytes of exposed *Drosophila* larvae. Interestingly, these values show a significant correlation ($p < 0.001$, $r = 0.529$) with the concentration increases (Fig. S3). In general, the genotoxicity of MNPLs is a hot topic mainly due to the role of genotoxicity as a surrogate biomarker of carcinogenesis. In this regard, exposure to polystyrene (PS) MNPLs showed DNA damage *via* the formation of micronuclei and nuclear buds (Poma et al., 2019), or the induction of significant levels of DNA strand breaks (Ballesteros et al., 2020). Interestingly, PS-NPL exposure mediated DNA damage, even at relatively low concentrations (Brandts et al., 2022). To mimic the effects of environmental MNPLs, true-to-life MNPLs obtained from PET water bottles have been used to determine their genotoxicity, showing positive effects (Roursgaard et al., 2022; Alaraby et al., 2023). Moreover, DNA damage could be generated by MNPLs aging (Chen et al., 2022).

Despite the lack of references on the potential genotoxicity of bioplastics MNPLs, we can assume a similar genotoxic behavior to MNPLs resulting from fossil fuel-based plastics. The mechanism of MNPLs-mediated genotoxicity seems to be closely related to inflammatory, oxidative stress, and DNA repair disturbances (Tagorti and Kaya, 2022). Nevertheless, the low levels of ROS observed in several cases of nano-sized materials would suggest the direct physical interaction with DNA, or with its replication machinery, as alternative mechanisms (Roursgaard et al., 2022; Joksimovic et al., 2022). It should emphasize the relevance of the used cells (hemocytes) present in the hemolymph, playing a relevant role in the phagocytosis of invading organisms. This ability means that they can easily internalize nanoparticles, as is the case of PLA-NPLs. A similar role is assigned to coelomocytes which are phagocytic leukocytes that appear in the bodies of animals that have a coelom, like sea urchins. In this last model, ZnO-NPs administered in the food provoked genotoxicity and transmissible effects to offspring due to DNA damage of male gametes (Manzo et al., 2017).

Our results would confirm the potential genotoxicity of PLA-NPLs, possibly mediated by oxidative stress, Accordingly, the observed genotoxicity of PLA-NPLs raises health concerns about bioplastics requiring further studies before extending their uses as alternative candidates to petroleum-based plastics.

4. Conclusions

The current study aimed to define the potential health hazards associated with the exposure to MNPLs of bioplastic origin. To such end, in-house nanoplastics from PLA pellets, as representative of bioplastics, were obtained and extensively characterized. In addition, by using *Drosophila*, as a representative *in vivo* model organism, a wide set of biomarkers of effects have been evaluated.

The advantage of using *Drosophila* is that it allows us to follow the journey of PLA-NPLs from its presence in the intestine lumen to its presence in hemolymph (as equivalent to human blood). Thus, PLA-

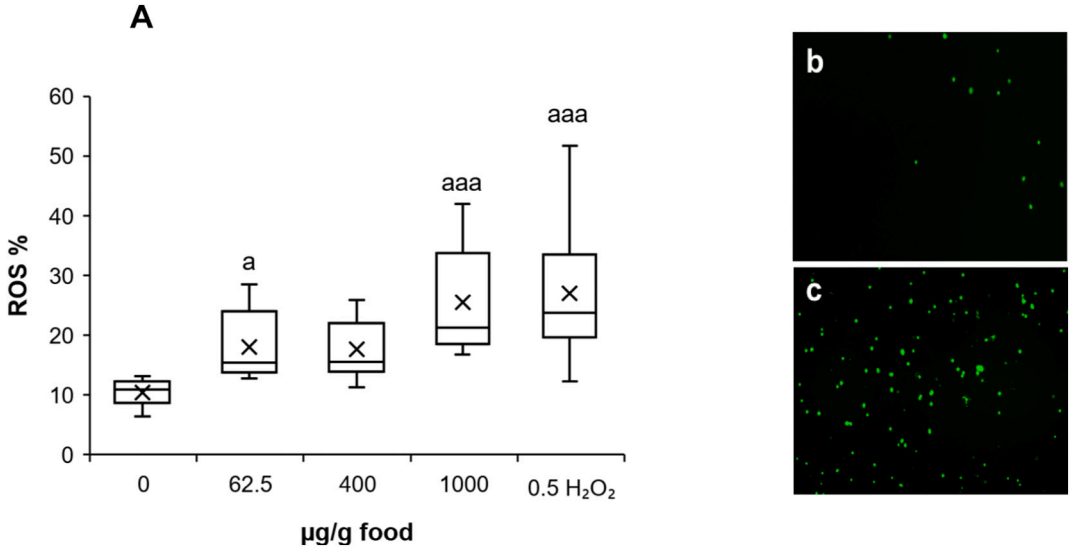

Fig. 7. Oxidative stress induced by PLA-NPLs exposure (A). Representative fluorescent images showing hemocytes with low (b) and high ROS levels (c). *$p < 0.05$ and ***$p < 0.001$, Multiple pairwise comparisons were performed using Kruskal-Wallis.

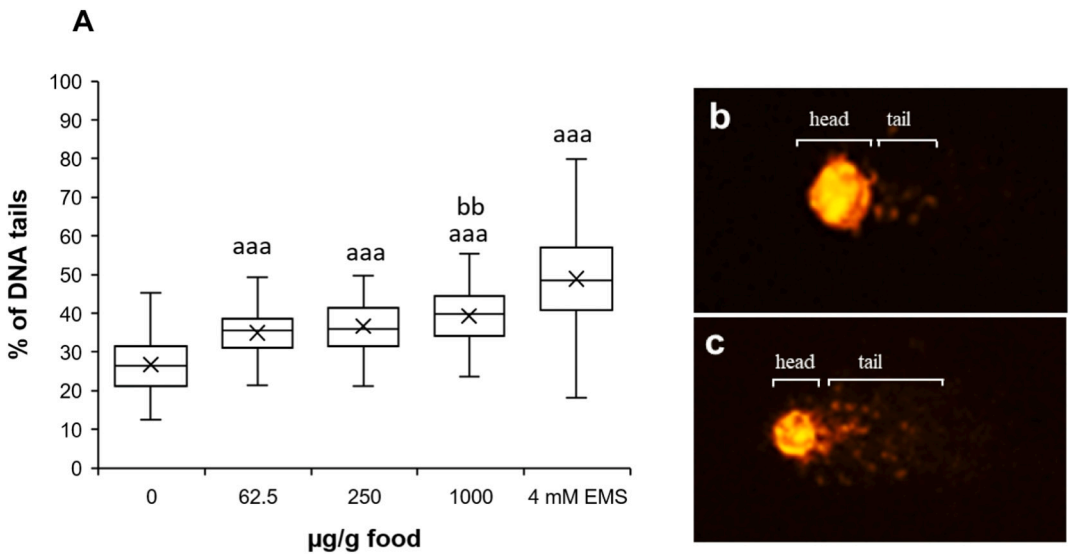

Fig. 8. Percentage of DNA in the comet tails with different concentrations of PLA-NPLs (A). Representative fluorescent images showed a single hemocyte with a low comet (b) or high comet tail (c). $^{aaa}p < 0.001$ *vs* negative control and $^{bb}p < 0.01$ *vs* 62.5 µg/g food PLA. Multiple pairwise comparisons were performed using Kruskal-Wallis.

NPLs have been shown to translocate the intestinal barrier, which is a red flag about their potential to be internalized into exposed organisms, including humans. Furthermore, such internalization triggers a wide molecular response affecting the expression of genes involved in different pathways, which is also a red flag regarding their potentially hazardous effects. In this context and considering that damage induction to cellular DNA can drastically compromise human health, our findings showing that PLA-NPLs exert an important genotoxic response, are of relevance to define the safety of PLA-NPLs exposure, and by extension of other MNPLs resulting from bioplastics degradation.

Thus, our study gives reasonable objections to the expanding uses of bioplastics. Consequently, further *in vitro* and *in vivo* studies are required to confirm the potential health risks associated with exposure to their MNPLs.

Despite the above indicated, and although the experiments were well designed, there are always aspects that can pose limitations to the obtained results (Sumpter et al., 2023).

Thus, i) although two experiments with different replicates and concentrations were used, it is obvious that more experiments and more individuals scored could improve the quality of the obtained data. ii) Although we have been working with nontoxic concentrations, no data exist on the range of concentrations present in the different environmental compartments. If we assume that the used concentrations are higher than those found in real scenarios, this would limit the relevance of the obtained data. Nevertheless, this did not affect the involved mechanisms of action. iii) Most of the studies only know the nominal concentrations used, and no information on the effective dose/concentration is known. In our case, although we do not know the concentration reaching cell/tissue target, we apport interesting data supporting cell internalization of PLA-NPLs and its ability to cross the intestinal barrier and deposit in hemolymph, as equivalent to blood in humans. iv) Although for some end-points there is uncertainty over how meaningful they are to individual health, genotoxicity, as a surrogate biomarker of carcinogenesis, plays a well-known role in human health.

CRediT authorship contribution statement

Mohamed Alaraby: Writing – original draft, Methodology, Data curation. **Doaa Abass:** Methodology, Data curation. **Marinella Farre:** Supervision. **Alba Hernández:** Writing – review & editing, Conceptualization. **Ricard Marcos:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alba Hernandez reports financial support, equipment, drugs, or supplies, and travel were provided by H2020 Food Security Sustainable Agriculture and Forestry Marine Maritime and Inland Water Research and the Bioeconomy. Alba Hernandez reports financial support, equipment, drugs, or supplies, and travel were provided by Ministry of Science Technology and Innovations. No has patent no pending to no. no If there are other authors, they 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170592.

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