

Research article

Biodegradation of polyvinyl chloride, polystyrene, and polylactic acid microplastics in *Tenebrio molitor* larvae: Physiological responses



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ABSTRACT

It is widely understood that microplastics (MPs) can induce various biological stresses in macroinvertebrates that are incapable of biodegrading plastics. However, the biodegradation and physiological responses of plastic-degrading macroinvertebrates toward MPs of different degradability levels remain unexplored. In this study, *Tenebrio molitor* larvae (mealworms) were selected as a model of plastics-degrading macroinvertebrate, and were tested against three common plastics of different degradability rankings: polyvinyl chloride (PVC), polystyrene (PS), and polylactic acid (PLA) MPs (size <300 µm). These three MPs were biodegraded with the rate sequence of PLA > PS > PVC, resulting in a reversed order of negative physiological responses (body weight loss, decreased survival, and biomass depletion) of mealworms. Simultaneously, the levels of reactive oxygen species (ROS), antioxidant enzyme activities, and lipid peroxidation were uniformly increased as polymer degradability decreased and intermediate toxicity increased. PVC MPs exhibited higher toxicity than the other two polymers. The oxidative stresses were effectively alleviated by supplementing co-diet bran. The *T. molitor* larvae fed with PLA plus bran showed sustainable growth without an increase in oxidative stress. The results provide new insights into the biotoxicity of MPs on macroinvertebrates and offer comprehensive information on the physiological stress responses of plastic-degrading macroinvertebrates during the biodegradation of plastics with different degradability levels.

1. Introduction

In 2021, production of petroleum-based plastics reached 390 million metric tons (PlasticsEurope, 2022). Six major polymer products, which contribute to those numbers, include polyethylene (PE), 31.3%; polypropylene (PP), 19.7%; polyvinyl chloride (PVC), 9.6%; polyethylene terephthalate (PET), 8.4%; polyurethane (PUR), 7.8%; and polystyrene (PS), 6.1% (PlasticsEurope, 2022). These plastics are resistant to degradation in the environment and have consequently been considered a major environmental concern for decades. Recently, microplastics (MPs) and nanoplastics (NPs) were listed as emerging pollutants in the

environment (Wang et al., 2021; Wu et al., 2017). Biodegradable plastics such as polylactic acid (PLA), poly(butylene adipate-co-terephthalate) (PBAT), polyhydroxyalkonates (PHA), etc., have been developed and applied as an alternative to petro-derived plastics (Haider et al., 2018; Lambert and Wagner, 2017), and contributed to about 1.5% of global plastics production in 2021 (PlasticsEurope, 2022). PLA, the most popular biodegradable/compostable polymer, reached 350,000 tons in global production in 2022, with an expected growth of 14.14% annually until 2035 (Chemanalyst, 2023). PLA biodegradation can be achieved under composting conditions (>60 °C, in the presence of moisture), but occurs very slowly under ambient environmental conditions (Chamas

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et al., 2020; Haider et al., 2018). Some researchers have expressed concern about the accumulation of discarded PLA wastes, and other biodegradable polymer products in the environment (Bagheri et al., 2017; Haider et al., 2018).

Plastic waste can be fragmented into MPs through biotic and abiotic reactions, leading to bioaccumulation in organisms, induction of various biotoxic effects, and disturbances of food webs in the environment (Lin et al., 2022; Petersen et al., 2022; Pfohl et al., 2022; Wang et al., 2022a). Macroinvertebrates ingesting plastic debris or MPs may simultaneously experience inflammatory responses and physical damage, possibly generating and releasing smaller-sized MPs or NPs. For example, PE MPs were found to be digestively triturated into NPs (less than 1 μm) in Antarctic krill (*Euphausia superba*), which increased the capacity of fragments for crossing biological barriers (Dawson et al., 2018). Earthworms (*Eisenia andrei*) exposed to different sizes of PE MPs also produced NPs (180–364 nm) in their intestines, which damaged spermatogenesis and coelomocyte viability (Kwak and An, 2021). Woodlice (*Porcellio scaber*) and enchytraeids (*Enchytraeus crypticus*) also ingested and biofragmented plastics (Jemec Kokalj et al., 2022). As micro(nano)plastics are emerging particulate anthropogenic contaminants, the physiological stress responses of macroinvertebrates to micro(nano)plastics exposure have also become an emerging concern.

Researchers have been contemplating innovative biodegradation techniques for the sustainable management and treatment of plastic wastes. During the last ten years, some macroinvertebrates (especially insect larvae) capable of biodegrading or digesting lignocellulosic materials or beeswax were found capable of biodegrading petroleum-based plastics via digestive enzymes in their gut. These plastic-degrading macroinvertebrates mainly belong to the darkling beetle family (Coleoptera: Tenebrionidae) and pyralid moth family (Lepidoptera: Pyralidae) (Wu and Criddle, 2021). *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) larvae, or yellow mealworms, are commercially available as animal feed (Hong et al., 2020). They bite, chew, and ingest plastic foam, film, and microplastic pieces, and are the first insect larvae found to demonstrate biodegradation of PS (Yang et al., 2015), with the larvae around the world demonstrating the same PS-degrading capabilities (Yang et al., 2018b). Besides PS, *T. molitor* larvae have shown to be capable of efficiently biodegrading a wide variety of other petroleum-based plastic products, including PE (Brandon et al., 2018), PS (Peng et al., 2019), PVC (Peng et al., 2020a), PP (Yang et al., 2020), and PUR (Liu et al., 2022a), through different gut-microbial-mediated mechanisms. They also biodegrade the biodegradable polymers such as PLA and poly(butylene adipate-co-terephthalate) (PBAT) products and assimilate the intermediates for energy supplementation (Peng et al., 2021, 2023). Thus, they are an ideal model insect species for investigating plastic biodegradation. Other commonly studied darkling beetles in this field are superworms (*Zophobas atratus*) and dark mealworms (*Tenebrio obscurus*) (Peng et al., 2019, 2022a).

Plastic biodegradation and conversion by macroinvertebrates, especially insects, comprise plastic biofragmentation processes via their mouthparts and intestines by biting, chewing, and gut contraction, which may generate smaller-sized particles such as MPs even NPs (So et al., 2022). To date, research on micro(nano)plastics-induced physiological stress responses in macroinvertebrates has primarily investigated the oxidative stress and metabolic dysbiosis in target macroinvertebrates without plastic-degrading abilities (e.g., Antarctic krill) (Dawson et al., 2018) or only with the ability of biodegrading degradable polymers (e.g., earthworms) (Meng et al., 2023), rarely providing insights into the macroinvertebrates with exceptional plastic-degrading abilities during plastic biodegradation. Furthermore, previously published studies typically reported the results of single polymers (e.g., PE MPs or PS NPs) (Li et al., 2021; Wang, H. et al., 2022a,b), but did not compare polymers with different ranked degradability and the influence of polymer degradability on stress or toxicology. Consequently, the oxidative stress and physiological responses of plastic-degrading macroinvertebrates to the ingestion and biodegradation of MPs with

different ranked polymer degradability remain unknown, and the scientific gap needs to be filled with a deep and comprehensive assessment.

Therefore, we hypothesize that a) physiological responses, including survival, growth, and development, of macroinvertebrates with exceptional plastic-degrading abilities to micro(nano)plastics exposure could differ from those of non-plastic-degrading macroinvertebrates; b) oxidative stress in plastic-degrading macroinvertebrates could be inversely related to the degradability of polymers; and c) the enhancement of endogenous metabolic activities by supplementing exogenous food source or energetic/nutrition-rich co-diet could alleviate the toxicity caused by micro(nano)plastics. To test these hypotheses, we selected *Tenebrio molitor* larvae as a model macroinvertebrate and then picked three common plastics with different ranked degradability, i.e., PVC, PS, and PLA, to comprehensively evaluate the stress responses and physiological performances of macroinvertebrates. We tested the physiological responses, including survival rate, average weight, and biomass nutrient content of *T. molitor* larvae, over 6 weeks of PVC, PS, and PLA feeding and then determined the plastic mass reduction, depolymerization, and size distribution of residual polymer particles in the frass. Biochemical indicators, including reactive oxygen species (ROS), antioxidant enzyme activities, and lipid peroxidation were systematically investigated to provide critical information to understanding of the oxidative stress in larvae during plastic biodegradation. Lastly, the effects of supplementing wheat bran on physiological performance and homeostasis were examined. The results of this study filled knowledge gaps in the toxicology of MPs to macroinvertebrates and provided comprehensive information on the physiological stress responses of plastic-degrading macroinvertebrates during the biodegradation of plastics with different ranked degradability.

2. Materials and methods

The experimental procedures and analytical methods followed previously established methods and protocols, which were recommended for the biodegradation of plastics in insect larvae (Wu and Criddle, 2021).

2.1. Materials and chemicals

PVC, PS, and PLA plastic powder products (size <300 μm) were purchased from Zhonglian Petrochemical Co., Guangdong, China (Supporting Information as Text S1). According to our laboratory analysis, the three plastic materials did not contain additives (phthalic acids <0.5 ppm) (Table S1) and were of high purity (Table S2). For PVC, PS, and PLA polymers, the number average molecular weight (M_n) was 68.4 ± 5.1 , 78.8 ± 3.3 , and 30.5 ± 2.3 kDa; the weight average molecular weight (M_w) was 122.8 ± 3.1 , 194.7 ± 5.4 , and 47.3 ± 1.8 kDa; and the size average molecular weight (M_z) was 170.1 ± 8.4 , 271.3 ± 12.0 , and 52.9 ± 1.9 kDa, respectively.

2.2. Preparation of insect larvae for microplastic exposure

Tenebrio molitor larvae were purchased from Binzhou Mealworm Co. (Shandong, China). All tested larvae were 3–4 larval instars (in the rapid growth period) with an initial average weight of 71.6 ± 2.2 mg and were sensitive to external abiotic disturbance (Table S3). Wheat bran was purchased from the same company. The element ratio of C: H: O: N in the bran was 40.1: 7.7: 47.3: 3.4 according to the analytical results using an elemental analyzer (Vario EL cube, Elementar, Germany) in our laboratory.

Eight treatments were prepared in triplicate based on the diet conditions (replicates, $n = 3$), i.e., PVC, PVC + bran, PS, PS + bran, PLA, PLA + bran, bran, and unfed. The test period lasted 6 weeks in order to avoid pupation of *T. molitor* larvae. For the supplementation of exogenous food source or co-diet (bran), the mass ratio of respective plastics versus wheat bran was selected at 1:5 (w/w) in accordance with

previous results (Text S2) (Peng et al., 2021; Yang et al., 2018a). The larvae were reared in smooth-walled glass containers (15 cm × 15 cm × 6 cm) as incubators. Each container was initiated with 100 depurated larvae that were chosen at random, along with 0.8 g of the respective feedstock (MPs only or MPs plus bran). When the feedstock was depleted, an additional 1.0 g of feedstock was supplemented to the container. The feedstock was provided limitlessly, and the exoskeletons of the larvae were removed from the container every day. Dead larvae were removed from the containers to prevent cannibalism and any possible intraspecific diseases. All glass containers were kept in an incubator maintained at 25 °C and 70% humidity. The experimental procedures and conditions followed previously established protocols (Wu and Criddle, 2021).

2.3. Evaluation of the physiological stress responses of *T. molitor* larvae

The changes in survival indexes, including the survival rates and average weight of larvae, were determined weekly by weighing and counting all *T. molitor* larvae in the containers. The total plastics consumed, total frass generated, and specific plastic consumption rates (SPCRs) were measured and calculated only at the end of the test (day 42) (Text S3).

After 6 weeks of continuous feeding, the biomass nutrient content of the larvae was measured (replicates, n = 3), including the water, total sugar, fat, crude fat (including fat, organic acids, fat-soluble vitamins, biochromes, etc.), protein, and crude protein (including protein and other nitrogen substances) contents, in order to further investigate the physiological stress responses of larvae after plastic biodegradation. The test protocols were based on the food safety testing standards (Text S4) (Tsouchatzis et al., 2022; Turck et al., 2021). The biomass nutrient content was further calculated and normalized to evaluate the nutrient status of larvae, with the result presented on the radar map.

2.4. Analytical methods

To collect egested frass for analyses, additional clean glass containers were prepared under the same incubation conditions (Text S5). The water-extracted fraction (C_w), ethanol-extracted fraction (C_e), and organic solvent-extracted fraction (C_o) of frass were determined via sequential extraction. Tetrahydrofuran (THF) was used for the extraction of PS and PVC polymers, and dichloromethane (DCM) for the PLA polymer. The respective plastic removal efficiency and estimated plastic mass reduction were calculated (Text S6) using established protocols (Peng et al., 2021, 2022a; Wu and Criddle, 2021).

To identify the depolymerization and biodegradation of the plastics, gel permeation chromatography (GPC) analysis was performed to determine the molecular weight changes (Wu and Criddle, 2021). In brief, the frass sample (0.20 g) was extracted with 20 mL of the organic solvent (THF for PS and PVC and DCM for PLA) at room temperature. After filtration, the solution was mixed and evaporated on a magnetic stirrer with gentle heating at 60 °C to concentrate the solution to about 5 mg/mL. The residual extract was then injected (200 µL) into the GPC analyzer (eluent flow rate: 0.8 mL/min, temperature: 30 °C). The polydispersity index was calculated as $PDI = M_w/M_n$ (Peng et al., 2022a, b). All GPC analyses were performed in triplicate. Other analytical methods, such as Fourier transform infrared (FT-IR) spectra and X-ray diffraction (XRD) analysis, could also be employed to characterize the biodegradation of microplastics in future studies (Hojjati-Najafabadi et al., 2023a, 2023b; Xia et al., 2023).

To determine the size distribution of the residual plastic particles, the frass was first mixed with a 30% H₂O₂ solution for 24 h at 60 °C to completely digest organic matter. The residual solids were rinsed with DI water and then filtered through a PTFE film filter (pore size: 0.2 µm). The filtered solids were carefully collected and used to determine the size distribution of residual plastics in frass using a laser light scattering particle sizer (Malvern Mastersizer 3000, Malvern Panalytical, UK) with

measurement range from 0.01 to 3500 µm.

2.5. Determination of ROS, antioxidant enzymes, and lipid peroxidation levels

The ROS content in larval biomass was analyzed using a DHE assay kit (HR8821) from Biolab Technology Co., Ltd. (Beijing, China). The intensity of ROS in larval biomass was expressed by fluorescence intensity per mg protein. The larvae under different feeding conditions (bran, PS, PS + bran, PVC, PVC + bran, PLA, and PLA + bran) were randomly selected, cleaned, ground by a glass grinder with a homogenate buffer, and then subjected to cell disruption using a cell disruptor. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was incubated at 37 °C for 30 min, and prepared for bicinchoninic acid (BCA) protein quantification and ROS fluorescence detection with excitation wavelength of 488–535 nm and emission wavelength of 610 nm. A fluorescence microplate reader (VICTOR Nivo Alpha Multimode microplate reader, PerkinElmer) was used to measure the ROS concentration.

The superoxide dismutase (SOD) in the larvae under different feeding conditions was analyzed using the SOD enzyme assay kit (KTB1030) from Abbkine Scientific Co., Ltd. (Shanghai, China). A microplate reader (Infinite M nano, Tecan Trading Co., Ltd.) with an absorbance of 450 nm was used to measure the concentration.

The glutathione-S-transferase (GST) in the larvae under different feeding conditions was analyzed using the GST enzyme assay kit (KTB1630) from Abbkine Scientific Co., Ltd. A microplate reader (Infinite M nano, Tecan Trading Co., Ltd.) with an absorbance of 340 nm was used to measure the concentration.

The catalase (CAT) in the larvae under different feeding conditions was analyzed using the CAT enzyme assay kit (KTB1040) from Abbkine Scientific Co., Ltd. (Shanghai, China). A microplate reader (Infinite M nano, Tecan Trading Co., Ltd.) with an absorbance of 450 nm was used to measure the concentration.

The lipid peroxidation levels of the larvae under different feeding conditions were determined by analyzing the malondialdehyde (MDA) in larvae using the MDA assay kit (KTB1050). The kit was purchased from Abbkine Scientific Co., Ltd. (Shanghai, China). A microplate reader (Infinite M nano, Tecan Trading Co., Ltd.) with absorbances of 532 nm and 600 nm was used to measure the concentration.

2.6. Statistics analysis

Statistical ANOVA was used to compare the physiological responses of larvae, SPCR, and estimated plastic mass reduction using Origin Pro 2021 (Origin Lab Corp., U.S.A.). The variations in the M_n , M_w , M_z , and polydispersity index were analyzed by pairwise comparisons using the student's t-test with Tukey's correction. All error values were reported as the mean ± standard deviation.

3. Results and discussion

3.1. Consumption and biodegradation of plastic polymers by *T. molitor* larvae

The plastic-degrading macroinvertebrate *T. molitor* larvae began to chew and ingest the PVC, PS, and PLA MPs after they were poured into the containers (Fig. 1a–c). The larvae exhibited a higher affinity for and consumption rate of PLA MPs with the sequence of PLA > PS > PVC, and the specific plastic consumption rate (SPCR) was 44.4 ± 2.2 , 40.0 ± 1.5 , and 17.3 ± 0.5 mg plastics $100 \text{ larvae}^{-1} \text{ d}^{-1}$, respectively, after the larvae fed the MPs as sole diet. The larvae consumed more polymers in the presence of co-diet bran, i.e., the consumption rate increased by 159%, 75%, and 133% for the larvae fed with PLA, PS, and PVC plus co-diet bran, respectively (Table S4), with a similar trend to previous reports (Brandon et al., 2018; Peng et al., 2019, 2020a, 2021; Yang et al.,

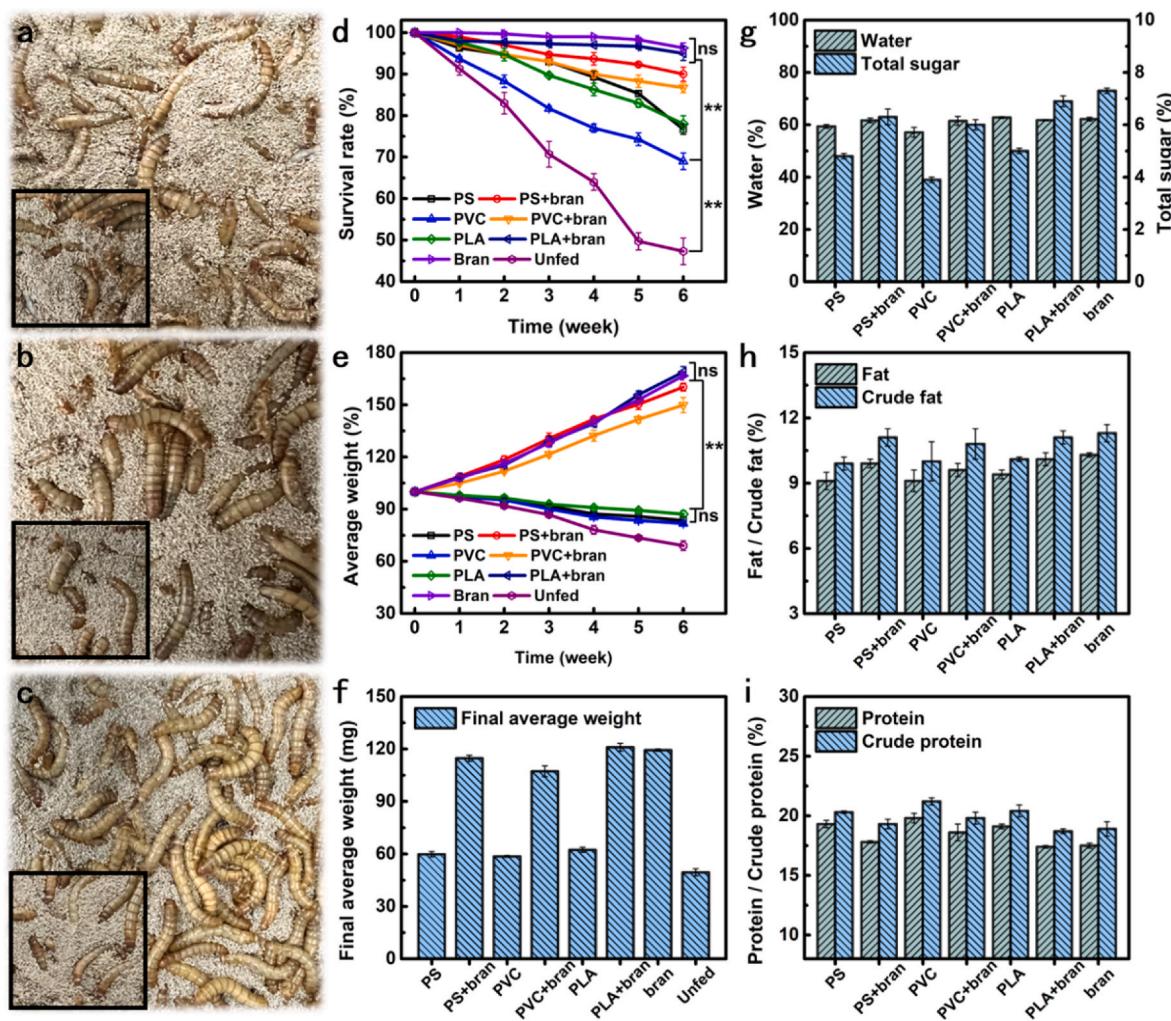


Fig. 1. The plastic-degrading *Tenebrio molitor* larvae fed with three microplastics with different ranked degradability (PVC, PS, and PLA) and the larval physiological responses to ingestion and biodegradation of the microplastics. **a-c** The larvae fed on the PS + bran, PVC + bran, and PLA + bran diets. The inserted images are the larvae fed with sole plastics diet. **d** Changes in survival rates (%) of the larvae over a 6-week period. Statistical significance was determined by Kaplan-Meier survival analysis. Symbols *, **, and ns indicate statistical significance ($p < 0.05$, $p < 0.001$, and no significant difference, respectively). **e** Changes in average weights of larvae over the 6-week period (%). **f** Final average weights of the larvae after the 6-week period (mg). **g** Final water and total sugar content (%) of the larvae under different diet conditions. **h** Final fat and crude fat content (%). and **i** Final protein and crude protein content (%) of the larval biomass.

2018a). The SPCR of the PLA + bran-fed larvae over 6 weeks was 115.3 ± 4.3 mg plastics 100 larvae $^{-1}$ d^{-1} , equivalent to 12.0 ± 0.4 mg plastics g larvae $^{-1}$ d^{-1} , which was about 200% and 60% higher than that of PVC + bran-fed larvae (40.3 ± 1.4 mg plastics 100 larvae $^{-1}$ d^{-1} or 4.5 ± 0.2 mg plastics g larvae $^{-1}$ d^{-1}) and PS + bran-fed larvae (70.0 ± 1.8 mg plastics 100 larvae $^{-1}$ d^{-1} or 7.5 ± 0.2 mg plastics g larvae $^{-1}$ d^{-1}), respectively (Fig. S1 and Table S4). The results indicated that the plastic consumption rate by the larvae was polymer degradability-dependent, with higher degradability and plastic consumption occurring in the presence of an energetic co-diet. Based on hydrophobicity, PVC is slightly more favorable for enzymatic attack than PS (Min et al., 2020). However, the higher SPCRs of PS compared to PVC could be attributed to the fact that the potential toxicity of the degradation intermediates and products of PS should be milder than that of PVC, which generates chlorinated organics after biodegradation (Peng et al., 2020a; Zhang, Z. et al., 2022a,b).

3.2. Physiological responses of *T. molitor* larvae to microplastics

During the 6-week incubation, the survival rates (SRs) of all groups fed with sole MPs were lower than the bran-fed control, i.e., bran ($96.3 \pm 1.2\%$), PLA ($78.0 \pm 2.0\%$), PS ($76.7 \pm 1.2\%$), and PVC ($69.2 \pm 2.0\%$),

but much higher than the unfed group ($47.3 \pm 3.2\%$). In the presence of co-diet bran, the SRs were $95.0 \pm 1.7\%$ for the PLA + bran group, $90.0 \pm 1.7\%$ for the PS + bran group, and $86.7 \pm 1.2\%$ for the PVC + bran group (Fig. 1d, Table S5). The higher SRs in the polymer-fed groups compared to the unfed group confirmed that all polymers provided energy sources to the larvae through the biodegradation of plastics. However, the lack of nutrients and minerals in plastics also negatively impacted the larval survivability, according to the results which showed that supplementation of energetic/nutrition-rich co-diet bran improved survivability (Brandon et al., 2018; Peng et al., 2019, 2021). There was no significant difference ($p > 0.05$) in the SRs between the larvae fed with bran and those fed with PLA + bran (Fig. 1d). PLA polymer is a hydrolyzable polymer, and is much easily digested and mineralized in the larval gut than persistent PS and PVC polymers. The findings indicate that biodegradation and mineralization of plastics with relatively high degradability (e.g., PLA) does not cause an obvious negative impact on the survival of the larvae when the exogenous food source is supplemented.

At the end of the test period, the SR of the PS + bran-fed larvae ($90.0 \pm 1.7\%$) was slightly lower than that of the bran only and the PLA + bran-fed larvae but higher than that of the PVC + bran-fed larvae ($86.7 \pm 1.2\%$) (Fig. S2 and Table S5). This suggested that the sequence of the negative

impact on SRs was PVC > PS > PLA, where the PLA had almost no negative impact. Previous studies have indicated that the extent of PVC mineralization by *T. molitor* larvae was limited, with only 2.9% of Cl-released from ingested PVC during biodegradation by *T. molitor* larvae and high levels of chlorinated intermediates accumulated (Peng et al., 2020a). These degradation intermediates and products could cause toxicity to the larvae and reduce survivability.

The change in larval average weight is another indicator of the physiological responses. During the 6-week period, the average weights of PLA + bran-fed, PS + bran-fed, PVC + bran-fed, and bran-fed larvae all increased progressively, while those unfed or fed on MPs only significantly decreased (Fig. 1e and Table S6), indicating that the exogenous food source or co-diet bran supported larval growth. When fed with MPs only, the reduction in average weight occurred in the following order: PLA (-12.9%) > PS (-16.5%) > PVC (-18.2%) > unfed (-30.9%). This confirmed previous findings that biodegradation of plastics as a sole diet supported larval life activity but not growth, and that the larvae consumed their stored biomass for survival (Peng et al., 2021; Yang et al., 2018a), which also suggested that the generation of chlorinated intermediates from PVC could have more negative impact on the larvae. When the co-diet was supplemented, the sequence of the increase in average weights was bran (66.7%) \approx PLA + bran (68.9%) > PS + bran (60.2%) > PVC + bran (49.9%). The final average weight of the PLA + bran-fed larvae (120.9 ± 2.2 mg) was not significantly ($p > 0.05$) different from that of the bran-fed larvae (119.4 ± 0.6 mg), but was higher than that of the PS + bran-fed and PVC + bran-fed larvae, i.e., 114.7 ± 1.6 mg and 107.3 ± 3.1 mg, respectively (Fig. 1f). The results suggested that plastics with a high polymer degradability (e.g., PLA) combined with an exogenous food source were more favorable for plastic-degrading larvae to maintain long-term growth and physiological function.

To further examine the physiological responses to the plastics with different rankings of degradability, changes in the biomass nutrient content of the larvae were determined. At the end of the 6-week test, the water content of the PVC-fed, PS-fed, and PLA-fed larvae changed $57.1 \pm 1.9\%$, to $59.4 \pm 0.7\%$, and $62.8 \pm 0.3\%$, respectively (Fig. 1g and Table S7), while that of the PVC + bran-fed, PS + bran-fed, and PLA + bran-fed larvae was maintained at $61.5 \pm 1.7\%$, $61.7 \pm 0.9\%$, and $61.8 \pm 0.2\%$, respectively. The data showed that the water content of the larvae fed with PS and PVC MPs only was lower than that of the PLA-fed ($62.8 \pm 0.3\%$) and bran-fed ($62.2 \pm 0.7\%$) groups, but no significant difference was observed among the three plastics plus bran-fed and bran-fed larvae ($p > 0.05$). The digestive processes of different plastics within the gut microenvironment were associated with distinctive enzymes and various hydrolysis reactions (Kim et al., 2020; Peng et al., 2022a). This finding revealed that exposure to MPs over a long period, such as hydrophobic plastics PS and PVC with relatively low degradability, could cause mild dehydration in the plastic-degrading larvae, thus resulting in higher mortality and growth inhibition (Fig. 1d).

The key nutrient contents, i.e., total sugar, fat, and crude fat, of the larvae fed on plastics only were significantly lower ($p < 0.05$) than those of the bran-fed larvae (Fig. 1g-i and Table S7), which indicated their malnutrition status. During the test period, the larvae fed on plastic-only diets did not obtain sufficient energy from the plastic polymers with complex polymer structures and high molecular weights as an exclusive food source. They had to consume their stored biomass and energy reserves to compensate for the lack of nutrients for survival but still accomplished the processes of plastic digestion and biodegradation. Notably, the levels of total sugar and fat contents of the PVC-fed larvae were the lowest among all groups (Table S7), which was associated with incomplete biodegradation of PVC to chlorinated organics in the larvae as observed previously (Peng et al., 2020a). By contrast, for the PS + bran, PVC + bran, and PLA + bran-fed groups, the nutrient contents of the larvae were maintained at relatively normal levels (Fig. 1g and h). The protein and crude protein contents of the larvae fed with plastic only were slightly higher than those fed with plastic plus bran and bran only

(Fig. 1i and Table S7). This was due to the loss of stored carbohydrate and fat under the malnutrition status, resulting in a relative increase in protein content in the larval biomass.

The overall nutrient contents of the larval biomass were normalized in a radar map to evaluate the physiological responses. After 6 weeks of continuous feeding, the nutrient contents of the PS-fed, PVC-fed, and PLA-fed larvae shrank (Fig. 2), while those of the larvae with co-diet bran remained healthy. The PLA + bran-fed larvae had the highest level of nutrient content, and their radar map almost overlapped the bran-fed larvae (Fig. 2). The nutrient status of the PVC + bran larvae (in the color olive) was worse than that of the PLA + bran-fed and the PS + bran-fed larvae. This confirmed that the negative response to MPs exposure by the plastic-degrading *T. molitor* larvae followed the sequence of PVC > PS > PLA, while co-diet bran alleviated the negative impact of MPs and improved the physiological performances of the larvae under MPs exposure.

In summary, the results demonstrated that the ingestion of MPs induced negative physiological responses in the plastic-degrading macroinvertebrate *T. molitor* larvae similar to those of macroinvertebrates without plastic-degrading capacity. Supplementation of an energetic co-diet alleviated the negative impact of exposure to MPs. The levels of negative physiological responses induced by the MPs followed the sequence of PVC > PS > PLA, which appeared dependent on polymer degradability and intermediate toxicity. Future research should determine the impact of major physicochemical properties (e.g., molecular weight, hydrolizability, polymerization, crystallinity, etc.) on plastic-degrading macroinvertebrates.

3.3. Plastic mass reduction, biodegradation, and residual particles

The plastic removal efficiencies of PLA, PS, and PVC were calculated as $85.2 \pm 1.2\%$, $67.7 \pm 1.8\%$, and $58.2 \pm 1.5\%$ by the larvae fed with plastics only, and $76.5 \pm 0.4\%$, $62.5 \pm 0.1\%$, and $49.6 \pm 1.0\%$ by the larvae fed with plastics plus bran (Fig. S4). The sequence of plastics removal efficiency was PLA > PS > PVC. In terms of removal rate, the larvae fed with co-diet bran showed significantly higher plastic mass reduction than those fed plastics only, similar to SPCRs (Table S4). The PLA + bran-fed larvae exhibited the highest plastic mass reduction (3672.0 ± 19.2 mg) compared with the larvae fed with PS + bran (1750.0 ± 2.8 mg) and PVC + bran (892.8 ± 18.0 mg) over the test period (Fig. S4a).

The depolymerization and biodegradation of plastics were

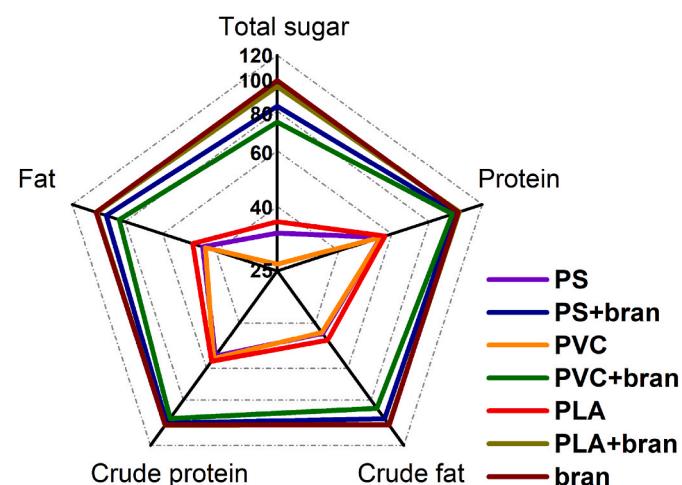


Fig. 2. The radar map for the comprehensive evaluation of biomass nutrient contents (total sugar, fat, crude fat, protein, and crude protein) of the larvae under seven diet conditions after 6 weeks. Bran-fed larvae served as the controls. The overall biomass nutrient contents are represented by the relative area on the radar map.

characterized using GPC analysis. A significant reduction in molecular weights (M_w , M_n , and M_z) was observed after the ingested PVC, PS, and PLA polymers passed through the larval intestine (Fig. 3a–c and Table S9). For PVC, PS, and PLA polymers, the M_w was decreased by $25.3 \pm 3.2\%$, $35.7 \pm 3.4\%$, and $29.0 \pm 6.1\%$, respectively, and the M_n was decreased by $29.1 \pm 3.1\%$, $48.6 \pm 2.9\%$, and $43.0 \pm 3.9\%$, respectively, indicating a broad depolymerization pattern for the three ingested polymers as both M_n and M_w decreased (Peng et al., 2020b; Yang et al., 2021). The PDI of the residual PVC, PS, and PLA polymers increased significantly after biodegradation, i.e., from 1.80, 2.47, and 1.55 to 1.90, 3.09, and 1.93, respectively. The reduction of M_n and M_w of PS was higher than that of PVC although both MPs were at a similar molecular weight range. The PDI results indicated the internal scission of the macromolecules of the polymers and increased the broadness of molecular weight in the residual polymers (Peng et al., 2022b). The results corroborated the efficient depolymerization and biodegradation of the ingested PVC, PS, and PLA polymers in the intestines of larvae.

3.4. The particle size distribution of residual polymers

The size-frequency distribution of residual plastics extracted from the frass was measured to determine the biofragmentation of the ingested MPs. Residual PS, PVC, and PLA plastic particles with different size distributions were observed in the egested frass after the biodegradation (Fig. 3d–f). The mean size of the residual polymers in the frass from larvae fed with PLA + bran, PS + bran, and PVC + bran ($35.3 \mu\text{m}$, $126.7 \mu\text{m}$, and $143.9 \mu\text{m}$) was larger than that from the larvae fed with PLA, PS, and PVC only ($21.2 \mu\text{m}$, $66.9 \mu\text{m}$, and $86.4 \mu\text{m}$) (Fig. 3d–f). The size of residual particles followed a sequence of PVC > PS > PLA, indicating that the particle mean size appeared to be polymer biodegradability-dependent. The size distribution of the three residual polymers suggested that the residual particular size in the gut was not as critical as other factors (biodegradability, intermediate toxicity) when it

came to negative impact on larval survival, as small-sized PLA did not show stronger negative impact on the larvae than larger-sized PS and PVC.

The PS, PVC, and PLA particles all fell in the size range of MPs (i.e., $>1 \mu\text{m}$), and NPs ($<1 \mu\text{m}$) were not determined by the laser particle sizer that had measurement range from 0.01 to $3500 \mu\text{m}$. The finding is of significance because NPs can induce more serious physiological stress and toxicity than MPs in organisms, including inactivating biomolecule function and triggering cellular biotoxicity (So et al., 2022; Wang et al., 2021). Unlike with observations of biofragmentation of the ingested MPs into NPs in the intestines of macroinvertebrates such as earthworms and Antarctic krill, which have poor or no plastic-degrading ability (Dawson et al., 2018; Kwak and An, 2021), the NPs or submicron-plastics were not found in the frass of plastic-degrading superworms (*Z. atratus*) fed with PS and PE foams (Peng et al., 2022a). In this study, our observations confirmed that NPs were not detected in the excrement of *T. molitor* larvae with preeminent plastic-degrading ability. We hypothesize that if plastic-degrading macroinvertebrates could rapidly biodegrade ingested plastic particles, and that the reaction rate was a function of surface area, then NPs would be degraded at a much faster rate than MPs without accumulation in the gut. Future research will be performed to investigate the particle size of residual plastics in the intestines and frass after biofragmentation and biodegradation by other plastic-degrading insects or macroinvertebrates.

3.5. The oxidative stress responses in larvae after plastic biodegradation

MPs and NPs share in the formation of ROS as they are frequently involved in induction of developmental toxicity, neurotoxicity, cytotoxicity, and oxidative stress (Hu and Palić, 2020). In this study, significantly higher levels of ROS were found in the larvae fed with solely PVC, PS, and PLA MPs (i.e., 6.04×10^6 , 4.85×10^6 , and $4.95 \times$

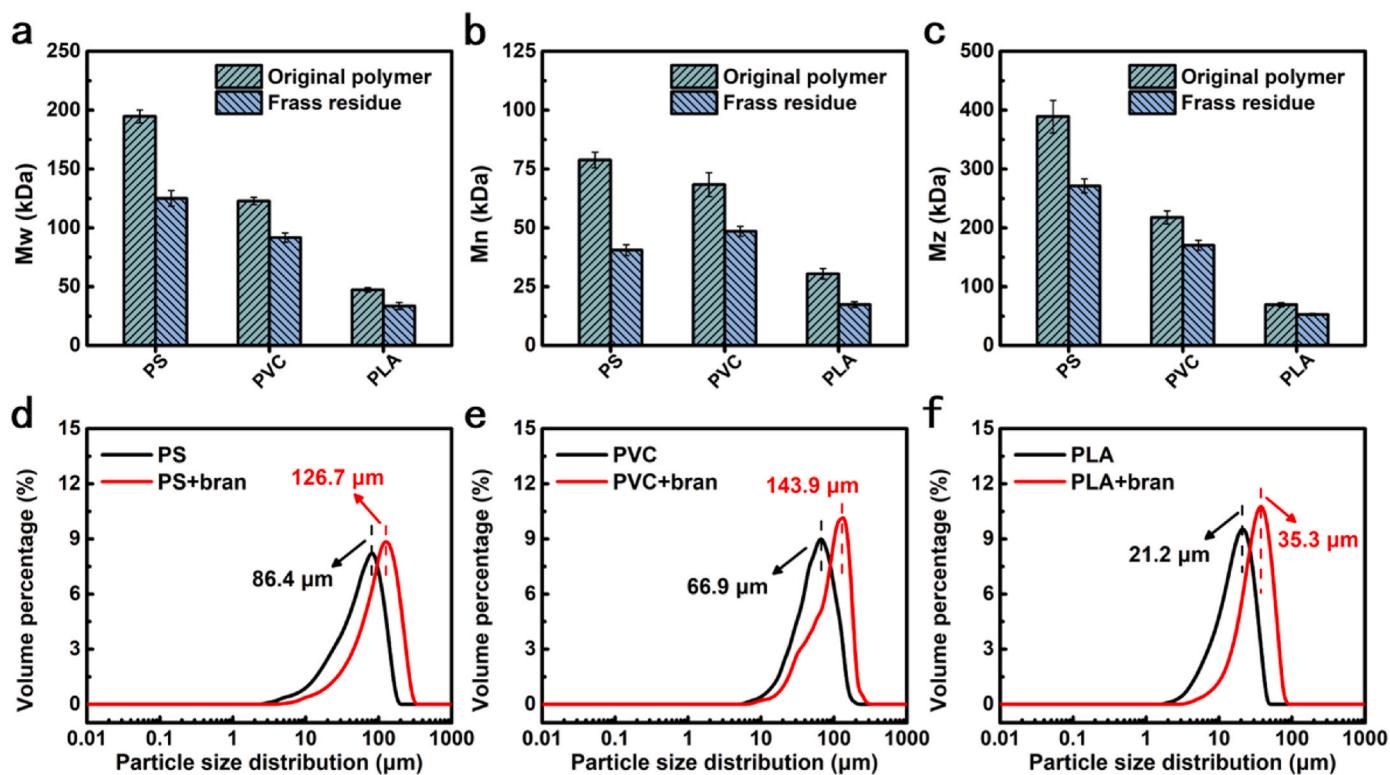


Fig. 3. Characterization of depolymerization and biofragmentation of the microplastics of PS, PVC, and PLA by *T. molitor* larvae. **a–c** Changes in M_w , M_n , and M_z values of the ingested PS, PVC, and PE MPs after passing through the intestinal tracts. **d–f** The size distribution of the residual PS, PVC, and PLA particles in the frass from the larvae fed with sole plastics versus plastics plus bran. The mean sizes of particles on a volume basis are marked.

10^6 fluorescence intensity/mg protein, respectively) versus 4.10×10^6 fluorescence intensity/mg protein in the bran-fed larvae (Fig. 4a). A remarkable ROS increase was also observed when earthworms (*Eisenia fetida*), macroinvertebrates with poor plastic-degrading ability, were exposed to PS MPs with different exposure concentrations of 0.1, 0.25, 0.5, 1.0, and 1.5 g/kg for 7 days (Liu et al., 2022b). The results of this study imply that MPs exposure induces oxidative stress in plastic-degrading *T. molitor* larvae just as they do in earthworms. In particular, the highest ROS was found in the PVC-fed larvae, which was consistent with the sequence of negative physiological responses of the larvae observed in this study (Fig. 1), which could be attributed to toxicity of both PVC MPs and their degraded chlorinated intermediates. The biodegradation of PVC and PS mainly occurs via oxidative depolymerization processes, while PLA polymers are broken down via enzymatic hydrolysis by polyester hydrolases, lipases, carboxylesterases, etc. (Inderthal et al., 2021; Zhang et al., 2022a,b). Biodegradation of PVC releases chlorinated intermediates and hydrogen chloride (Peng et al., 2020a; Zhang, Z. et al., 2022a,b). Therefore, the MPs-induced oxidative stress in the PVC-fed larvae could be higher than that induced by PLA and PS MPs. The increase in ROS could also be partially attributed to activation or upregulated genes for PVC, PS, and PLA degradation. The observation of generation of ROS in the gut of superworms (*Z. atratus*), another number of darkling beetle, during PS biodegradation was reported (Chen et al., 2023).

Antioxidant enzymes such as SOD, GST, and CAT play an important role in protecting tissues against ROS damage. Exposure to MPs increased ROS and resulted in SOD, GST, and CAT reactions as a signal (Hu and Palić, 2020). To understand the activity of antioxidant enzymes in larvae against oxidative stress, we measured the contents of SOD, GST, and CAT to access their enzymatic activities for eliminating ROS in organisms (Li et al., 2021). The contents of SOD in the larvae fed with sole PLA, PS, and PVC MPs all increased (Fig. 4b), and were 116.5 ± 7.0 ,

108.5 ± 7.7 , and 158.2 ± 13.1 U/g fresh weight, respectively, versus 59.4 ± 3.2 U/g fresh weight in the bran-fed larvae. In organisms, SOD aids in the elimination of the superoxide anion (O_2^-) by converting O_2^- into H_2O_2 and O_2 (Hu and Palić, 2020; Wu et al., 2011). Therefore, the results confirmed that the MPs also induced significant antioxidant defenses in plastics-degrading *T. molitor* larvae depending on the rankings of polymer degradability. Furthermore, the addition of the co-diet bran reduced the content of SOD (Fig. 4b). The results suggested that the oxidative stress responses to plastic MPs were effectively alleviated by enhancing metabolic activities via the feeding of a co-diet. In particular, no significant difference was observed between the SOD contents of the bran-fed and PLA + bran-fed larvae, indicating that biodegradation of PLA with a nutrition-rich co-diet (bran) did not induce significant antioxidant defenses in the larvae.

The enzymatic activities of GST, which scavenges the metabolites of lipid peroxidation and helps alleviate oxidative damage (Hu and Palić, 2020; Wu et al., 2011), exhibited a similar trend to that of SOD in the larvae (Fig. 4c). The CAT activities of the larvae under all diet conditions except for the PVC-fed groups were not statistically different (Fig. 4d), which was consistent with the observation that only the PVC-fed larvae had high mortality and negative physiological responses to MPs associated with serious internal oxidative damage.

Free radicals would attack the cellular structure and biomacromolecule function (e.g., membrane lipids) if the antioxidant enzymes failed to clear the excess ROS generated (Shao et al., 2018). To further investigate the oxidative stress responses in the larvae during biodegradation of the plastics, the MDA contents in the larvae were determined (Fig. 4e). Consistent with the results of the ROS levels and antioxidant enzyme activities, the ingestion and biodegradation of MPs resulted in a significant increase in the MDA contents in the larvae with the order of the increase being PVC > PS > PLA > bran. The co-diet reduced the level of lipid peroxidation and oxidative damage. In

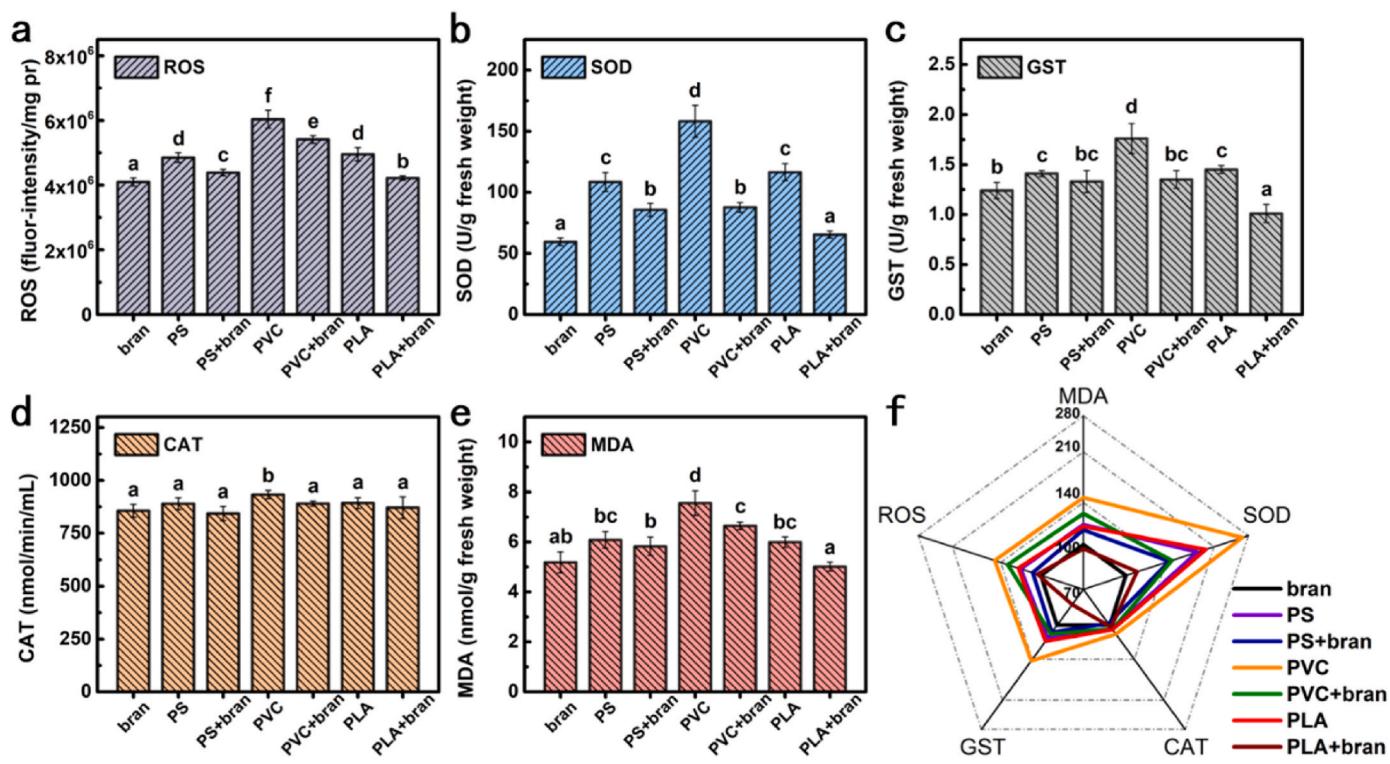


Fig. 4. Comparison of the reactive oxygen species (ROS), antioxidant enzymes, and lipid peroxidation levels in the larvae under seven different diet conditions at the end of the 6-week period. Bran-fed larvae served as the controls. **a-e** The contents of ROS (fluorescence intensity/mg protein), superoxide dismutase (SOD, U/g fresh weight), glutathione-S-transferase (GST, U/g fresh weight), catalase (CAT, nmol/min/mL), and malondialdehyde (MDA, nmol/g fresh weight) in the larvae. Different letters above the columns indicate significant differences. **f** Radar map showing the overall physiological stress responses of the larvae to PS, PVC, and PLA microplastics. The levels of oxidative stress in the larvae are represented by the relative area.

particular, the level of PLA + bran-fed larvae was the close to that of bran-fed larvae. However, the MDA content of the PVC + bran-fed larvae was still statistically higher than that of the bran-fed control ($p < 0.05$), which was attributed to the fact that the activities of the antioxidant enzymes did not accommodate the generation rate of ROS, thereby resulting in the exacerbation of lipid peroxidation in the larvae.

The overall antioxidant response of the larvae to oxidative stress was comprehensively evaluated using the radar map (Fig. 4f). Generally, the levels of oxidative stress responses induced by MPs exposure followed the sequence of PVC > PS > PLA. Combined with the physiological responses (Fig. 1), the PVC-fed larvae exhibited the most serious oxidative damage and dysbiosis, while those of the PS-fed and PLA-fed larvae were visually at similar levels. The results supported the conclusion that oxidative stress caused by MPs was polymer biodegradability-dependent. The supplementation of the exogenous food source (i.e., co-diet bran) significantly reduced the oxidative stress and damage in larvae. In particular, the PS + bran-fed and PLA + bran-fed larvae exhibited no statistically significant oxidative damage, and the PLA + bran-fed larvae showed the best performance (Fig. 4e and f). Previously, we proposed a circular approach for PLA waste management via resource recovery of used PLA as the feedstock for insect biomass production using *T. molitor* larvae (Peng et al., 2021). The findings in this study support the notion that PLA plus bran can be a long-term sustainable option for rearing *T. molitor* larvae using PLA wastes as supplementary feed without negative impact or toxicity to the *T. molitor* larvae.

4. Conclusions and future implications

Based on the results of this study and previous literatures, a schematic diagram of the physiological responses of *Tenebrio molitor* larvae after ingestion and biodegradation of the microplastics PVC, PS, and PLA is summarized as Fig. 5 with the conclusions expounded upon below:

a) The plastics-degrading macroinvertebrates, *Tenebrio molitor* larvae, effectively biodegraded PVC, PS, and PLA microplastics over a 42-

day period. The biodegradation was supported by mass reduction and molecular weight reduction. The biodegradability of the tested polymer MPs followed the sequence of PLA > PS > PVC.

- b) Ingestion and biodegradation of plastics MPs caused negative physiological responses in the plastic-degrading *T. molitor* larvae, including weight loss, decreased survival rates, biomass depletion, and oxidative damage. The sequence of the survival rates of *T. molitor* larvae of PLA-fed, PS-fed, and PVC-fed larvae was $78.0 \pm 2.0\% > 76.7 \pm 1.2\% > 69.2 \pm 2.0\%$, indicating a decrease in survival rates correlated with polymer biodegradability.
- c) Supplementation of co-diet bran significantly enhanced consumption and mass reduction rates of all three plastics and alleviated oxidative stress and toxicity evaluated using ROS levels and antioxidant enzymatic activities.
- d) Biofragmentation of ingested MPs occurred but the residual particles remained at micrometer size during plastics biodegradation. NPs was not detected, which was different from the previous observation of formation of NPs by non-plastic-degrading macroinvertebrates, and thus reduced risk of NPs toxicity.

In this study, for the first time, we discovered that MPs-induced biotoxicity and oxidative stress levels in the plastic-degrading macroinvertebrates associated with polymer biodegradability. Biofragmentation of tested MPs into NPs was not observed. The discoveries fill knowledge gaps of MPs biotoxicity because previous studies on stress responses and oxidative damage in macroinvertebrates induced by micro(nano)plastics primarily focused on macroinvertebrates with or without extremely poor plastic-degrading abilities.

The physiological responses of the plastic-degrading insect larvae are polymer degradability-dependent. This indicates caution must be applied when it comes to using the results obtained from single polymers for toxicity and risk assessment. The supplementation of an exogenous food source (wheat bran) improved the larval survival rate and biomass nutrient content, enhanced plastic consumption and biodegradation, and alleviated the levels of oxidative stress in the plastic-degrading *T. molitor* larvae. For the future study, other major persistent plastics,

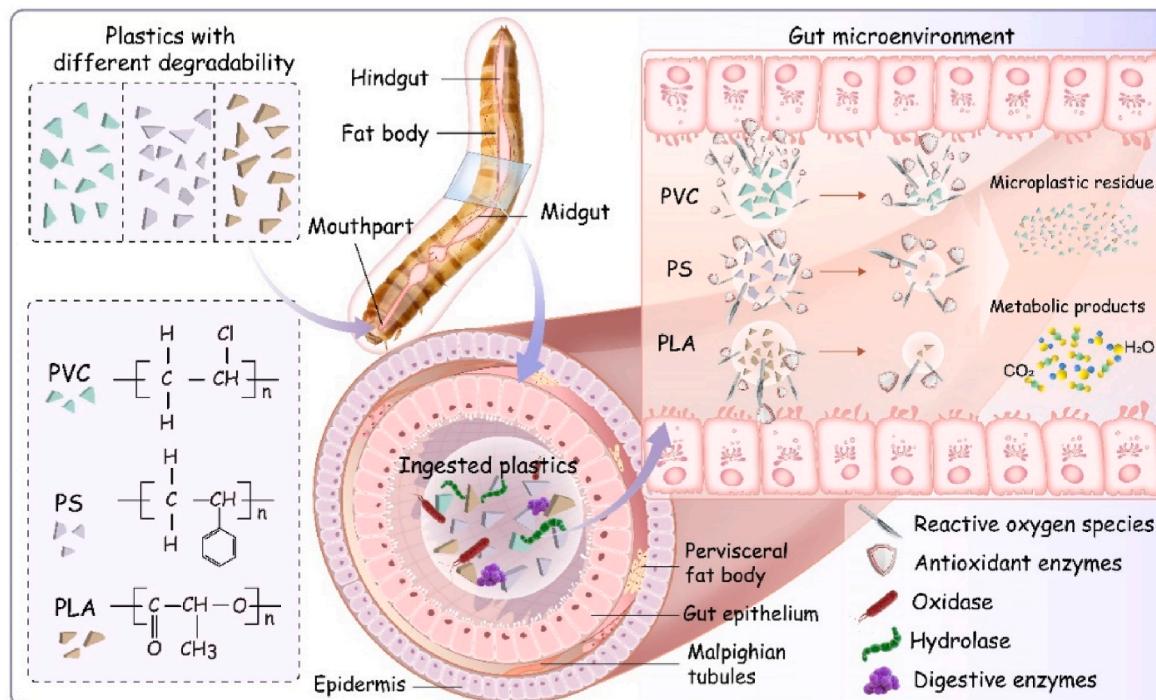


Fig. 5. A schematic diagram of the physiological responses of *Tenebrio molitor* larvae after ingestion and biodegradation of microplastics with different biodegradability.

e.g., PE, PP, PUR, PET, and biodegradable polymers, e.g., PBAT, should be tested to rank their physiological impacts and health risks. Research should also assess the physiological stress responses of other plastics-degrading insects versus non-plastic-degrading macroinvertebrates.

Credit author statement

B.-Y.P. performed most of the experiments and analyses and drafted the manuscript. Y.S. performed some data analysis, provided key technical guidance, and co-wrote the manuscript. P.L. and S.R.Y. supervised data analysis and revised the manuscript. Y.Z.X. performed some experiments on larval breeding and some analysis on antioxidant enzymes. J.B.C. and X.F.Z. were responsible for technical help and funding acquisition. W.-M.W. designed the experiment, supervised data analysis, and drafted and revised the manuscript. Y.L.Z. designed the experiment, supervised data analysis, provided funding, and revised the manuscript.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2023.118818>.

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