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Effects of Polyethylene Microplastic Particle Size and Concentration on Earthworm Fitness and Gut Microbial Functional Potential

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Abstract

Polyethylene microplastics (PE MPs) occur widely in soils, but how particle size and concentration relate to earthworm physiological responses and gut bacterial communities remains unclear. We exposed Eisenia fetida to four nominal PE particle sizes (15, 45, 100, and 200 μm) at three concentrations (0.5%, 2%, and 5%, *w*/*w*) for 30 days. Mortality, container-level mean body-weight change, cocoon production, gut bacterial communities, and PICRUSt2-predicted functional potential were assessed. Mean body-weight change was lower in all 12 PE MP treatments than in the control (Dunnett-adjusted $p < 0.001$), with the most negative mean value (−19.50 ± 3.80%) observed at 15 μm and 5%. Particle size and concentration had significant main effects on body-weight change, but no significant interaction. Mortality and cocoon production did not differ significantly from the control after adjustment. Neither Chao1 richness nor Shannon diversity differed significantly among groups. Global bacterial community differences were detected (PERMANOVA, $R^2 = 0.393$, $p = 0.0001$), although unequal dispersion (PERMDISP, $p = 0.0069$) and nonsignificant BH-adjusted pairwise comparisons limited their interpretation. None of the ten dominant phyla or genera tested differed significantly among groups after false-discovery-rate correction, while LEfSe yielded exploratory discriminatory taxa. Only three KEGG Level 2 and six Level 3 treatment–control comparisons met the BH-adjusted significance threshold within their respective comparison families. Overall, container-level body-weight change showed the most consistent treatment–control differences, whereas evidence for treatment-specific gut bacterial and predicted functional changes was more limited under these high-exposure laboratory conditions.

Keywords: *Eisenia fetida*; soil ecotoxicology; cocoon production; 16S rRNA amplicons; PICRUSt2

Academic Editors: Lianguo Chen and Fayuan Wang

Received: 18 September 2026
Revised: 17 October 2026
Accepted: 28 October 2026
Published: 30 October 2026

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1. Introduction

Plastics have become ubiquitous materials in modern society because of their broad applications and high durability [1–3]. Large amounts of plastic waste gradually fragment in the environment, generating microplastics (MPs; particle size < 5 mm). These particles are now widely distributed across terrestrial and aquatic environments [1,2]. Agricultural

soils are important sinks for MPs and may receive substantial inputs from human activities [4]. Major sources include the degradation of plastic mulch films [5], organic fertilizer application [6], and sewage sludge reuse [7]. Microplastics have also been widely detected in inland waters, providing potential pathways for their redistribution across environmental compartments [8,9]. Long-term plastic film mulching has been associated with changes in soil microbial communities [10], while studies in aquatic environments have documented a range of ecotoxicological effects [11]. Recent evidence further indicates that microplastic contamination can influence soil health and biological processes [12]. In particular, polyethylene (PE) microplastics have been shown to alter soil microbial community assembly and ecosystem multifunctionality [13]. These findings highlight the ecological relevance of MP contamination, although its biological effects may vary with particle properties and exposure conditions.

The potential relationships between MP exposure, gut microbiota, and host physiology have received increasing attention. In earthworms, combined exposure to mulch-derived MPs and cadmium has been associated with physiological changes, although the effects of MPs cannot be isolated from those of cadmium in such experiments [14]. Evidence from vertebrates also suggests that gut microorganisms can influence host metabolic processes. Bäckhed et al. [15] demonstrated that the gut microbiota contributes to the regulation of fat storage in mice. Subsequent studies reported that polystyrene MPs altered gut bacterial communities and metabolic indicators in mice [16,17]. Similarly, studies in zebrafish and marine fish have described intestinal injury, microbial disturbance, and metabolic responses following MP exposure [18,19]. However, these findings largely concern vertebrates and cannot be directly extrapolated to earthworms. Although the internal distribution and potential toxicity of MPs have also been reviewed in the context of human health [20], the relationships among MP exposure, gut bacterial communities, and physiological responses in soil invertebrates require further investigation.

Earthworms play important roles in soil ecosystems and are widely used as model organisms in terrestrial ecotoxicology [21,22]. Through the ingestion of soil particles and organic matter, they may be exposed to MPs accumulated in terrestrial environments [23]. Previous studies have reported adverse physiological responses to MP exposure, including tissue damage and oxidative stress. MP exposure has also been associated with changes in earthworm gut bacterial communities. For example, Wang et al. [24] reported reduced arsenic accumulation and altered gut bacterial composition in earthworms exposed to MPs. Li et al. [25] examined the ecotoxicological effects of different particle-size ranges of industrial-grade PE and polypropylene microplastics on *Eisenia fetida*, providing evidence that particle-size-related responses warrant further investigation. Although these studies demonstrate that physiological and microbial responses may occur under MP exposure, the respective effects of PE particle size and exposure concentration have not been fully characterized within a single factorial experimental framework that also considers gut bacterial communities and their predicted functional potential. Moreover, whether organism-level responses are accompanied by corresponding differences in gut bacterial composition and inferred functional profiles remains uncertain.

In this study, *Eisenia fetida* was exposed to PE MPs of four nominal particle sizes (15, 45, 100, and 200 μm) at three concentrations (0.5%, 2.0%, and 5.0%, *w*/*w*) for 30 days. Using a 4 × 3 factorial design with an additional unamended control, we aimed to (i) determine the effects of particle size, concentration, and their interaction on earthworm mortality, biomass change, and cocoon production; (ii) examine treatment-associated variation in gut bacterial diversity and community composition; and (iii) evaluate differences in microbial functional potential inferred from 16S rRNA gene data using PICRUSt2. By integrating organism-level endpoints with gut bacterial community analysis and functional

prediction, this study characterizes responses across controlled PE MP exposure conditions without assuming a causal relationship between microbial changes and earthworm physiology.

2. Materials and Methods

2.1. Polyethylene Microplastics, Soil, and Earthworms

PE MPs with four nominal particle sizes (15, 45, 100, and 200 μm) were custom-ordered from Suzhou Xipuneng Plastic Co., Ltd. (Suzhou, China). Particles were rinsed three times with ultrapure water, immersed in 70% ethanol (Shandong Lircon Medical Technology Co., Ltd., Dezhou, China) for 30 min, rinsed again, and dried at 60 °C. They were then stored in clean, covered containers. The supplier did not provide batch-specific characterization reports. Therefore, the reported particle sizes are nominal, and particle-size distributions, polymer identity, morphology, and additive content were not independently verified.

Artificial soil was prepared by mixing peat moss, kaolin clay, and quartz sand (all obtained from Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China) at a dry-mass ratio of 1:2:7 (10:20:70), following the formulation in OECD Test Guideline 222 [26] and Zhang et al. [27]. Calcium carbonate ($CaCO_3$) was added to adjust pH toward the guideline target of 6.0 ± 0.5, and moisture was set to approximately 60% of maximum water-holding capacity (WHC). The standardized substrate reduced background variation among treatments. However, final pH and baseline soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), NH_4^+-N, NO_3^--N, and available phosphorus (AP) were not measured; the target pH should therefore not be interpreted as a verified value.

Adult *Eisenia fetida* with well-developed clitella and intact body surfaces were selected for the experiment. Earthworms were acclimated in clean substrate for 7 days under controlled laboratory conditions (20 ± 2 °C, 60–80% relative humidity, and a 12 h light/12 h dark photoperiod). Pre-decomposed cow manure was provided as food during acclimation. Before exposure, the earthworms were placed on moist filter paper for 24 h to allow gut depuration. Healthy and active individuals were then selected for the exposure experiment.

2.2. Exposure Experiment

Four nominal particle sizes (15, 45, 100, and 200 μm) were crossed with three PE MP concentrations (0.5%, 2%, and 5%, *w*/*w*; 5, 20, and 50 g kg^{-1} dry soil). The four size classes span 15–200 μm to compare smaller and larger nominal fractions within a single factorial experiment; size-fractionated PE exposures have also been examined in earthworms [25]. The concentrations represent elevated experimental loadings rather than typical agricultural background levels. The resulting 12 PE MP treatments were compared with an unamended control (CK). At equal mass, smaller particles may also differ in particle number and total surface area; these contributions could not be separated experimentally.

For convenience, treatments were designated as XSL, XSM, and XSH for 15 μm PE MPs; SL, SM, and SH for 45 μm PE MPs; ML, MM, and MH for 100 μm PE MPs; and LL, LM, and LH for 200 μm PE MPs, representing low (0.50%), medium (2.0%), and high (5.0%) concentrations, respectively. Each treatment included three independent biological replicates. The treatment codes and corresponding nominal particle sizes and concentrations are summarized in Table 1.

Table 1. Nominal polyethylene microplastic exposure groups and treatment codes.

Group	Nominal Particle Size (μm)	Concentration (% *w*/*w*)
CK	—	0

XSL	15	0.5
XSM	15	2.0
XSH	15	5.0
SL	45	0.5
SM	45	2.0
SH	45	5.0
ML	100	0.5
MM	100	2.0
MH	100	5.0
LL	200	0.5
LM	200	2.0
LH	200	5.0

PE MPs were added to the soil in successive portions and mixed thoroughly after each addition to improve homogeneity. Each container received 1.0 kg dry-weight-equivalent soil plus 5, 20, or 50 g PE MPs, as assigned. The mixtures were placed in 1.0 L glass containers, each holding ten acclimated adult *Eisenia fetida*. The study comprised 13 groups, each with three independent containers (39 containers and 390 worms in total).

The 30-day exposure was conducted at 20 ± 2 °C, 60–80% relative humidity, and a 12 h light/12 h dark cycle. Every seven days, all containers, including CK, received approximately 5 g of pre-decomposed, air-dried, finely ground cow manure. Soil moisture was maintained near 60% WHC by weighing and adding ultrapure water. Containers were covered with breathable gauze to limit escape while allowing gas exchange. Survival and visible behavior were monitored during exposure.

2.3. Assessment of Biomass Change, Mortality, and Cocoon Production

Earthworm biomass change, mortality, and cocoon production were assessed after 30 days of exposure. Before exposure, all ten earthworms in each container were individually weighed, and their mean body weight was calculated as the initial mean body weight ($\overline{W_0}$). At the end of the experiment, surviving earthworms were collected, gently rinsed with ultrapure water, depurated on moist filter paper for 24 h, and individually weighed. The final mean body weight ($\overline{W_{30}}$) was calculated from the surviving individuals in each container. The biomass change rate was calculated as follows:

$$\text{Biomass change}\ (\%) = (\overline{W_{30}} - \overline{W_0})\ /\overline{W_0} \times 100$$

where $\overline{W_0}$ represents the initial mean body weight of all ten earthworms introduced into each container, and $\overline{W_{30}}$ represents the final mean body weight of the individuals surviving after 30 days. Dead individuals were excluded from the final mean body-weight calculation. Each container was treated as an independent experimental unit.

Mortality was calculated as the percentage of dead individuals relative to the initial number of earthworms introduced into each container:

$$\text{Mortality}\ (\%) = N_d/N_0 \times 100$$

where N_0 represents the initial number of earthworms, and N_d represents the number of dead individuals recorded at the end of the exposure period.

Cocoons recovered from each container at day 30 were counted as the reproductive endpoint. Juveniles were not quantified and were not combined with cocoon counts. This short-term measure of cocoon production was not intended to replace the standardized OECD 222 reproduction test, which assesses juvenile output over a longer period.

2.4. Gut Microbiota Sequencing and Analysis

At day 30, one surviving earthworm was collected from each experimental container for gut bacterial analysis. Each sample represented an individual earthworm, and no pooling was performed. A total of 39 gut samples were obtained from the 13 experimental groups, with three independent container-level replicates per group. The selected earthworms were rinsed with sterile water and dissected aseptically. The collected gut material was snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

DNA was extracted from gut samples using the FastDNA SPIN Kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The V3–V4 region of the bacterial 16S rRNA gene was amplified using primers 338F and 806R. Amplicon sequencing was performed by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China), using the Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, CA, USA) with a 2 × 250 bp paired-end sequencing configuration. Sequencing reads were quality-filtered and denoised using DADA2 within QIIME 2 (version 2019.4) to generate amplicon sequence variants (ASVs), which were subsequently assigned taxonomy using the SILVA database (release 132). The resulting ASV table was rarefied to 74,426 sequences per sample for the reported community analyses.

Chao1 and Shannon indices summarized bacterial richness and diversity. Bray–Curtis dissimilarity among rarefied ASV profiles was visualized with principal coordinate analysis (PCoA). PERMANOVA tested overall and factorial community patterns, and PERMDISP assessed heterogeneity in within-group dispersion (Section 2.7).

Phylum- and genus-level relative abundances were summarized for each group. Linear discriminant analysis effect size (LEfSe, version 1.1.01) was used as an exploratory screen for treatment-discriminatory taxa; an LDA threshold alone was not considered evidence of confirmed differential abundance.

2.5. Prediction of Gut Microbial Functional Potential

The functional potential of gut microbial communities was predicted based on 16S rRNA gene sequencing data using PICRUSt2 (version 2.3.0). The predicted metagenomic profiles were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to obtain functional categories at different hierarchical levels.

Predicted KEGG Level 2 and Level 3 profiles were compared between each PE MP treatment and CK using Welch's *t*-tests. A total of 36 Level 2 categories and 192 Level 3 pathways were included in the comparisons. Benjamini–Hochberg (BH) correction was applied separately within each of the 12 treatment–control comparisons and at each KEGG level, with adjusted $q < 0.05$ considered statistically significant within the corresponding comparison. Unadjusted $p < 0.05$ was used to identify nominal differences, which were distinguished from FDR-adjusted results. These profiles represent predicted genomic potential, not measured pathway activity. Therefore, differences in predicted pathway abundance were not interpreted as direct evidence of altered microbial metabolic activity or earthworm physiological functions.

2.6. Quality Assurance and Contamination Control

Containers and instruments were cleaned before use to minimize contamination during experimental preparation and sample handling. The unamended control (CK) was maintained under the same experimental conditions as the PE MP treatments, except that no PE MPs were added. Sterile handling procedures were followed during gut dissection and DNA preparation. Gut material was promptly frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

DNA extraction blanks and PCR no-template controls (NTCs) were included to monitor potential contamination during DNA extraction and amplification, respectively. Both

types of negative controls were examined by agarose gel electrophoresis and subjected to 16S rRNA gene amplicon sequencing. No detectable target amplification was observed in the negative controls, and sequencing-based quality-control analysis did not identify detectable bacterial contamination.

2.7. Statistical Analysis

Analyses were conducted in R 4.4.2. Values are presented as mean ± standard deviation (SD) unless noted otherwise. Each container was an independent experimental unit (n = 3 per group); measurements from worms housed together were summarized at the container level to avoid pseudoreplication.

Particle size, concentration, and their interaction were tested in factorial models fitted to the 12 PE MP treatments. CK was excluded because it had no assigned PE particle size or positive exposure concentration. Mortality was modeled from dead/surviving counts using a binomial generalized linear model (GLM); biomass change was analyzed using a Gaussian linear model; and cocoon counts were analyzed using a Poisson GLM. Type II tests assessed model terms, and model adequacy was evaluated by examining residual normality for the Gaussian model and Pearson dispersion for the binomial and Poisson models.

For treatment–control comparisons, all 13 groups were included in separate endpoint-specific models, and the 12 PE MP treatments were compared with CK using Dunnett-type contrasts with multivariate-t adjustment. Tukey-adjusted estimated marginal means were used for pairwise comparisons among concentrations within each particle-size class and among particle-size marginal means averaged across concentrations. Marginal particle-size comparisons were interpreted when the interaction was not significant.

Chao1 and Shannon indices were compared across all 13 groups using Kruskal–Wallis tests. Factorial linear models assessed particle size, concentration, and their interaction across the 12 PE MP treatments. Bray–Curtis dissimilarity was calculated from rarefied ASVs and visualized by PCoA with Lingoes correction. Overall and factorial PERMANOVA and PERMDISP used 9999 permutations. Factorial PERMANOVA used sequential tests (size before concentration), so term estimates depend on that order. Exact pairwise PERMANOVA was applied to the 13 groups, with BH adjustment performed across all 78 pairwise comparisons. The 12 treatment–CK comparisons were also evaluated as a separate comparison family. With three samples per group, each 3-versus-3 exact test has only 20 possible allocations, limiting the smallest attainable p value to 0.1 under the reported scheme. PERMANOVA results were interpreted alongside PERMDISP to account for potential differences in within-group dispersion.

Kruskal–Wallis tests compared the ten most abundant phyla and genera across groups, followed by separate BH adjustments at each taxonomic rank. LEfSe was applied exploratorily using unadjusted $p < 0.05$ and LDA score ≥ 2.0. Because these LEfSe findings were not adjusted for multiple testing, they are reported as candidate discriminatory taxa rather than validated biomarkers.

For PICRUSt2-predicted KEGG Level 2 categories and Level 3 pathways, each PE MP treatment was compared with CK using Welch's t-tests. BH adjustment was performed separately within each treatment–control comparison and KEGG level. Unadjusted $p < 0.05$ was considered nominal significance, whereas $q < 0.05$ indicated significance within the corresponding BH-adjusted comparison family.

3. Results

3.1. Effects of Polyethylene Microplastics on Earthworm Survival, Biomass, and Cocoon Production

Mean mortality varied from 10.00% to 33.33% across PE MP treatments, compared with 10.00 ± 10.00% in CK (Figure 1a). MH (100 μm, 5%) had the highest mean mortality. No factorial effect was significant (size, $p = 0.312$; concentration, $p = 0.777$; interaction, $p = 0.650$), and no treatment differed significantly from CK after Dunnett adjustment (all adjusted $p > 0.05$). Summary statistics, factorial test results, and Dunnett-adjusted treatment–control comparisons are provided in Tables S1–S3.

The container-level biomass-change index showed clearer treatment–control differences (Figure 1b). CK showed a positive mean biomass change of 7.22 ± 4.06%, whereas all PE MP treatments had negative values, ranging from −2.55 ± 0.51% in LL (200 μm, 0.5%) to −19.50 ± 3.80% in XSH (15 μm, 5%). All 12 treatment–CK comparisons were significant (Dunnett-adjusted $p < 0.001$). Particle size ($p = 2.85 \times 10^{-9}$) and concentration ($p = 1.42 \times 10^{-7}$) had significant main effects, but their interaction did not ($p = 0.486$). Negative biomass changes were generally greater at smaller nominal particle sizes and higher concentrations. All six pairwise comparisons among particle-size marginal means were significant (Tukey-adjusted $p < 0.05$). Detailed concentration and particle-size contrasts are provided in Tables S4–S6.

Mean cocoon production was 4.00 ± 1.00 per container in CK and 1.33–9.33 in the PE MP treatments (Figure 1c). SH (45 μm, 5%) had the highest mean (9.33 ± 1.53). Particle size ($p = 7.90 \times 10^{-9}$) and concentration ($p = 0.013$) had significant main effects, with no interaction ($p = 0.975$). Within the 45 μm treatments, SH exceeded SL (Tukey-adjusted $p = 0.024$). However, no individual PE MP treatment differed significantly from CK after Dunnett adjustment (all adjusted $p > 0.05$).

3.2. Alpha and Beta Diversity of the Earthworm Gut Microbiota

The 16S dataset comprised 39 gut bacterial samples, three per group. Neither Chao1 nor Shannon differed significantly across the 13 groups (Kruskal–Wallis: $p = 0.952$ and 0.170, respectively; Figure 2A,B). Within the 12 PE MP treatments, neither index showed significant effects of particle size, concentration, or their interaction (all $p > 0.05$). Sample-level alpha diversity indices and the corresponding statistical analyses are provided in Tables S7–S9.

The Bray–Curtis PCoA showed substantial overlap among groups, although some samples were separated in the ordination (Figure 2C); PCoA1 and PCoA2 accounted for 16.27% and 9.55% of variation, respectively. The global PERMANOVA was significant ($R^2 = 0.393$, $p = 0.0001$). Sequential factorial PERMANOVA across PE treatments also detected size ($R^2 = 0.109$, $p = 0.012$), concentration ($R^2 = 0.071$, $p = 0.042$), and interaction ($R^2 = 0.188$, $p = 0.049$) terms. However, these sequential tests depended on the order in which factors were entered, and the results required consideration of differences in multivariate dispersion. Detailed global and factorial PERMANOVA results are presented in Tables S10 and S11.

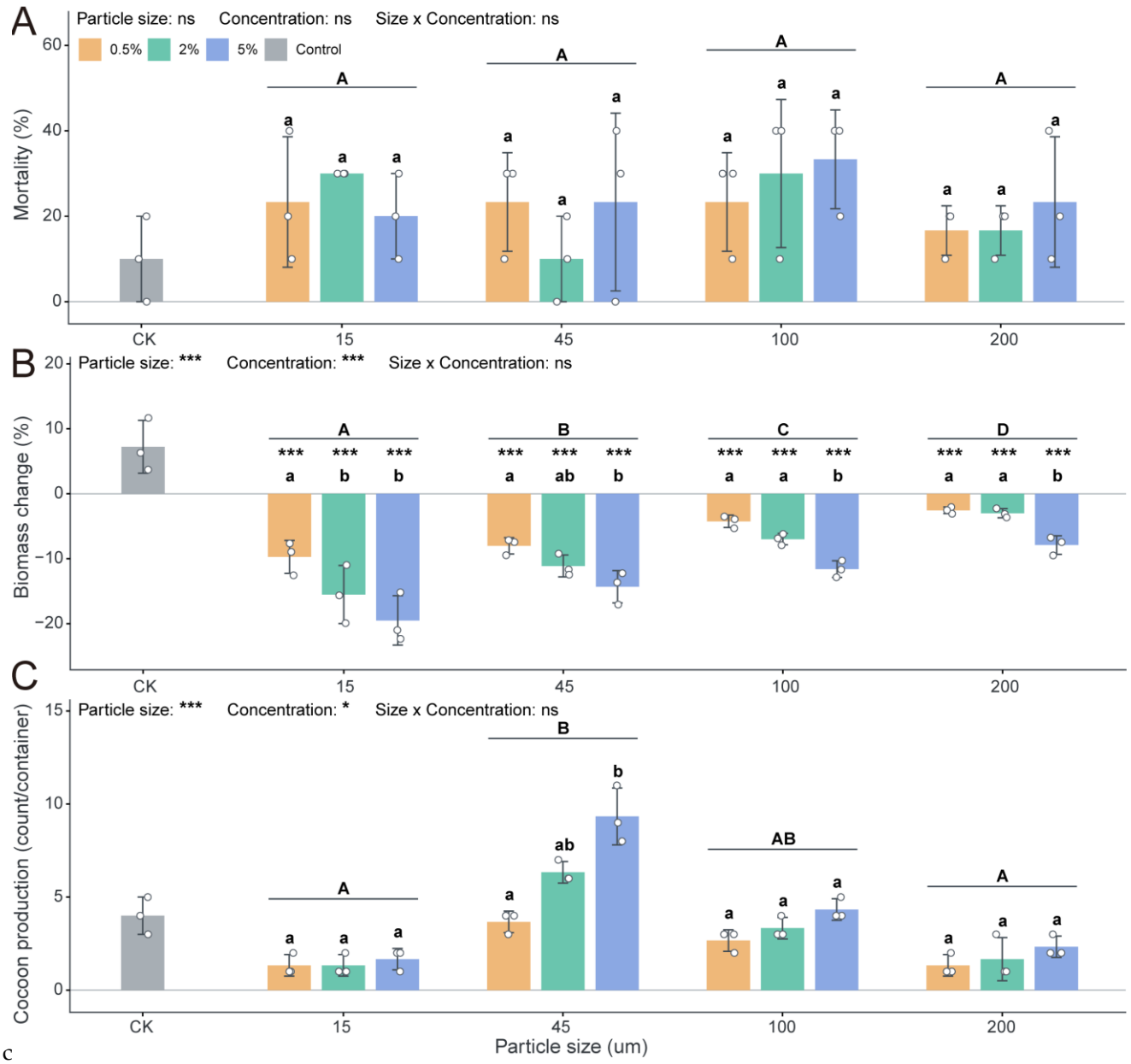


Figure 1. Effects of polyethylene microplastics (PE MPs) with different particle sizes and concentrations on earthworm performance after 30 days of exposure. (**A**) Mortality (%); (**B**) biomass change (%); and (**C**) cocoon production (cocoons per container). Bars represent the mean ± standard deviation (SD), and open circles represent individual experimental containers (n = 3 per treatment). Different lowercase letters indicate significant differences among concentrations within each particle-size class, whereas uppercase letters indicate differences among particle sizes averaged across concentrations (Tukey-adjusted $p < 0.05$). Asterisks indicate significant differences from the control (CK) after Dunnett adjustment (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). CK was excluded from the factorial analyses.

Multivariate dispersion differed significantly among groups (PERMDISP: $F = 3.205$, $p = 0.0069$), indicating that the significant PERMANOVA results may reflect differences in within-group variability as well as group centroids. None of the 78 exact pairwise tests were significant after BH adjustment (all adjusted $p > 0.05$), including the 12 treatment–CK comparisons. With only three samples per group, the minimum attainable p value of

0.1 in the exact pairwise tests further limited the ability to identify treatment-specific differences. PERMDISP and pairwise PERMANOVA results, together with sample-level PCoA coordinates and dispersion values, are provided in Tables S12–S15.

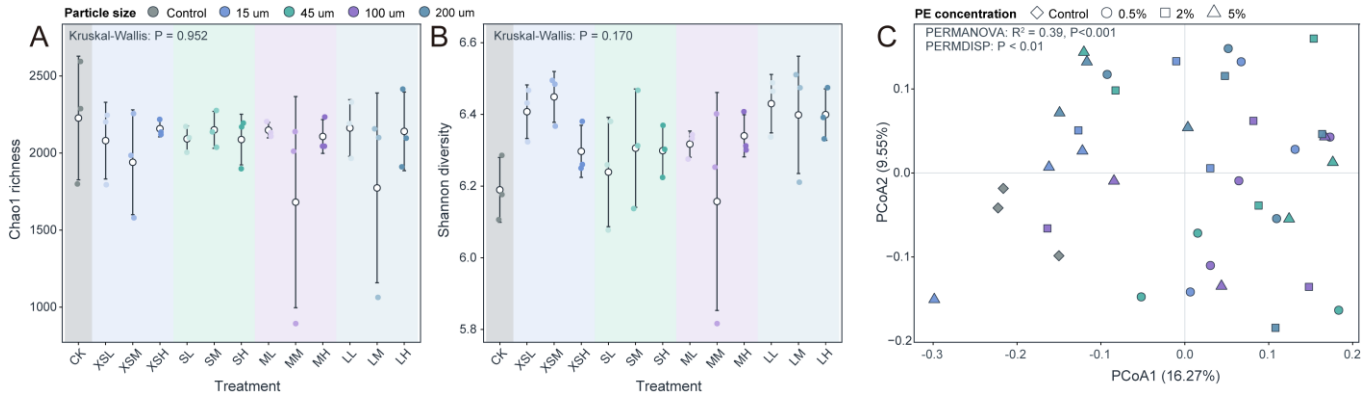


Figure 2. Effects of PE MP exposure on alpha and beta diversity of earthworm gut microbiota. (**A**) Chao1 richness index; (**B**) Shannon diversity index; and (**C**) principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. Each point represents an individual gut microbial sample ($n = 3$ per treatment). PERMANOVA and PERMDISP results are shown in panel (**C**).

3.3. Taxonomic Composition of the Earthworm Gut Microbiota

Taxonomic assignment coverage of the rarefied 16S data is summarized in Table S16. Actinobacteriota, Firmicutes, and Proteobacteria were the most abundant phyla in CK, with mean relative abundances of 38.87%, 20.30%, and 15.16%, respectively (Figure 3A). Firmicutes had numerically higher relative abundances in XSM (34.54%) and SM (36.21%) than in CK (20.30%), whereas Actinobacteriota remained common throughout. Other phyla, including Chloroflexi and Patescibacteria, also varied numerically among groups. Sample-level and treatment-level phylum abundances are provided in Tables S17 and S18.

Demequina, Ilumatobacter, Microbacterium, and Bacillus were among the most abundant assigned genera (Figure 3B). Demequina represented 12.42% in CK and 8.49% in LL, while Microbacterium represented 4.44% in CK and 10.83% in SL. Clostridium_sensu_stricto_1, Romboutsia, and JG30-KF-CM45 also varied numerically, without a consistent pattern across nominal particle sizes or concentrations. Corresponding genus-level abundances are provided in Tables S19 and S20.

Kruskal–Wallis tests detected no significant differences among the 13 groups for any of the ten most abundant phyla or ten most abundant genera after BH correction (all $q > 0.05$; Table S21). Although some taxa showed nominal $p < 0.05$ before correction, these differences did not remain significant after BH adjustment. Thus, the observed abundance differences are descriptive and do not establish treatment-specific enrichment or depletion of individual taxa.

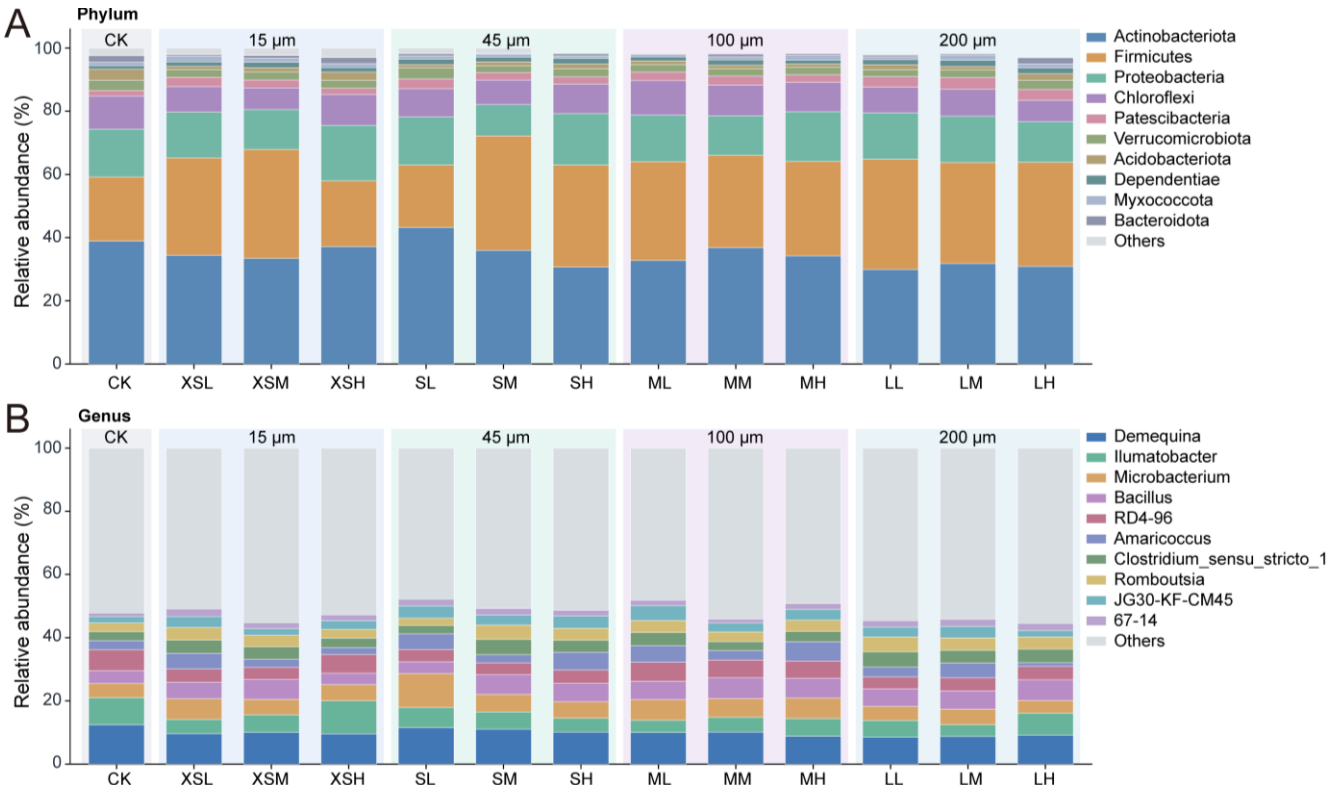


Figure 3. Gut bacterial taxonomic profiles under PE MP exposure: (**A**) dominant phyla and (**B**) dominant genera. Bars show group means from three independent containers. Less abundant taxa not displayed individually were combined as 'Others'; sample-level abundances are provided in Tables S17 and S19. Colors correspond to the bacterial taxa identified in the legends of panels (A,B).

3.4. Exploratory Identification of Discriminatory Bacterial Taxa

The archived LEfSe output comprised 164 taxonomic records across the 13 experimental groups, of which 105 met both screening criteria (unadjusted $p < 0.05$ and LDA score ≥ 2.0) (Figure 4; Table S22). The remaining 59 records had LDA scores below 2.0 and were excluded from the threshold-qualified set. None of the ten dominant phyla or genera were significant in the separate BH-adjusted Kruskal–Wallis analyses.

The 105 threshold-qualified records spanned several phyla, including Actinobacteriota, Firmicutes, and Proteobacteria, but these exploratory signals did not override the FDR-adjusted results for dominant taxa.

At finer taxonomic levels, candidates included Microbacteriaceae, *Nocardioides simplex*, Sphingomonadaceae, and lineages assigned to JG30-KF-CM45. Several records represented different taxonomic ranks within related bacterial lineages and should not be interpreted as independent biomarkers. Because LEfSe results were not corrected for multiple testing and each group contained only three replicates, these taxa should not be treated as confirmed treatment-specific biomarkers.

Taken together, LEfSe provides hypotheses for follow-up testing rather than firm evidence of changes in specific bacterial lineages.

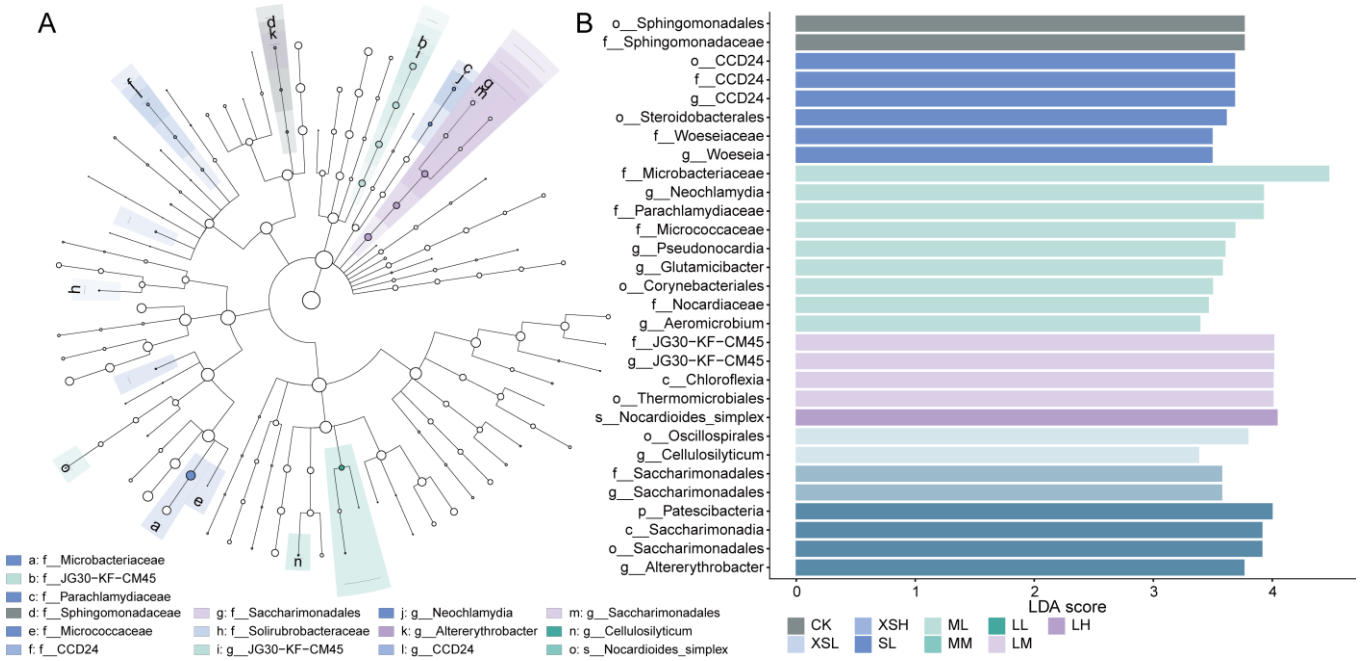


Figure 4. Exploratory LEfSe analysis of gut bacterial taxa: (**A**) discriminant cladogram and (**B**) representative taxa ranked by LDA score. Screening criteria were unadjusted $p < 0.05$ and LDA score ≥ 2.0. Of the 164 archived taxonomic records, 105 met these criteria (Table S22). No FDR correction was applied, so the plotted taxa are candidate indicators, not confirmed biomarkers.

3.5. Predicted Functional Profiles of the Earthworm Gut Microbiota

PICRUSt2 inferred 36 KEGG Level 2 categories and 192 Level 3 pathways from the 16S profiles (Figure 5). Amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins were prominent Level 2 categories. Most predicted profiles were similar across groups, with only a few treatment–CK comparisons meeting the BH-adjusted significance threshold within their respective comparison families.

At KEGG Level 2, three treatment–category comparisons met the within-comparison BH-adjusted threshold ($q < 0.05$). Relative to CK, predicted carbohydrate metabolism was lower in XSM (15 µm, 2%; $\log_2$FC = −0.052, $q = 0.013$), predicted amino acid metabolism was lower in SH (45 µm, 5%; $\log_2$FC = −0.066, $q = 0.017$), and the category annotated as 'Digestive system' was lower in ML (100 µm, 0.5%; $\log_2$FC = −1.165, $q = 0.047$). The last annotation should not be interpreted as a direct measure of earthworm digestion. Complete treatment–control comparisons for KEGG Level 2 categories are provided in Table S23.

At KEGG Level 3, 413 of 2304 treatment–pathway tests had nominal $p < 0.05$, but only six met the BH-adjusted significance threshold within their respective treatment–CK comparisons. Five occurred in ML, where predicted secondary bile acid biosynthesis was higher ($\log_2$FC = 0.575, $q = 0.047$), whereas aminobenzoate degradation ($\log_2$FC = −0.140, $q = 0.047$), caprolactam degradation ($\log_2$FC = −0.265, $q = 0.047$), ribosome biogenesis in eukaryotes ($\log_2$FC = −0.193, $q = 0.047$), and protein digestion and absorption ($\log_2$FC = −1.208, $q = 0.047$) were lower. The remaining significant comparison occurred in SH, where the predicted abundance of the pathway annotated as 'Epithelial cell signaling in *Helicobacter pylori* infection' was higher ($\log_2$FC = 0.360, $q = 0.019$). Disease- or host-related pathway names are KEGG annotations, not evidence of infection or altered host physiology. Complete treatment–control comparisons for KEGG Level 3 pathways are provided in Table S24.

Other pathways, including the citrate cycle, pyruvate metabolism, vitamin B6 metabolism, branched-chain amino acid biosynthesis, and protein export, showed only nominal

differences in some treatment comparisons. Overall, the results indicate a limited number of treatment-specific differences in predicted functional potential under the applied correction scheme, rather than widespread inhibition of bacterial metabolism. The pathway selections and plotting summaries used for Figure 5 are documented in Tables S25–S27, and treatment-level functional response trends are summarized in Table S28.

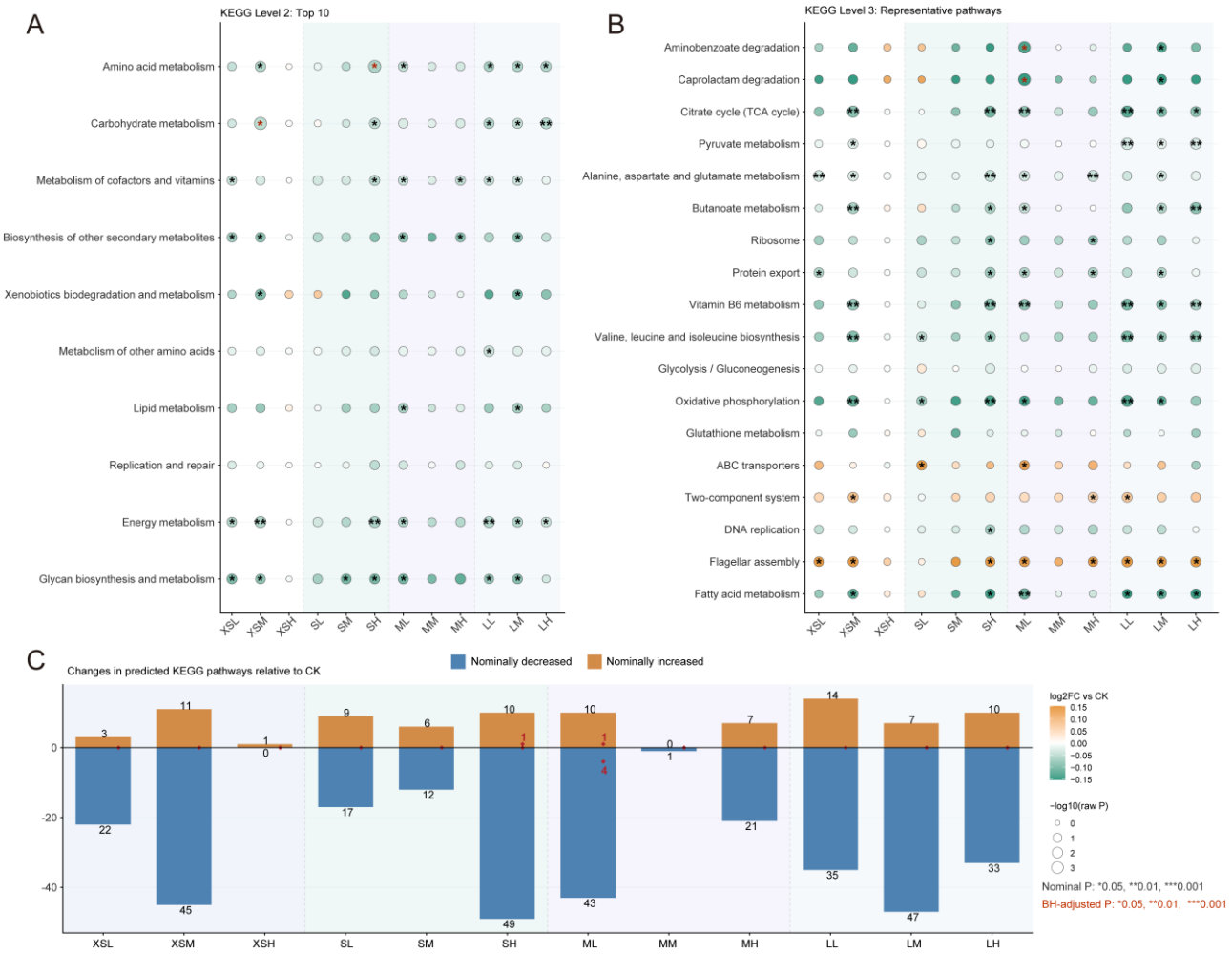


Figure 5. PICRUSt2-predicted KEGG functional profiles under PE MP exposure. (**A**) Ten representative KEGG Level 2 categories and (**B**) selected Level 3 pathways, displayed as treatment–CK comparisons; symbol size indicates nominal significance and color indicates the direction of change. (**C**) Numbers of Level 3 pathways with nominal increases or decreases relative to CK; red markers denote comparisons remaining significant after BH correction. Nominal and FDR-adjusted significance are distinguished in the plot; only corrected $q < 0.05$ results are treated as significant in the text.

4. Discussion

Across the measured endpoints, biomass change showed the clearest response to PE MP exposure, whereas mortality and cocoon production had no significant treatment–CK contrasts. Bacterial alpha diversity showed no detectable differences, and overall beta-diversity patterns varied alongside unequal dispersion. Only a few PICRUSt2-predicted pathways remained significant after correction. These contrasting findings argue for assessing organismal and gut bacterial responses separately rather than assuming they change together.

4.1. Differential Responses of Earthworm Growth, Survival, and Cocoon Production to PE Microplastics

Biomass change was the most consistent organism-level response: every PE MP treatment had lower biomass change than CK, with generally more negative values at smaller nominal sizes and higher concentrations. Earlier earthworm studies have linked PE exposure to ingestion-related stress, tissue injury, oxidative damage, and altered metabolism [25,28–31]. These mechanisms could increase maintenance costs or reduce energy available for growth, consistent with the energy-limited tolerance framework [32]. However, our experiment did not measure ingestion, oxidative stress, or energy metabolism, so these remain possible explanations.

The observed changes in mean body weight were broadly consistent with reports of PE-associated biomass reductions [28,33] and a meta-analysis showing an overall negative effect of microplastics on earthworm growth [34]. Other studies have reported little weight change despite biochemical responses [35], or different responses across polymer types and loading levels [36]. Here, size and concentration showed clear main effects on biomass change, but no significant interaction; the data therefore do not support a synergistic effect. Model-based marginal main-effect contrasts and their 95% confidence intervals are provided in Table S29. Equal amounts of cow manure were supplied to all containers, but intake was not measured. Feeding behavior and substrate accessibility may still have influenced biomass outcomes. Furthermore, because the initial mean body weight included all ten earthworms, whereas the final mean was calculated only from surviving individuals, the reported biomass change reflects a comparison of container-level means rather than paired growth of individual worms. Selective mortality may therefore have influenced this endpoint.

Mortality was less informative during the 30-day exposure: none of the treatment–CK differences remained significant. Earlier work likewise shows that sublethal responses may occur before clear survival effects [33,37], whereas longer exposures have sometimes increased mortality [28]. Our results therefore support biomass change as the more consistent short-term endpoint, without excluding lethal effects under other conditions. However, the absence of significant treatment–control differences should not be interpreted as evidence that mortality was unaffected, particularly given the limited number of replicate containers.

Cocoon counts varied with size and concentration, but no treatment differed significantly from CK after correction. The high mean in SH cannot therefore be interpreted as reproductive stimulation. Cocoon output may depend on adult condition, reproductive allocation, or the number of surviving adults, none of which was tested mechanistically here. Published reproductive responses also vary among experiments [34,35,37,38]. Importantly, cocoons alone do not measure reproductive success because hatching and juvenile production were not recorded [26]. The 30-day exposure was shorter than a standardized OECD 222 reproduction test.

Together, these outcomes show why mortality alone is insufficient to characterize short-term PE MP responses. The container-level biomass-change index showed more consistent treatment–control differences than mortality or cocoon production, although these endpoints describe different aspects of earthworm performance.

Exposure levels are an important limitation. Our 5–50 g kg^{-1} PE MP treatments (5000–50,000 mg kg^{-1}) substantially exceeded the concentrations estimated in agricultural soil surveys, including means or ranges of 6.79 mg kg^{-1} [39] and 3.12–7.99 mg kg^{-1} [40]. Those estimates used different sampling and analytical approaches, but they underline that our treatments are high-loading scenarios. At these levels, physical changes in the substrate and access to food may also influence body-weight outcomes. The observed effect magnitudes should not be extrapolated directly to typical farmland exposure.

Artificial soil provided a controlled experimental substrate but differs from natural soil. Because its final pH, nutrients, and other baseline properties were not measured, substrate-related contributions to the responses cannot be assessed directly. Field-relevant concentrations and well-characterized natural soils are needed to test the generality of these findings.

The particle-size comparison also has limitations. Only nominal PE sizes were available; batch-specific size distributions, polymer identity, morphology, and additives were not independently verified. Equal mass loadings of different sizes also imply different particle numbers and surface areas, assuming otherwise comparable particle properties. Our findings therefore identify differences among nominal size treatments, not the independent effects of size, particle number, or surface area on toxicity.

4.2. Gut Microbial Community Variation Under PE Microplastic Exposure

Gut bacterial richness and Shannon diversity showed no significant differences among the experimental groups, despite variation in overall community patterns. Soil ingestion continually introduces microorganisms into the earthworm gut, where local conditions may favor some lineages over others [41,42]. Relative-abundance differences can therefore occur without detectable changes in richness or evenness. Although community resistance has been proposed as a possible explanation for limited diversity responses [43], nonsignificant alpha-diversity tests alone cannot demonstrate community resistance or stability.

Earlier studies reported little change in gut diversity after HDPE or PP exposure [44], or microbial diversity that remained stable despite other physiological changes under PS exposure [45]. Other work found shifts in particular bacterial groups after LDPE treatment [46] or more pronounced diversity changes [47], consistent with broader syntheses [34]. Such variation may reflect earthworm species, MP properties, exposure duration, and environmental context. Niche differences along the soil–gut–cast continuum may also contribute to variation in gut bacterial communities independently of MP exposure [48].

Although global PERMANOVA was significant, PERMDISP also detected unequal dispersion. The overall pattern may therefore reflect differences in within-group variability as well as in group centroids [49]. No individual treatment differed significantly from CK after BH correction of the exact pairwise PERMANOVA tests. With $n = 3$ per group, the minimum attainable two-group exact p was 0.1, limiting treatment-level inference. Thus, the overall beta-diversity differences should not be interpreted as evidence that each PE MP treatment produced a distinct shift in gut bacterial composition.

Gut conditions can select among ingested microorganisms [41,42], while PE MP exposure might alter substrate properties or the bacterial pool entering the gut. Particle ingestion and passage could also differ among individuals [47,48,50], although these were not measured. Importantly, PE MPs were not quantified in gut contents, so actual ingestion and potential differences in particle uptake among nominal size classes remain unverified. The soil bacterial community was not monitored, so changes in bacteria entering the gut cannot be separated from changes occurring within it. Actinobacteriota, Firmicutes, and Proteobacteria remained among the most abundant phyla, and none of the ten most abundant phyla or genera differed significantly among groups after FDR correction. Relative-abundance data also cannot establish absolute cell-number changes [51]. LEfSe candidates, including Microbacteriaceae and Sphingomonadaceae, thus remain exploratory rather than validated treatment indicators.

Overall, gut bacterial communities showed variation in beta-diversity patterns, but the adjusted analyses provided limited evidence of treatment-specific changes in individ-

ual dominant taxa or pairwise community composition. The observations justify examining microbial responses alongside organismal endpoints but do not establish that gut bacterial changes caused the observed differences in container-level biomass change.

4.3. Selective Responses of Predicted Gut Microbial Functions to PE Microplastic Exposure

Compared with the consistent treatment–CK differences in the container-level biomass-change index, PICRUSt2 analysis identified relatively few differences in predicted gut bacterial functions that met the BH-adjusted significance threshold within individual treatment–control comparisons. These differences occurred in particular treatments rather than across a clear size or concentration gradient. The findings therefore provide limited evidence for a consistent size- or concentration-dependent shift in predicted functional profiles.

Predicted carbohydrate metabolism was slightly lower in XSM, and predicted amino acid metabolism was lower in SH. These categories concern the potential processing of carbon compounds and cellular building blocks. Previous studies have linked earthworm-associated bacteria to carbon processing [52] and reported functional-profile differences following PS [53] or PE mulch-derived MPs [54]. However, our small, treatment-specific differences do not demonstrate broad suppression of nutrient-related microbial functions.

Other studies have directly measured altered tissue, enzyme, or metabolite responses after MP exposure [31,35,45]. Those outcomes are not equivalent to the 16S-based pathway predictions reported here. Lower inferred category abundances therefore do not show that microbial metabolic activity or nutrient assimilation by the earthworms declined.

At Level 3, five of the six comparisons meeting the within-comparison BH-adjusted significance threshold occurred in ML (100 μm, 0.5%). These included lower predicted aminobenzoate and caprolactam degradation, as well as higher predicted secondary bile acid biosynthesis. The remaining significant comparison occurred in SH (45 μm, 5%), involving a pathway annotated as epithelial cell signaling in *Helicobacter pylori* infection. Such pathway labels do not show that the corresponding compounds were present or metabolized. Moreover, annotations referring to eukaryotic ribosome biogenesis, host digestion, or bacterial infection should not be interpreted as evidence of altered earthworm physiology. Because KEGG combines annotations from diverse organisms [55], these labels must be interpreted cautiously. The concentration of significant comparisons in ML also argues against a simple dose–response pattern.

Shared metabolic capabilities across different bacteria could partly explain the limited variation in predicted profiles [56,57], but functional redundancy was not directly examined. PICRUSt2 infers potential functions from marker-gene phylogeny and reference genomes [58,59]; comparisons with shotgun metagenomics show important limitations [60]. The accuracy of these predictions also depends on how well the sampled bacterial lineages are represented in reference genomes. Thus, few significant predictions do not prove unchanged metabolism. Equally, nominal results for the citrate cycle, pyruvate metabolism, or protein export do not support claims of broad pathway inhibition. These findings should also be considered in light of the three biological replicates per treatment and the use of separate BH corrections for individual treatment–control comparisons.

The consistent differences in container-level biomass change, together with the relatively limited differences in predicted bacterial functions, indicate that these endpoints did not show parallel treatment responses. However, this comparison cannot establish a mechanistic relationship between gut bacterial functional potential and earthworm body-weight change. Whether gut bacteria contribute to PE-associated differences in the biomass-change index remains unresolved and will require direct functional and host measurements.

5. Conclusions

During this 30-day artificial-soil experiment, all 12 PE MP treatments showed lower container-level mean body-weight change than CK. Particle size and concentration had significant main effects on this index, but their interaction was not significant. Mortality and cocoon production showed no significant individual treatment–control differences after adjustment. Neither Chao1 richness nor Shannon diversity differed significantly among groups. Although the global PERMANOVA was significant, PERMDISP also detected unequal dispersion, and no exact pairwise comparison remained significant after BH correction. PICRUSt2 predictions showed three significant KEGG Level 2 and six Level 3 treatment–CK comparisons after BH correction within the respective comparison families; these results do not demonstrate changes in actual microbial metabolic activity. Thus, the body-weight change index was the most consistent treatment–control endpoint, although its calculation from initially introduced and surviving worms may have been influenced by selective mortality. No causal link between gut bacterial changes and body-weight responses was established. The high PE loadings, nominal particle sizes, artificial soil, limited replication, and missing baseline soil measurements constrain field extrapolation. Future studies should combine field-relevant exposures and characterized particles with measurements of particle ingestion, soil and gut microbiota, microbial activity, and host physiology.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/toxics14110971/s1. Table S1: Earthworm physiological endpoints by treatment group (mean ± SD; three independent containers per group); Table S2: Factorial effects of particle size, concentration and their interaction on earthworm physiological endpoints; Table S3: Dunnett-adjusted treatment–control comparisons for physiological endpoints; Table S4: Concentration contrasts within particle-size classes; Table S5: Marginal particle-size contrasts; Table S6: Particle-size contrasts within concentration classes; Table S7: Sample-level bacterial alpha diversity indices; Table S8: Factorial analysis of alpha diversity; Table S9: Kruskal–Wallis test of alpha diversity; Table S10: PERMANOVA results; Table S11: Factorial PERMANOVA results; Table S12: PERMDISP results; Table S13: Pairwise PERMANOVA comparisons; Table S14: PCoA coordinates; Table S15: Dispersion values for individual samples; Table S16: Rank-specific taxonomic assignment coverage of rarefied 16S data; Table S17: Dominant bacterial phyla at sample level; Table S18: Dominant bacterial phyla by treatment group; Table S19: Dominant bacterial genera at sample level; Table S20: Dominant bacterial genera by treatment group; Table S21: Kruskal–Wallis and FDR results for dominant taxa; Table S22: Complete exploratory LEfSe output and threshold-screening status; Table S23: Differential KEGG Level 2 pathways; Table S24: Differential KEGG Level 3 pathways; Table S25: KEGG Level 3 pathways selected for Figure 5; Table S26: KEGG Level 2 summary used for Figure 5; Table S27: KEGG Level 3 summary used for Figure 5; Table S28: Functional response trends among PE MP treatments; Table S29: Model-based marginal main-effect contrasts with 95% confidence intervals.

Author Contributions: Conceptualization, H.W. and Y.W.; methodology, Y.Z. and H.W.; software, X.C.; validation, Y.Z. and Y.H.; formal analysis, L.C., X.C. and Y.L.; investigation, L.C., Y.Z. and Y.H.; resources, Y.W.; data curation, L.C. and Y.H.; writing—original draft preparation, L.C.; writing—review and editing, Y.W.; supervision, H.W.; funding acquisition, Y.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, grant number 32500400, and the Fundamental Research Funds for the Central Universities, grant number 2572025AW22.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw 16S rRNA gene amplicon sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1530731. The supporting data are available in the GitHub repository at https://github.com/ink430/earthworm-polyethylene-microplastics-data (accessed on 27 October 2026).

Conflicts of Interest: The authors declare no conflicts of interest.

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