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Microplastics exert minor influence on bacterial community succession during the aging of earthworm (*Lumbricus terrestris*) casts

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ABSTRACT

The soil microbiome, which is shaped by gut-related activities of earthworms, is affected by microplastic contamination. However, the influence of microplastics on earthworm gut and cast microbiomes has been poorly explored. Here, we investigated the influence of microplastics (1% in soil, w/w) on soil physicochemical properties and bacterial communities during gut passage and cast aging of *Lumbricus terrestris*. Microplastics used in agricultural film production were selected, i.e., low density polyethylene, polylactic acid and polybutylene adipate terephthalate (PBAT). Different niches, including pre-ingestion soil, gut content and aged casts (from 0 to 180 days), were studied. Results showed that microplastics possibly enhanced the gut passage-derived difference between pre-ingestion soil and fresh cast in terms of pH, ammonium, nitrate and nitrite, and dissolved organic carbon. But such effects mostly faded out after 180 days of aging. The composition, as well as the alpha and beta diversity of both the total (DNA) and active (RNA) bacterial communities were decisively shaped by their niche (R^2: 0.22–0.63, $p < 0.001$, PERMANOVA), rather than the presence/absence or the types of MPs. Nevertheless, biomarkers indicative of PBAT treatment were identified, and functional prediction for the active community showed that bacterial communities of this treatment had higher potentials for hydrocarbon degradation (4.9–7.8 times that of the microplastic-free treatment in gut and aged casts). We also identified a "Soil-related core community" and a "Gut-related core community" (contributing to 39.2%–50.2% of the cast microbiome), which possibly neutralized microplastic impacts and maintained the structure and function of bacterial communities during the soil–gut–cast transit. Our findings indicate that the tested microplastics exerted a minor influence on the bacterial communities during the cast aging process, microplastics in aged casts might not necessarily have significant additional influence on the soil microbiome when they are incorporated into soils. Future studies testing different soils, polymers, and earthworm species, under field conditions are recommended to help enhance current knowledge of the influence of microplastics on earthworm cast microbiomes.

1. Introduction

Microplastics (MPs) of varying sizes, shapes, colors and polymer types have been found in almost every corner of the Earth, including the Arctic and Antarctica (Aves et al., 2022; Bergmann et al., 2022). Intensive research has been conducted on the impacts of MPs in aquatic systems following early reports of contamination of UK beaches (Thompson et al., 2004). Studies focusing on the impacts of MPs in terrestrial systems, however, are few and far between. Over the last few years, there have been more studies looking at plastic debris and how it can affect the composition and function of soil microbial communities (Meng et al., 2019; Ng et al., 2021). The presence of MPs in soils can alter the soil biophysical environment by changing soil physicochemical properties(De Souza Machado et al., 2018; Qi et al., 2020a). Biodegradable microplastics have been found to create hotspots with enhanced carbon and nutrient turnovers in soils (Zhou et al., 2021), and they also release labile compounds that favor the growth of certain groups of microbes (Meng et al., 2023b). Researchers have argued that the "soil plastisphere"—the micro-environment directly under the influence of plastic debris—can function as hotspots of antibiotic resistance genes and potential pathogens (Zhu et al., 2022; Rillig et al., 2023). On a macro scale, MPs can also potentially affect soil geochemical cycles by shaping soil microbial communities. High dosages of polyethylene MPs (5%, w/w), for example, were found to decrease N_2O

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emissions in fertilized soils in the short term by changing the abundance of key bacterial taxa (Ren et al., 2020). Another study found that hydrogen production in isopod guts was stimulated by polylactic acid (PLA) MPs but inhibited by polyethylene terephthalate (PET) and polystyrene (PS) MPs (Hink et al., 2023).

Earthworms play a vital role in shaping the composition of the soil microbiome as well as the ecological functions of soils (Phillips et al., 2019). Gut-related processes of earthworms include ingestion, digestion and casting. These processes can stimulate ingested soil microorganisms, leading to potential changes in element cycles (Drake and Horn, 2007). The presence of MPs in casts (feces) excreted by earthworms has been reported (Huerta Lwanga et al., 2017), demonstrating the need to better understand the influence of these particles on the gut microbiome. Most existing studies have reported that MPs in general show negligible impacts on the earthworm gut microbiome (Cheng et al., 2021; Yu et al., 2022a; Adhikari et al., 2023; Tang et al., 2023). However, others have reported that MPs of certain polymers (e.g., PS) do exert additional effects on the gut, such as affecting the occurrence of antibiotic resistance genes (Xu and Yu, 2021) and causing translocation in gut bacteria (Li et al., 2022).

Earthworm casts, however, have been largely overlooked in studies of MP influence on the soil microbiome. Earthworm casts are normally more fertile than bulk soils (Van Groenigen et al., 2019). Once casts are deposited in soils, they undergo accelerated aging which leads to significant changes in their microbial compositions and physicochemical properties (Aira et al., 2005). Aged casts will eventually be incorporated back into soils due to natural weathering or animal activities, releasing nutrients and microorganisms that can alter the soil microbiome. The legacy effects of earthworm gut transit on the soil microbiome can be long-lasting, e.g., beyond 168 days for *Lumbricus terrestris*. (Yang et al., 2024). Previous studies have also indicated that the composition of the bacterial community in casts follows a time- and nutrient-dependent succession during aging (Aira et al., 2019), highlighting a potential time-dependent effect of casts on the soil microbiome.

To the best of our knowledge, there is no available information on how MPs affect physicochemical properties and bacterial community succession of casts during aging. In addition, although a few studies have provided DNA-based profiling of MP-affected bacterial communities in soils and earthworm guts, there is still an urgent need to better link such findings with actual changes in the function by clearly separating the active (RNA) part from the total (DNA) community. To address these issues, the current study aimed to (1) explore the potential influence of MPs on the physicochemical properties of earthworm cast, and (2) reveal the impacts of MPs on the composition and function of bacterial communities residing in the earthworm gut and casts by targeting both the total (DNA) and the active community (RNA). With this study, we tried to provide realistic and comprehensive information that can help create a holistic picture of MP impacts on bacterial community succession during the soil–gut–cast transit.

2. Materials and methods

2.1. Soil, earthworms and microplastics

Soil containing no detectable levels of MPs was collected from the fields of Unifarm, Wageningen University & Research (the Netherlands). The collected soil was air dried and then screened with a metal sieve (2 mm). The soil consisted of 3.2% clay, 50.0 % silt and 46.8% sand (sandy loam). The soil pH was 6.2 (1:5, w/v, extracted with water) and contained 3.8% organic matter (loss on ignition). To simulate the aging of earthworm casts deposited on the soil surface (Photo S1), *Lumbricus terrestris,* a widespread anecic species, was selected for cast production. Adult worms with a clear clitellum and similar body weights were handpicked and purged in the dark for 48 h prior to the experiment (4.03 ± 0.12 g, n = 48). Three polymers commonly used to produce agricultural mulch films were selected for the experiment. They are fossil-based non-biodegradable low-density polyethylene (LDPE, Dow™ LDPE 310E), bio-based compostable PLA (NatureWorks® Ingeo™ Biopolymer 2003D), and fossil-based biodegradable polybutylene adipate terephthalate (PBAT, Ecoflex® F Blend C1200). MPs were lab-prepared following a cryogenic fragmentation method described by Meng et al. (2023a). MPs obtained from the cryogenic fragmentation were screened with metal sieves (212 μm and 350 μm mesh). The fraction remaining between the two sieves was collected for experiment. The final average sizes of MPs were 419 ± 160 μm for LDPE, 465 ± 107 μm for PLA and 338 ± 215 μm for PBAT. Detailed size distributions are provided in Fig. S1.

2.2. Cast production and cast aging

To produce and collect uniform casts, we carried out the cast production and aging in microcosms in the lab (Photo S2). Four treatments were established for the experiment. The control treatment (Control) was established using MP-free soil, while for the LDPE, PLA and PBAT treatments, soil spiked with 1% (dw/dw) of the corresponding MPs was used. For each treatment, different niches were selected for detailed study: pre-ingestion soil (Soil), earthworm gut content (Gut), freshly produced cast (C0), and cast aged for 15 (C15), 60 (C60) and 180 (C180) days.

Glass Petri Dishes (⌀120 mm × 20 mm) were used as cast production units (CPUs). 50 g of dry MP-free soil or dry MP-spiked soil was fully mixed in a sealed glass jar and transferred to the CPU. The water content was adjusted to 25% using distilled water. Six units were prepared for each treatment (total of 24 Petri Dishes). All units were pre-incubated for 7 days. 3 g of soil was collected as pre-ingestion soil (Soil) from each Petri Dish before two worms (weighed together) were transferred to each CPU. The cast production was carried out at 16 °C in the dark (to get higher cast production). Cast collection was carried out every 24 h, allowing the moisture of fresh casts to evaporate for a few hours (up to 24 h). Almost all casts collected from the CPUs during cast production were already firm and stable (Photo S3). Special attention was paid during the cast collection to ensure that the cast integrity was maintained. Most of the fresh casts collected (at least 3.0 g, including casts collected from other units if the weight was not adequate) were immediately transferred to the cast aging units (described below) while a small portion was collected and sampled as fresh casts (C0). As a result, the C0 samples were collected during the 6-day cast production, which could ensure the representativeness for casts produced on different days. No mortality of earthworms was recorded and by the end of the cast production, worms were collected, rinsed with distilled water, dried with paper tissue, and preserved at −20 °C before gut content extraction was conducted. The presence of MPs (1%, dw/dw) did not have significant influence on the cast production and growth of *Lumbricus terretris* during the 6-day cast production (Data S1).

Cast aging was performed in straight specimen containers (30 mL, polypropylene). The containers were filled with 20 g of dry MP-free soil and a piece of cotton gauze was carefully placed on top of the soil. The water content was then adjusted to 25% using distilled water. The purpose of covering the soil with cotton gauze was to help separate aged casts from the soil during sampling. All the containers were pre-incubated at 16 °C in the dark for 7 days before use. After placing the fresh casts into the containers, the containers were wrapped with parafilm to avoid excessive evaporation while maintaining air exchange. For each treatment, the aging of cast was carried out for 15 days (C15), 60 days (C60) and 180 days (C180). For each time point, six units were established with approximately 3 g of casts per unit.

2.3. Sampling

Pre-ingestion soil (Soil) was collected just before earthworms were added to the CPUs. Fresh (C0) and aged (C15, C60 and C180) casts were collected with metal tweezers and spatulas, tools were cleaned and

disinfected with 70% ethanol after each use. The collected soil and cast samples were divided into two portions: one portion was immediately stored in sterile Eppendorf microcentrifuge tubes (2 mL) at −80 °C and the other portion was weighed and stored in glass vials (20 mL) then dried in the oven at 40 °C for 48 h.

Earthworms kept at −20 °C were slowly defrosted in the biosafety cabinet. The dissection of earthworms was conducted using sterile surgical scissors and nails inside a biosafety cabinet, following Barois et al. (1993). The dorsal side of the worm was cut and the entire gut content of each worm was collected and deposited into sterile Eppendorf microcentrifuge tubes (2 mL). Collected gut contents were kept at −80 °C for nucleic acid extraction.

2.4. Chemical analysis

For aged casts (C15, C60 and C180) at least 18 g (3 g × 6 replicates, fresh weight) were collected for each treatment. Approximately 40 g of fresh casts (C0) were collected for each treatment across the six-day cast production. Since most of the oven-dried samples were used for other MP-related measurements (paper in preparation), we had to merge the replicates to get enough material for the chemical analysis. Soil and cast pH were measured from water extracts with a ratio of 1:5 (m:v) using a pH meter (Fisherbrand™ accumet™ FE150). The moisture contents were calculated based on the gravimetric difference before and after being dried in the oven (40 °C, 48 h). Oven dried samples were gently crushed with glass rods and completely passed through a 2 mm sieve. Samples were then shaken in a ratio of 1:10 (m:v) for 2 h with a 0.01M calcium chloride solution following ISO 14255:1998. After the centrifugation and filtration of the suspension, the concentrations of nitrate and nitrite (N-NO_x), ammonium (N–NH_4) and dissolved organic carbon (DOC) in the extract were measured using a segmented flow analyzer (SFA, Skalar San++). The detection limits for N-NO_x, N–NH_4 and DOC were 0.5 mg kg^{-1}, 1 mg kg^{-1}, and 3 mg kg^{-1}, respectively.

2.5. Nucleic acid extraction and reverse–transcription polymerase chain reaction (RT–PCR)

The extraction of total DNA and RNA was conducted following a protocol previously reported by Harkes et al. (2019). The protocol was scaled down to fit the 250–350 mg samples into the Eppendorf microcentrifuge tubes (2 mL). Detailed procedures are described in Text S1. The quantity and quality of obtained DNA and RNA were determined by NanoDrop™ One (Thermo Scientific™) and Qubit 4 Fluorometers (Thermo Scientific™), respectively. DNA was successfully extracted from all niches, however, we failed to extract enough RNA from C0. Therefore, C0 was excluded from the downstream analysis to be introduced below. The final extracts were kept at −80 °C for future use.

The synthesis of complementary DNA (cDNA) from the extracted total RNA (RT–PCR) was carried out using the Maxima First Stand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific™) following the user manual. The final products were kept at −80 °C for future use.

2.6. 16S amplicon sequencing and bioinformatic analysis

The V3–V4 region of the 16S rRNA gene was amplified using the 341F (CCTAYGGGRBGCASCAG)–806R (GGACTACNNGGGTATCTAAT) primer set. Polymerase chain reaction (PCR) was carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 0.2 μM of forward and reverse primers, and about 10 ng DNA or cDNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s. The PCR products of proper size were selected through 2% agarose gel electrophoresis. The same amount of PCR products from each sample were pooled, end-repaired, A-tailed, and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform (PE250) at Novogene Co., Ltd.

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (v1.2.11, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Quality filtering on the raw tags was performed using the fastp software (version 0.23.1) to obtain high-quality clean tags (Chen et al., 2018). Chimera sequences were detected and removed with UCHIME. Denoise was performed with DADA2 to obtain initial ASVs (amplicon sequence variants). Species annotation was performed with QIIME2 software against the Silva (v138.1) database. Finally, a total of 10,046,679 sequences (avg 75,539 ± 6152 per sample) passed all quality filters and were assigned to ASVs. Sequence data have been uploaded to NCBI Sequence Read Archive (SRA) with accession number PRJNA1063260.

2.7. Community analysis and statistics

Data analysis was performed on R (v4.2.3) using package “microeco” (v1.3.0) (Liu et al., 2021). ASVs not assigned in the Kingdom “k_Archaea” or “k_Bacteria” were removed. ASVs with the taxonomic assignment “mitochondria” or “chloroplast” were considered to be contaminants and were removed. Rarefaction was not conducted as the sequencing depths of all samples have reached saturation (Fig. S2) to retain as much information as possible (Willis, 2019). The abundance-based coverage estimator index (ACE) and Shannon index were calculated to estimate the richness and diversity of communities. Principal coordinate analysis (PCoA) based on Bray–Curtis and Unweighed Unifrac distance was performed to profile the similarity and dissimilarity between samples and tested with permutational multivariate analysis of variance (PERMANOVA). To study the contribution of soil microbiome to the cast microbiome, we defined a “Soil-related core community”, the members (ASVs) of which occurred (sequence > 0) in all samples belonging to pre-ingestion soil and gut. To explore the contribution of indigenous earthworm gut microbiome to the cast microbiome, we defined a “Gut-related core community”, the members (ASVs) of which did not occur (sequence = 0) in any pre-ingestion soil sample but in > 80% of the gut samples. The analysis of core community was conducted at both the DNA and RNA levels. Biomarkers indicative of certain niches or treatments were explored using Linear discriminant analysis (LDA) Effect Size (LEfSE) at ASV level (Segata et al., 2011), and an LDA score of > 2 was used as the filter. Community functional prediction was performed only for the active community (RNA) using FAPROTAX (Louca et al., 2016). The normality of data was checked with the Shapiro–Wilk test. For data following normal distribution, inter-niche or inter-treatment comparison was conducted with one-way analysis of variance (ANOVA). For data not following normal distribution, the Kruskal–Wallis H-test was used. The significance level was set as $\alpha = 0.05$. Data visualization was conducted on R (v4.2.3) using package “microeco” (v1.3.0) and Python (v3.12.0) using the Seaborn (v0.13.1) and Matplotlib (v3.5.3) library.

3. Results

3.1. Physicochemical properties of the soil and cast

The moisture content of casts stayed stable during the 180-day aging process (Fig. 1A) and there was no significant difference between treatments (one-way ANOVA, $p > 0.05$) within the same niche. The only fluctuation was observed between C0 and C15 with LDPE, where the moisture of C15 was significantly higher than C0 ($p < 0.05$). The pH values of C0 were remarkably higher than Soil, and the addition of MPs in general led to higher pH values (7.35–7.50) compared to Control (6.89) (Fig. 1B). Such difference gradually disappeared from C0 to C60. However, with extended aging up to 180 days, a slightly lower pH was found for PBAT (6.23) compared to other treatments (6.46–6.53).

The addition of MPs did not change the trend of N–NH_4 and DOC

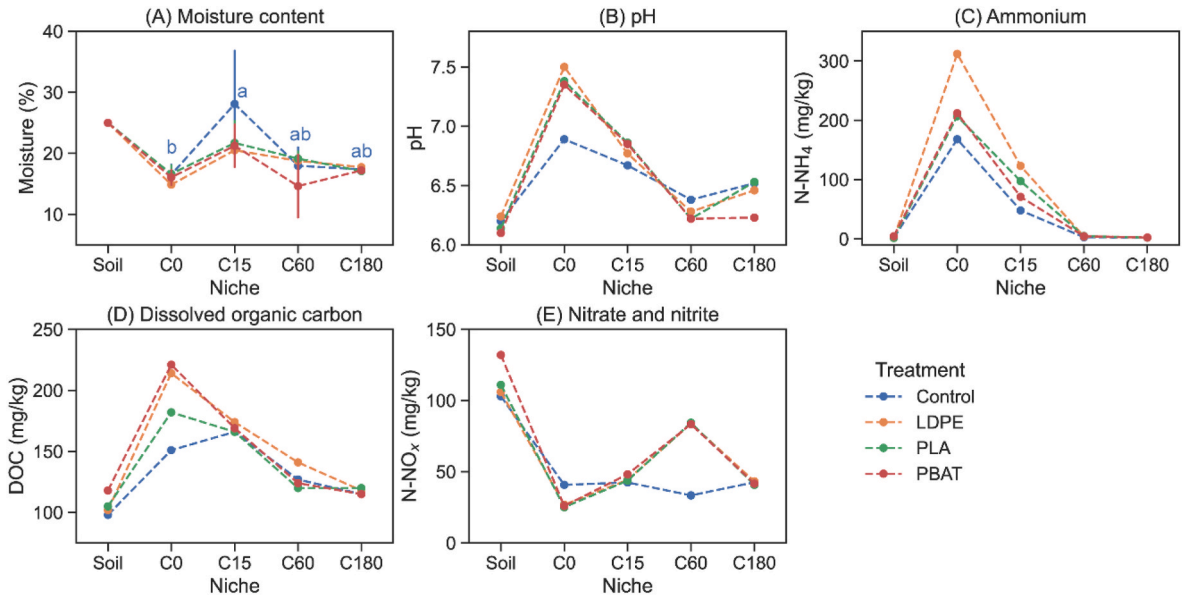

Fig. 1. Physicochemical properties of pre-ingestion soil (Soil) and casts in different treatments. C0–C180: casts aged for 0–180 days. The effects of treatments and niches on the moisture of casts were tested separately with one-way ANOVA. Significant difference was labeled (if any) with lowercase letters. Due to insufficient sample size, the values of pH, ammonium (N–NH_4), nitrate and nitrite (N-NO_x) and dissolved organic carbon (DOC) were obtained from one sample pooled from all replicates within the same niche. Detection limits for pH, N–NH_4, N-NO_x, and DOC were 0.01, 1 mg kg^{-1}, 0.5 mg kg^{-1}, and 3 mg kg^{-1}, respectively.

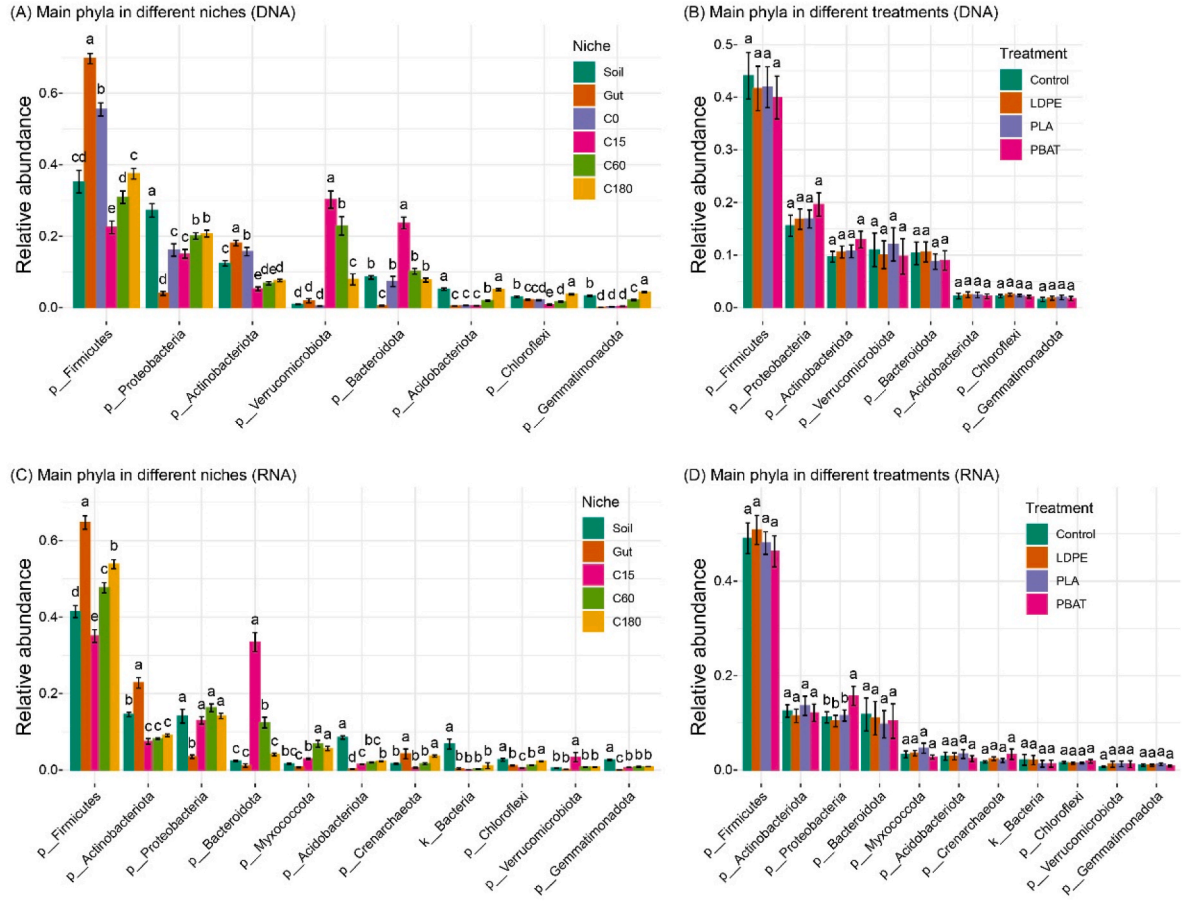

Fig. 2. (A & C) Relative abundances of main phyla in different niches during the soil–gut–cast transit across all treatments at DNA and RNA levels. (B & D) Relative abundances of main phyla in different treatments across all niches at DNA and RNA levels. Significant difference in relative abundance was tested with one-way ANOVA and labeled with lowercase letters. Only phyla with relative abundances > 0.01 were considered as main phyla and displayed. Soil: pre-ingestion soil. Gut: earthworm gut content. C0, C15, C60 and C180 represent earthworm casts aged for 0, 15, 60 and 180 days, respectively.

(Fig. 1, C–D) during gut transit and cast aging. Contents of N–NH_4 (168–312 mg kg^{-1}) and DOC (151–221 mg kg^{-1}) in C0 were significantly higher than Soil (N–NH_4: 1.7–4.6 mg kg^{-1}, DOC: 98–118 mg kg^{-1}), and the contents of C0 in MP-addition treatments (i.e. LDPE, PLA and PBAT) were 23–86% (N–NH_4) and 21–46% (DOC) higher than those for Control. During the aging process, the N–NH_4 and DOC contents in all treatments declined sharply to a similar level after 60 days and stayed stable in the late aging state. The N-NO_x contents substantially declined after gut transit in all treatments (Fig. 1E) and larger reductions were observed for MP-addition treatments (−80% to −75%) as compared to Control (−60%). The N-NO_x content of Control stayed stable throughout the 180-day aging, whereas those of LDPE, PLA and PBAT gradually increased in the first 60 days and reached ~84 mg kg^{-1}, which was more than twice that of Control (33 mg kg^{-1}). From C60 to C180, the N-NO_x contents of MP-addition treatments decreased to a level similar to Control.

3.2. Bacterial community compositions of different niches under different treatments

Most detected phyla were shared by both the 16S DNA sequencing and the 16S cDNA sequencing, except 6 phyla with extremely low relative abundances (Table S1). Differential abundance tests showed that the relative abundances of main phyla altered significantly during the soil–gut–cast transit at both DNA and RNA levels (Fig. 2, A & C). In Soil, phyla including Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota and Acidobacteriota dominated the community, accounting for 54.7–97.2% (DNA) and 72.1–95.8% (RNA) of the total sequences across all samples (Fig. S3). While in Gut, the ratios of sequences belonging to Firmicutes (e.g., *Bacillus, Sporosarcina, Paenibacillus*) and Actinobacteriota increased significantly (ANOVA, $p < 0.05$) compared to Soil, accounting for 82.5–92.2% (DNA) and 75.2–93.0% (RNA) of the total sequences (Fig. S4). During cast aging, bacterial communities in the cast developed in a clear aging time-dependent manner (Gut–C180). The relative abundances of gut-predominating phyla e.g. Firmicutes and Actinobacteriota declined sharply in the first 15 days ($p < 0.05$), but later increased steadily from C15 to C180. While phyla that were suppressed in the gut, such as Verrucomicrobiota and Bacteroidota experienced a boom from Gut to C15 (from 0.11% to 33.7% for Verrucomicrobiota, from 0.24% to 36.3% for Bacteroidota) and then decreased gradually from C15 to C180. Surprisingly, it was found that MPs did not show any significant influence on the relative abundances of main phyla at DNA level (Fig. 2, B), whereas at RNA level PBAT MPs was associated with significantly higher relative abundance of Proteobacteria ($p < 0.05$, Fig. 2, D).

3.3. Bacterial community richness and diversity

Principal coordinate analysis (PCoA) at ASV level (Fig. 3) showed clear separation between communities derived from DNA and RNA (Table S2, Bray–Curtis: $R^2 = 0.14$, $p < 0.001$; Unweighed Unifrac: $R^2 = 0.07$, $p < 0.001$). PERMANOVA analysis showed that both the total and active bacterial communities were significantly separated by the niche rather than the treatment (Table 1). Pairwise PERNANOVA based on Bray–Curtis distance (Table S3) and Unweighed Unifrac distance (Table S4) further showed that each niche forms a unique microenvironment where the bacterial community composition differs significantly from the rest.

Across all samples, the total (DNA) community richness (ACE: 1317 ± 286) was significantly higher than the active (RNA) community richness (ACE: 1061 ± 141) (Kruskal–Wallis H-test, $p < 0.001$), while the diversity (Shannon index) of the total community did not differ significantly from the active community. The community richness and diversity at DNA and RNA level under different treatments were compared within each niche (Fig. S5) and across all niches (Fig. 4, C–D). It turned out that the alpha diversity of bacterial communities was not affected by the addition of MPs. However, significant differences in richness and diversity were found between different niches within each treatment (Fig. S6) and across all treatments (Fig. 4, A–B). It was also obvious that the total community in general exhibited larger inter-niche differences than the active community (Fig. S6 & Fig. 4, A–B). The richness and diversity of the total bacterial communities decreased significantly from Soil to Gut and reached the lowest in C15 ($p < 0.05$). While from C15 to C180, the richness and diversity increased gradually and finally recovered to similar levels as Soil. In contrast, results of

Table 1
Main PERMANOVA analysis for different groups on the total (DNA) and active (RNA) communities.

Measure	Groups	DNA		RNA	
		R^2	P	R^2	P
Bray-Curtis	Niche	0.63457	0.001	0.58499	0.001
	Treatment	0.02646	0.909	0.0366	0.862
Unweighed Unifrac	Niche	0.2257	0.001	0.31152	0.001
	Treatment	0.03771	0.928	0.03831	1

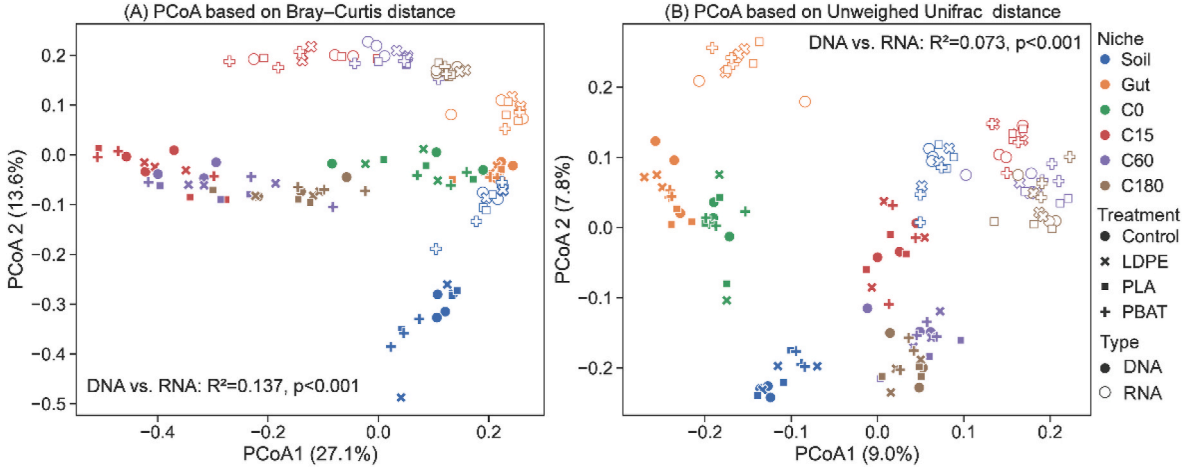

Fig. 3. Principal coordinate analysis (PCoA) of bacterial communities at ASV level. (A) PCoA profiling based on Bray–Curtis distance. (B) PCoA profiling based on Unweighed Unifrac distance. Samples of different niches and treatments are distinguished by different colors and symbols. Solid and hollow symbols represent total community (DNA) and active community (RNA), respectively. Soil: pre-ingestion soil. Gut: earthworm gut content. C0, C15, C60 and C180 represent earthworm casts aged for 0, 15, 60 and 180 days, respectively.

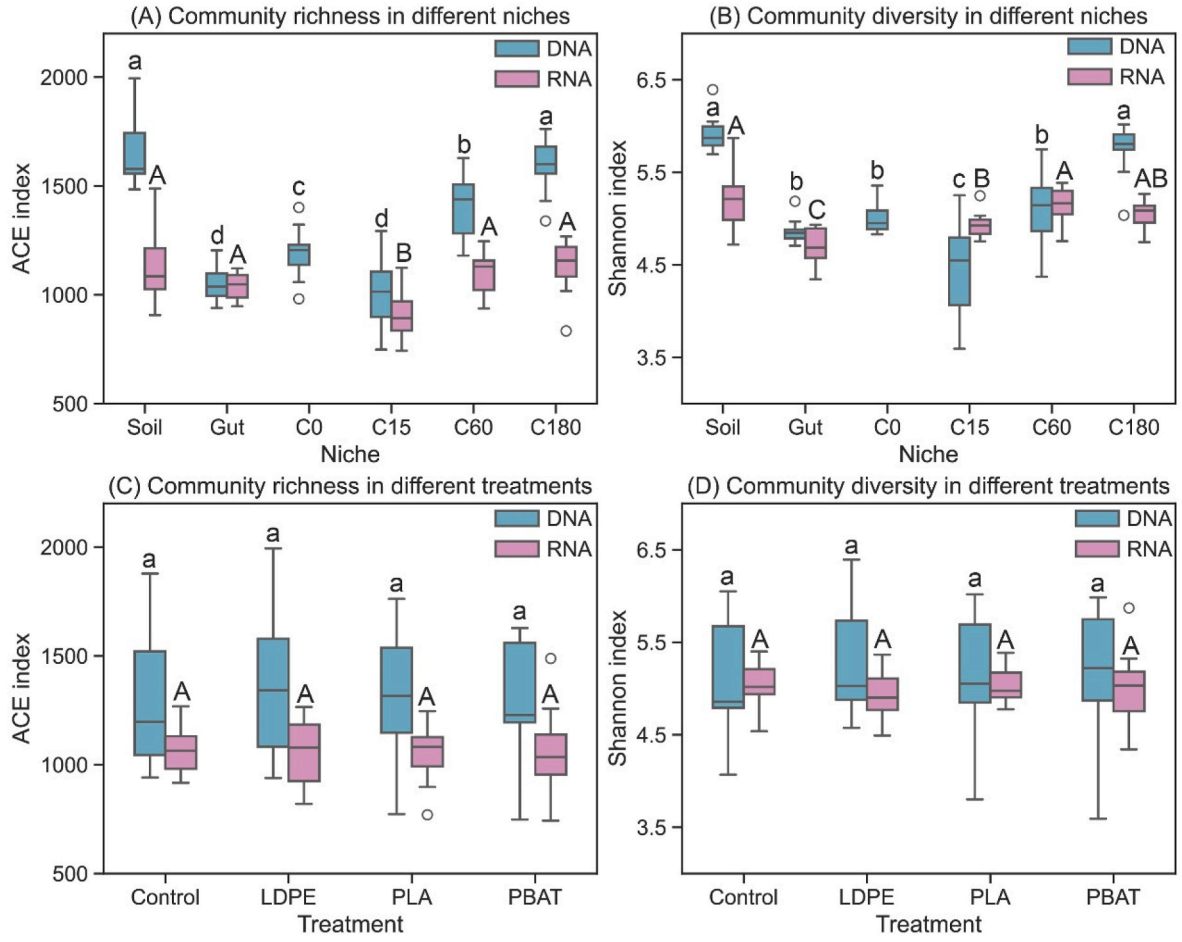

Fig. 4. Community richness (ACE index) and diversity (Shannon index) at ASV level. (A–B) Alpha diversity indexes in different niches. (C–D) Alpha diversity indexes in different treatments. Blue and pink colors represent DNA- and RNA-based results, respectively. Significant differences tested with one-way ANOVA were labeled with lowercase and uppercase letters for DNA and RNA results, respectively. C0, C15, C60 and C180 represent earthworm casts aged for 0, 15, 60 and 180 days.

active community only showed that the community richness in C15 was significantly lower than other niches ($p < 0.05$), and the community diversity was lower than other niches in Gut ($p < 0.05$).

3.4. Core community and biomarkers for different niches and treatments

The dynamics of the Soil-related core community (SCC) during the soil–gut–cast transit were displayed in Fig. 5. At DNA level, the SCC was comprised of 62 ASVs (Data S2) mostly belonging to Firmicutes (e.g. *Bacillus*, *Sporosarcina*, and *Lysinibacillus*) and Actinobacteriota (e.g. *Gaiella* and *Nocardioides*), which accounted for 20.5% (C15)–60.9% (Gut) of the entire community across different niches (Fig. 5, A). The SCC at RNA level was comprised of 80 ASVs (Data S2) mostly belonging to Firmicutes (e.g. *Bacillus*), Actinobacteriota (e.g. *Conexibacter*, *Gaiella*, and *Nocardioides*), and Proteobacteria (e.g. *Skermanella*), which accounted for 32.7% (C15)–60.9% (Gut) of the entire community across different niches (Fig. 5, B). In addition, 31 ASVs were shared by SCCs at both DNA and RNA levels (Data S2). At DNA level, the Gut-related total core community (GCC) consisted of 4 ASVs belonging to Verrucomicrobiota (*Luteolibacter*) and Actinobacteriota (*Nocardioides*) (Data S3), accounting for 0.1% (C0)–25.6% (C15) of the entire community across the gut–cast transit (Fig. 5, A). The GCC at RNA level consisted of 11 ASVs belonging to Firmicutes (e.g. *Candidatus_Lumbricincola*) and Actinobacteriota (*Nocardioides*) (Data S3), but making up a smaller portion of the entire community 0.05% (C180)–2.5% (Gut) (Fig. 5, B).In general, the SCCs at both DNA and RNA levels first prospered from pre-ingestion soil to earthworm gut, then experienced a substantial decline during the early stage of cast aging (Gut–C15) and recovered gradually in the late stage of cast aging (C15–C180). By contrast, the GCC at DNA level experienced a booming from Gut to C15 and maintained a considerable level throughout the 180-day aging. It is noteworthy that the SCC and GCC at DNA level together accounted for 39.2% (C180)–50.2% (C0) of the entire cast microbiome during the cast aging process.

Given the clear separation of samples by different niches on PCoA plots (Fig. 3) and the significant changes in soil/cast physicochemical properties, it is not surprising that thousands of ASVs were identified as biomarkers for different niches (Data S4 & Data S5). At DNA level, we identified 356, 160, 104, 87, 77 and 263 ASVs that are indicative of Soil, Gut, C0, C15, C60 and C180, respectively. While at RNA level, there were 337, 56, 57, 64 and 99 biomarkers for Soil, Gut, C15, C60 and C180, respectively. It was found that *Sphingomonas* and *Bryobacter* were the most indicative genera for Soil at DNA and RNA level, respectively (Fig. 5, C–D). In the earthworm gut *Bacillus* was the most distinct biomarker for both the total and active community, while *Candidatus_Lumbricincola* was a biomarker only found at RNA level. C0 was characterized by the flourishing of *Aeromonas* and *Paenibacillus*. During the cast aging, biomarkers of C15, C60 and C180 were mostly members of Phylum Proteobacteria, Verrucomicrobiota, Bacteroidota and Chloroflexi.

Only a few taxa were identified as biomarkers for MPs. Specifically, 12 ASVs belonging to 8 genera were identified as biomarkers for PBAT at DNA level and 6 ASVs under 5 genera were identified as biomarkers for PBAT at RNA level (Table S5). Among them, 5 biomarkers belonging to Oxalobacteraceae, Comamonadaceae, *Cupriavidus* and *Bacteriovorax*

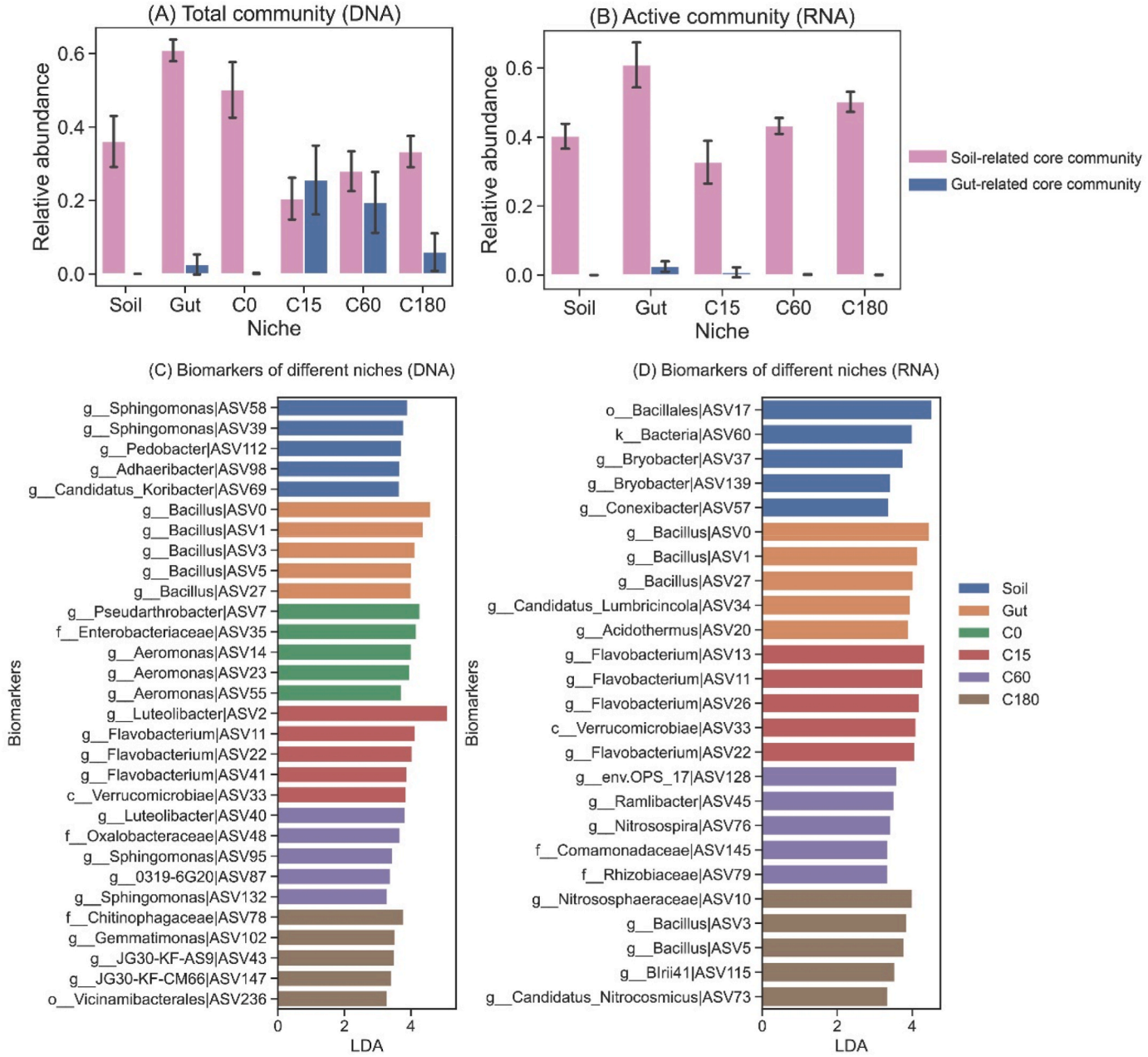

Fig. 5. Dynamics of soil-related core community and gut-related core community during the soil–gut–cast transit at (A) DNA level and (B) RNA level. The taxonomic compositions of the defined core communities can be found in Data S2 and Data S3. Bars represent average relative abundances calculated from 12 samples of each niche and error bars represent standard deviations. (C-D) Biomarkers (LEfSE) indicative of different niches at (C) DNA level and (D) RNA level. For each niche, only ASVs with top 5 LDA scores were displayed. The full biomarker lists at DNA and RNA level can be found in Data S4 and Data S5. Soil: pre-ingestion soil. Gut: earthworm gut content. C0, C15, C60 and C180 represent earthworm casts aged for 0, 15, 60 and 180 days, respectively.

were shared by both the total and active community. No biomarker was found for other treatments.

3.5. *Functional groups of active bacterial communities in different treatments and niches*

Functional prediction was only performed for the active bacterial community (RNA) using FAPROTAX and several functions related to nitrogen cycling, degradation of hydrocarbon and energy sources were selected and are displayed in Fig. 6. We did not find any significant influence of MP addition on functions related to nitrogen cycling. The addition of PBAT MPs significantly promoted the potential of active bacterial communities for aromatic/aliphatic hydrocarbon degradation in most niches ($p < 0.05$, Fig. 6, E–H), and such influence was also evident across all niches (Fig. S6, E–H). Notably, the aromatic/aliphatic hydrocarbon degradation potentials of Gut, C60 and C180 with PBAT were 6.3–9.4 times that of Control (Fig. 6, E & G), and the hydrocarbon degradation potentials of Gut, C60 and C180 with PBAT were 4.9–7.8 times that of Control (Fig. 6, H). In addition, PBAT MPs also increased the abundance of bacteria associated with aerobic chemoheterotrophy in the soil ($p < 0.05$, Fig. 6, I).

In contrast, almost all predicted functions were affected by the niche (Fig. S7). Bacterial communities in Gut and C180 showed significantly higher potential for nitrification compared to Soil, C15 and C60 ($p < 0.05$, Figs. S7 and A). Function groups associated with denitrification and aerobic chemoheterotropy were more abundant in C15 and C60 (p

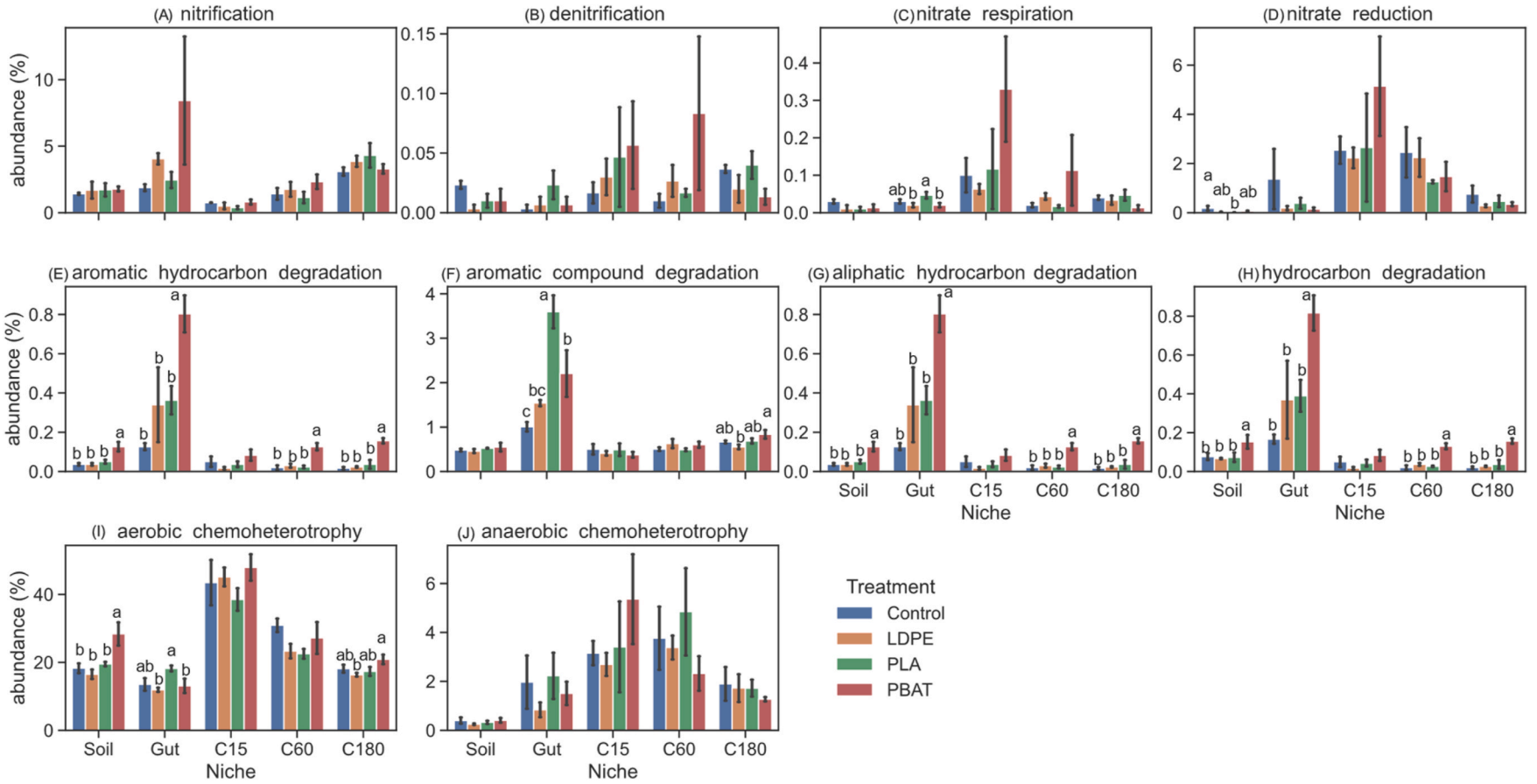

Fig. 6. Effects of microplastic-addition on the active bacterial functional groups in different niches. (A–D) functions related to nitrogen cycling. (E–H) functions related to the degradation capacity of hydrocarbon compounds. (I–J) functions related to energy sources. Differences in abundance of bacterial functional groups between treatments were tested for each niche (one-way ANOVA) and labeled with lowercase letters. Error bars represent standard errors (n = 3). Soil: pre-ingestion soil. Gut: earthworm gut content. C0, C15, C60 and C180 represent earthworm casts aged for 0, 15, 60 and 180 days, respectively.

< 0.05, Fig. S7 C–D). Gut hosted significantly more bacteria potentially able to degrade aromatic/aliphatic hydrocarbon compounds than any other niche ($p < 0.05$, Fig. S7, E–H). Abundances of anaerobic chemoheterotropy in different niches were as follows, C15 ≈ C60 > Gut ≈ C180 > Soil (Figs. S7 and J).

4. Discussion

4.1. MPs affected physicochemical properties of the earthworm cast

It was a pity that the physicochemical properties were measured from one sample pooled from all replicates due to insufficient sample size. As a result, we could not perform any statistics on these data. Nonetheless, the numerical difference in these parameters between the MP-addition treatments and Control (Fig. 1) was evident, considering the detection limits of applied analytical methods (pH: 0.01, N–NH_4: 1 mg kg^{-1}, N-NO_x: 0.5 mg kg^{-1}, DOC: 3 mg kg^{-1}). Existing research studying similar cast physicochemical properties could also show that the analytical errors of these physicochemical properties were usually within ranges that do not undermine the difference observed in our study (Aira et al., 2005; Jouquet et al., 2008; Clause et al., 2014; Shi et al., 2019). Our study showed that gut passage largely increased the pH, N–NH_4 and DOC contents but reduced the N-NO_x contents (Fig. 1) in the fresh cast compared to pre-ingestion soil, which is consistent with previous findings (Horn et al., 2003; Van Groenigen et al., 2019; Vos et al., 2019). It has been reported that earthworm guts contain significantly more water-soluble polysaccharides and amino acids than the surrounding soils (Drake and Horn, 2007), and earthworm casts are characterized by higher pH, Ca^{2+}, Mg^{2+} and K^{+} contents (Jouquet et al., 2008). Although nitrate and nitrite were not measured separately, the reduction of N-NO_x and the increase of N–NH_4 in the fresh cast was evident, and such changes could be attributed to the fact that the earthworm gut is an anaerobic and reductive microenvironment ideal for the reduction of nitrate (Horn et al., 2003; Zhou et al., 2019). Despite the limitations of the data, it seems that the presence of MPs in general enhanced the effect of gut processes on soils, leading to an even higher increase in the pH, N–NH_4 and DOC contents, and a larger decrease in the N-NO_x contents. However, more experimental data is needed to further confirm this phenomenon.

The sharp reduction of N–NH_4 and DOC contents from C0 to C60 in our study was in consistent with previous findings (Decaëns, 2000; Aira et al., 2005; Bottinelli et al., 2020). The development of N-NO_x contents in Control was also similar to previous field observations (Decaëns, 2000). During cast aging, MPs did not have much influence on the development of DOC, N–NH_4 and pH, except that the pH of PBAT did not increase from C60 to C180 as happened in other treatments. It could be that PBAT MPs experienced some hydrolysis, and the hydrolysates led to the slightly lowered pH value. The different trends of N-NO_x development between Control and MP-addition treatments might be attributed to the increased soil/cast porosity caused by MPs (Qi et al., 2020a), which could enhance aeration and promote nitrification.

Little information is available for MP influence on the physicochemical properties of earthworm gut content and cast; however, some studies have reported relevant findings for soils. For example, MPs in the form of fragments and foams were reported to increase the soil pH (Zhao et al., 2021), and PE MPs were found to increase the DOC content in the soil (Liu et al., 2017). One recent study has also shown that the presence of PP MPs (> 0.5%) could accelerate soil nitrification (Guo et al., 2023). However, contrasting findings were also reported, where MPs from PE were found to inhibit nitrification in the soil by directly affecting the enzyme activity (Lan et al., 2024). Although the effects of MPs on soils might provide useful reference, earthworm gut and cast are more complex microenvironments. Therefore, more in-depth studies are needed to elucidate MP effects on the physicochemical properties of earthworm gut and cast.

4.2. PBAT microplastics have minor influence on bacterial communities in earthworm gut and casts

The current study provides key information that adds to the holistic picture of how MPs associated with plastic mulch films affect bacterial community compositions and functions during an earthworm's ingestion–digestion–casting process. Overall, we report that the addition of LDPE, PLA and PBAT MPs to the soil at a dosage of 1% (w/w) did not significantly shift the total (DNA) community compositions in different niches, while PBAT MPs exerted a minor influence on the active (RNA) community compositions and functions.

The influence of MPs on the soil microbiome has recently been intensively investigated. Varying the polymer types (conventional plastics, biodegradable plastics), particle sizes (nanoplastics, microplastics), soil types, dosages (environmental concentrations, high concentrations), and incubation time (short-term, long-term), among other parameters, researchers have mostly reported that MPs can exert influence on the soil microbiome in different environments, e.g., crop rhizospheres (Qi et al., 2020b; Meng et al., 2023), forest soils (Ng et al., 2021) and vegetable farmlands (Beriot et al., 2023). A meta-analysis based on existing literature suggested that soil biota exposed to MPs under high concentrations and for long periods are more likely to experience negative effects on their structures and functions (Liu et al., 2023). Several factors, such as the relatively short incubation time (7 days), moderate dosage (1%, w/w) and the types of MPs (pure MPs free of additives), might explain why MPs addition did not exert significant influence on soil bacterial communities in our study.

However, less information is available concerning the influence of MPs on the earthworm gut and cast microbiomes. Several studies have reported that MPs (e.g. PS and PLA) significantly shifted the gut microbiome of *Eisenia fetida* (Xu and Yu, 2021; Li et al., 2022; Holzinger et al., 2023), while others reported that MPs showed negligible effects on the gut microbiome of *Eisenia fetida*, *Lumbricus terrestris* and *Metaphire guillelmi* (Cheng et al., 2021; Yu et al., 2022b; Adhikari et al., 2023; Tang et al., 2023). Furthermore, the influence of MPs on the earthworm cast microbiome remains largely unknown. To date, the only available information is from Adhikari et al. (2023), where the authors also reported that LDPE and PBAT MPs did not significantly affect the bacterial communities in the cast of *Lumbricus terrestris* (cast aging was not performed but was equivalent to 0–20 days old by our definition) using a larger experimental scale (mesocosm) and a different feeding strategy. By extending the aging process to 180 days and profiling both the total and active bacterial communities, our study further suggests that MPs (LDPE, PLA and PBAT) in the cast might not have significant additional influence on the soil bacterial community when the aged casts are finally incorporated into the soil.

An interesting finding was that some functional groups related to aromatic/aliphatic hydrocarbon degradation and aerobic chemoheterotrophy were significantly enhanced by PBAT MPs in most niches (Fig. 6, E–I), and several taxa were identified as PBAT biomarkers across all niches (Table S5). Previous studies have reported that PBAT MPs could undergo fragmentation and slight degradation during passage through earthworm guts (Adhikari et al., 2023; Meng et al., 2023a). PBAT is a copolyester of adipic acid (aliphatic acid), 1,4-butanediol (aliphatic alcohol) and terephthalic acid (aromatic acid). Therefore, it is possible that PBAT MPs either experienced slight hydrolysis or released trace amounts of PBAT monomers and/or oligomers during the cast aging process, which stimulated the growth of bacteria capable of utilizing PBAT hydrolysates. Most PBAT biomarkers identified by LEfSE were Proteobacteria (Table S5), a phylum possessing large plastic-degrading potential (Zrimec et al., 2021). A few recent studies have also found that PBAT is associated with an increase in Proteobacteria abundance (Liu et al., 2022; Chen et al., 2024; Han et al., 2024). In addition, one of the PBAT biomarkers identified in our study, ASV443 (f_Comamonadaceae), was previously reported as a potential PBAT degrader (Han et al., 2021). It was found that an enrichment of potential

PBAT bacterial degraders by 2.8–5.7% was able to substantially foster the degradation of PBAT films in certain soils (Han et al., 2021). While in another study, researchers found that the significant enrichment of specific genera can affect the dissipation potential of polycyclic aromatic hydrocarbon in different soils (Ren et al., 2016). Although the relative abundances of these PBAT biomarkers were not high, on average accounting for 1.4% and 0.52% of the total (DNA) and active (RNA) community, respectively, a slight change in certain microbes can also lead to the potential change in functions of the microbiome.

4.3. Earthworm gut process has a dominating influence on the composition and function of bacterial communities in the cast

The digestion–casting–aging processes exerted decisive influence on the bacterial communities in different niches (Fig. 3; Table 1). The digestion process (Soil–Gut) is accompanied by significant enzymatic activity in the gut, where ingested microorganisms may perish or flourish according to their physiological requirements (Aira et al., 2022). This explains why the community diversity declined in Gut compared to Soil (Fig. 4). Significant enrichment of Firmicutes and Actinobacteriota occurred in Gut (Fig. 2), making their members main biomarkers for Gut (Data S4 & S5). This could be attributed to a few things. First, most microorganisms belonging to Firmicutes and Actinobacteriota can produce endospores or spores that help them survive the digestion process. Second, the earthworm gut is a microaerophilic or even anaerobic microenvironment (Drake and Horn, 2007), therefore, facultative and/or obligate anaerobic microorganisms from phyla Firmicutes and Actinobacteriota can better survive in the gut.

During the aging of cast (C0–C180), the richness and diversity of bacterial communities in the cast first experienced a decline up to C15 then gradually increased to a level similar to Soil (Fig. 4, A–B), indicating a potential division of the cast aging process into 0–15 days and 15–180 days. As biomarkers of C15 (Fig. 5, C–D), members of Verrucomicrobiota carries genes capable of degrading stable polysaccharides (Orellana et al., 2022) and Bacteroidota specializes in the degradation of complex organic matter, especially in the form of polysaccharides (Wolińska et al., 2017). Their flourishing in C15 could be related to the adequate carbon pool in C0 and C15 (Fig. 1, D). The biomarkers of C60 and C180, in contrast, were mainly oligotrophic bacteria belonging to Acidobacteriota, Chloroflexi and Myxococcota. The division in the cast aging process (0–15 days and 15–180 days) coincided with the dynamics of the measured cast physicochemical properties, where C15 was seen as a transition point from fast to slow in terms of the changes in $N–NH_4$, DOC and pH (Fig. 1). Our findings were consistent with the previous report by Aira et al. (2019), where the bacterial communities in the cast of *Aporrectodea caliginosa* were grouped into 0–7 days and 15–60 days.

We identified a soil-related core community which exists and functions during the soil–gut–cast transit, where substantial biochemical processes occur (Fig. 5 & Data S2). This core community accounted for considerable proportions of the total and active community and was mainly comprised of Firmicutes, Actinobacteriota, the members of which contributed significantly to the biomarkers for Gut. The stability of this core community might have guaranteed the resistance of the whole community to the disturbance caused by contaminants. Such hypothesis has been demonstrated by a previous work where keystone taxa shared by soil and earthworm gut helped resist chlordane stress (Zhu et al., 2021).

In addition, the influence of earthworm gut bacteria on the cast bacterial community was revealed (Fig. 5, & Data S3). Among others, the *Candidatus_lumbricincola* was an eye-catching genus, which was first detected in the gut of the earthworm family Lumbricidae (Nechitaylo et al., 2009). Classic views are that the microbial composition of the earthworm gut reflects that of ingested soil or plant residues (Curry and Schmidt, 2007), this is to some extent echoed by the identification of the soil-related core community. However, the contribution of bacteria exclusively inhabiting the earthworm gut to the cast bacterial community was also evident, especially in the early stage of cast aging (0–60 days).

4.4. Conclusion and prospects

Our research presents one of the first attempts to provide a holistic picture of the effects of MPs on bacterial communities during the ingestion–digestion–casting process of earthworms. The observed alteration in the physicochemical properties indicate that the tested MPs (LDPE, PLA and PBAT) might enlarge the difference in pH, ammonium, nitrate/nitrite, and DOC contents between the pre-ingestion soil and the fresh cast, and such effects would gradually disappear with extended cast aging. However, such phenomenon needs to be better confirmed with more robust experimental data. It was also found that the bacterial community composition (both DNA and RNA) and the richness and diversity were decisively shaped by the niche, instead of the addition of the tested MPs. The soil-related core community and gut-related community identified in our research both significantly contributed to the main body of the cast microbiome, which was likely to have buffered the effects caused by MPs. Nevertheless, biomarkers were identified for PBAT and PBAT increased the predicted community functions related to hydrocarbon degradation. In general, our findings indicate that MP-containing cast might not have significant additional influence on the soil microbial community when they are incorporated into the soil. However, there is still a lack of knowledge on how MPs affect the microbiome composition and function in earthworm casts. Future research focusing on the community metabolic activity and the enzymatic activity in earthworm gut and cast, as has been explored in the soil (Song et al., 2023; Lan et al., 2024), will provide more explicit knowledge on MP influence on these niches. In addition, the influence of MPs might be limited to only the small sphere surrounding the particles. More in-depth knowledge might be obtained by zooming in on MPs hotspots (Zhou et al., 2021) or defining the target niche at the level of the (micro)plastisphere (Rillig et al., 2023). Finally, as the current study was conducted in a microcosm system, long-term field-scale studies are needed to generate more comprehensive and realistic information by testing the combinations of different earthworm species, soil types and MPs.

CRediT authorship contribution statement

Ke Meng: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Paula Harkes:** Writing – review & editing, Methodology, Investigation. **Esperanza Huerta Lwanga:** Writing – review & editing, Supervision, Conceptualization. **Violette Geissen:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109480.

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