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Original Research Article

Epidermal microorganisms contributed to the toxic mechanism of nZVI and TCEP in earthworms by robbing metal elements and nutrients

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ABSTRACT

Disrupting effects of pollutants on symbiotic microbiota have been regarded as an important mechanism of host toxicity, with most current research focusing on the intestinal microbiota. In fact, the epidermal microbiota, which participates in the nutrient exchange between hosts and environments, could play a crucial role in host toxicity via community changes. To compare the contributions of intestinal and epidermal symbiotic microorganisms to host toxicity, this study designed single and combined scenarios of soil contamination [nano zero-valent iron (nZVI) and tris (2-chloroethyl) phosphate (TCEP)], and revealed the coupling mechanisms between intestinal/epidermal symbiotic bacterial communities and earthworm toxicological endpoints. Microbiome analysis showed that 15% of intestinal microbes were highly correlated with host endpoints, compared to 45% of epidermal microbes showing a similar correlation. Functional comparisons revealed that key species on the epidermis were mainly heterotrophic microbes with genetic abilities to utilize metal elements and carbohydrate nutrients. Further verifications demonstrated that when facing the co-contamination of nZVI and TCEP, certain symbiotic microorganisms became dominant and consumed zinc, copper, and manganese along with saccharides and amino acids, which may be responsible for the nutritional deficiencies in the host earthworms. The findings can enrich the understanding of the coupling relationship between symbiotic microorganisms and host toxicity, highlighting the importance of epidermal microorganisms in host resistance to environmental pollution.

1. Introduction

Organisms in nature do not exist independently but in the form of symbionts with microorganisms [1,2]. As the second genome of the host, symbiotic microorganisms, including both probiotics and pathogens, can regulate host health and thus contribute to the vulnerability of the host under environmental stresses [3]. In the soil environment, earthworms and drilosphere microorganisms coexist as classic and widespread symbionts, and their interactions deeply influence earthworm tolerance to soil contamination [4]. Generally, symbiotic microorganisms are harbored both in the gut and on the epidermis. Intestinal microorganisms play important roles in pathogen inhibition and the maintenance of intestinal barrier functions, which have garnered much research attention in recent years [5,6]. Different from the gut, the epidermis serves as a vital barrier against external environmental threats, with its mucus layer providing an abundant nutritional source and stable microhabitat for symbiotic bacterial colonization [7]. However, due to the complexity and challenges associated with symbiotic microorganism separation and identification, existing toxicology studies have mostly focused on the direct impact of pollutants on the host, leaving the role of symbiotic microorganisms in host toxicity obscure.

Essentially, when contaminants induce host toxicity by disrupting symbiotic microorganisms, the final effects depend on the community changes of symbiotic microorganisms along with their functions. Certain microorganisms may strengthen or weaken the resistance of the host to external pollutants by modulating nutrient cycling, absorption of essential elements, and pollutant metabolism [8–11]. For example, intestinal microbes have been observed to affect host nutrient cycling through the

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synthesis of vitamins, short-chain fatty acids, and various gut hormones [8]. Previous research has shown significant increases in the abundance of beneficial microbiomes, such as *Blautia* and *Bifidobacterium*, induced by allicin (diallylthiosulfinate), in maintaining glucose homeostasis and ameliorating hepatic steatosis [11]. Concurrently, symbiotic microorganisms also regulate the absorption and homeostasis of essential host elements, influencing the pollution tolerance of the host via specific functional proteins. For instance, iron is essential to host immunity, and bacteria can manage intracellular iron storage and release at the molecular level through various receptors, thereby affecting host iron homeostasis and health [12]. On the other hand, recent findings showed that carbon/nitrogen metabolites generated by earthworms, such as S-(2-hydroxyethyl) glutathione, 16-hydroxypalmitic acid, and formamide, could be particularly utilized by microorganisms when exposed to polychlorinated biphenyls (PCBs) at environmental concentrations in soil [13]. As favorable carbon/nitrogen sources for microorganisms, these substances stimulate the colonization of the PCB-degrading bacteria *Novosphingobium* and *Achromobacter* in the gut, thus bolstering the resistance of earthworms to PCB pollution in soil. At present, toxicological studies on symbiotic microorganisms are merely focused on gut flora, while the response mode of epidermal microorganisms to pollutants, along with their contribution to host toxicity, remains largely unclear. Systematic comparisons using big data tools are urgently needed, especially on the structure–function relationship between the community composition of epidermal microorganisms and their biological interactions with the host.

In the present study, we hypothesized that epidermal microorganisms, which play a crucial role in mediating direct contact between the host and the environment, may contribute to host toxicity via their community responses under environmental stresses. To distinguish the contributions of intestinal and epidermal symbiotic microorganisms to host toxicity, we designed single and combined scenarios of soil contamination using nano zero-valent iron (nZVI) and tris (2-chloroethyl) phosphate (TCEP) as representatives of emerging nanoparticulates and organic contaminants [14,15], and investigated the coupling mechanisms between the intestinal/epidermal symbiotic bacterial communities and the earthworm physiochemical endpoints using 16S rRNA sequencing and metagenomic analysis techniques. After identifying alterations in the community structure, we screened out key microorganisms in the intestine and epidermis, and verified the relationship between their genetic functions and the related host endpoints under co-contamination conditions.

2. Materials and methods

2.1. Exposure experiment and host toxicity assays

Earthworms (*Eisenia fetida*) were provided by a farm in Jiaxing, China. Since TCEP pollution has been threatening the agriculture near e-waste dismantling area, paddy soil was collected from a farmland in Hangzhou, China, and was used as the culture matrix. TCEP and nZVI may coexist during nZVI-based soil remediation, which was mimicked as a typical coexposure scenario [16,17]. nZVI with an approximate diameter of 80 nm was obtained from Hongwu Material Technology (Guangzhou, China). The characterizations of soil and nZVI are shown in Supplementary material Text S1. TCEP and its internal standard *d*12-TCEP were purchased from Toronto Research Chemicals Company (Toronto, Canada). All organic solvents used in the study were of analytical grade. The experiment followed the Organization for Economic Cooperation and Development (OECD) Guideline 222. TCEP and nZVI were added to the soil to perform a 4 × 4 factorial experiment, reaching desired concentrations of 50, 500, and 5,000 μg/kg and 50, 500, and 5,000 mg/kg, respectively [15]. The concentration gradient was chosen according to the ambient concentration of TCEP and the application dose of nZVI during typical soil remediations [16–18]. A 28-d exposure was conducted in beakers, as detailed in Text S2. After exposure, ten earthworms were collected from each group and kept on moist filter paper for 24 h to clear their intestinal contents. Physiological endpoints, including weight gain rate and food ingestion rate, were measured, and biochemical indicators, including superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione S-transferase (GST), reduced glutathione (GSH), and acetyl-cholinesterase (AchE), were determined, as detailed in Text S3, Fig. S2 and our previous study [19].

2.2. Microbiome analysis of symbiotic microorganisms

Microbiota sampling from the secretion of the intestine and epidermis was performed under sterile conditions. After exposure, earthworms were cleaned, freeze-dried, and preserved at −80 °C. The sterilized earthworm was secured and incised to extract intestinal contents. The secretion of intestine and epidermis of three earthworms were collected as one sample, and stored at −80 °C for future analysis and sequencing. Each treatment group contained three replicates. Subsequent microbial sequencing was conducted by Majorbio Bio-Pharm Technology Co. (Shanghai, China). In detail, genomic DNA extracted from the samples was first analyzed via 1% agarose gel electrophoresis, and only samples with an OD260/280 value over 1.8 were used. After that, a quantitative real-time polymerase chain reaction (qPCR) was used for the amplification and purification of the product. The product was then examined and quantified using a fluorescence quantification system, and the Illumina library was constructed and sequenced. Resulting PE reads were joined based on overlap while concurrently conducting sequence quality control and filtration. A series of statistical and visual analyses, including the Venn diagram (Fig. S3), Principal Component Analysis (PCA) (Fig. S4), hierarchical clustering tree (Fig. S5), and Community heatmap analysis (Fig. S6), were carried out post-sample differentiation using the Majorbio Cloud Platform online tool (https://cloud.majorbio.com/page/tools/).

2.3. Determination of functional gene abundance

According to the above microbiome analysis, epidermal microorganisms showing high correlations with host toxicity were identified. Among these, *Brevundimonas*, *Microbacterium*, *Mesorhizobium*, and *Ensifer* are well-known microorganisms, and their reported biological functions are summarized in Table S1 based on the literature search. A heatmap was plotted by https://www.bioinformatics.com.cn, an online platform for visualization of the relationship between the epidermal microorganisms. To further quantify the microbial functions, genomic DNA was analyzed using qPCR performed on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The degenerate primers of 10 genes [*Fur*, Ferric uptake regulator; *Zur*, Zinc uptake regulator; *ZnuA*, Zinc ABC transporter substrate-binding protein; *CueR*, Cu(I)-responsive transcriptional regulator; *ScaC*, scaffoldin anchoring protein C; *MntR*, Mn^{2+} transporter; *Bgl*, beta-glucosidase; *LacZ*, beta-galactosidase; *NocR*, nucleoid occlusion protein regulatory protein; *PabcT*, Peptide ABC transporter] related to metal-responsive proteins and saccharides/amino acids uptake were designed using PRIMER 5.0 software based on the common sequences of detected species (*Brevundimonas*, *Microbacterium*, *Mesorhizobium*, *Ensifer*, etc.) from the NCBI database (Table S2). The relative abundance was calculated using the $2^{-\Delta\Delta Ct}$ method [20] and was visualized using a heatmap according to the data in Table S3.

2.4. Determination of TCEP contents

The quantification of TCEP in earthworms was accomplished via extraction with acetonitrile. The process was initiated by incorporating 0.2 g of earthworm tissue with 2 mL of acetonitrile and 20 ng of *d*12-TCEP employed as an internal standard. The subsequent supernatant was then subjected to an ultrasonic extraction for 30 min, followed by a 10-min centrifugation (3,000 rpm). This process was iteratively performed

thrice, after which the cumulated supernatant was desiccated to near-dryness under a nitrogen flow of 1.0 mL/min. The resulting residue was then re-dissolved with a 1:1 acetonitrile-water mix, followed by dilution to a predetermined range after filtration via a 0.22 μm organic PTFE filter. The TCEP analysis was conducted using the ACQUITY UPLC I-Class system (Milford, MA, USA) on ACQUITY UPLC BEH C18 columns (2.1 mm × 100 mm × 1.7 μm) coupled with an AB Sciex QTrap 5500 system (Foster City, CA, USA) using the multiple reaction monitoring (MRM) acquisition mode under positive iron mode (ESI+). An elution gradient was then implemented, using 50% formic acid in water (A) and 50% formic acid in acetonitrile (B), at a flow rate of 0.2 mL/min, maintained for 8 min.

2.5. Determination of elements

Considering symbiotic microorganisms could regulate the absorption and homeostasis of essential host elements and therefore contribute to host toxicity, non-metallic elements and metallic elements, including Na, K, Ca, Mg, P, Zn, Mn, Fe, and Cu, were selected, and their alterations in earthworms were measured after exposure to nZVI, TCEP, and their combination (nZVI-TCEP). In each treatment group, earthworm bodies from three individuals were comprehensively digested employing 6.0 mL concentrated HNO_3 and 2.0 mL H_2O_2 in a CEM Mars 4 microwave digestion system (USA). The acid was reduced to a volume before being brought up to a 50 mL volume using 2% dilute HNO_3 for measurements. Quantification of Na, K, Ca, Mg, P, Zn, Mn, Fe, and Cu was conducted using an inductively coupled plasma mass spectrometer (ICP–MS) (PerkinElmer NexION 300X, USA).

2.6. Determination of saccharides and amino acids

In each treatment group, three earthworms were homogenized manually in a vitreous tissue homogenizer with phosphate-buffered saline (1:9). Homogenates were centrifuged at 4 °C for 15 min at 3,000 rpm. D-glucose, lactose, and glutamine were measured using reagent kits from Jiangcheng Bioengineering Institute (Nanjing, China) and Abnova Corporation (Wuhan, China), following the manufacturer's instructions. In detail, D-glucose was measured by the glucose-oxidase method [21]. Lactose was broken down into galactose and glucose by β-galactosidase and then quantified by the glucose oxidase method [21]. The measurement of glutamine was based on the signal at 565 nm when glutamine was hydrolyzed to glutamate [22].

2.7. Data analyses

Data were analyzed using SPSS 26.0 and presented as mean ± standard deviation (SD). Three replicates were used for each treatment group in microbiome, gene abundance, and chemical/element assays. Normality and variance homogeneity were verified before conducting a one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test. Correlations between different strains and physiochemical indicators were evaluated by the Pearson correlation coefficient. The DIAMOND software tool (https://github.com/bbuchfink/diamond) was used to compare our non-redundant gene set with the NR database, with species annotation derived from the corresponding taxonomic database. The Kruskal–Wallis H rank sum test was used to determine significant species across treatments.

3. Results and discussion

3.1. Distinct structures and responses of intestinal and epidermal bacteria

According to the annotation of the microbiome, 45 and 242 phyla were identified in the intestine and on the epidermis, respectively, indicating that the species richness on the epidermis was higher than that in the intestine. Dissimilarities were observed between the intestinal bacteria and epidermal bacteria of earthworms (Fig. 1A). Owing to the intestinal microenvironment, the bacteria primarily (over 80% in total) included Chloroflexi, Proteobacteria, Acidobacteriota, Actinobacteriota, Firmicutes, and Bacteroidota. For the epidermal microorganisms, similar phyla (except Actinobacteriota) were found. The percentages of Proteobacteria, Actinobacteriota, and Bacteroidota on the epidermis were 2.1–5.6 times higher than those in the intestine, while Chloroflexi and Firmicutes were less dominant. A few significant changes were found in intestinal and epidermal microbial communities at the phylum level after exposure to nZVI, TCEP, and their combination. For example, compared with the microbial community of the control group, the relative abundance of Proteobacteria slightly increased from 15.2% to 23.4% after TCEP exposure, whereas Firmicutes respectively decreased from 10.9% to 2.4% and 5.1% after TCEP exposure and nZVI-TCEP coexposure. Firmicutes on the epidermis also decreased from 4.5% to 2.2% after nZVI-TCEP coexposure. These findings indicate a dysbiosis of the microbial community in the intestine and epidermis under the contaminated condition, highlighting the vulnerability of specific phylum such as Firmicutes [23,24]. Overall, the intestinal and epidermal microbiomes shared certain dominant species at the phylum level, while the structures of the two bacterial communities and their responses to soil contamination were distinct.

Cluster analyses were used to compare the response patterns of bacteria communities after exposure to nZVI, TCEP, and their combination (nZVI-TCEP). As shown in Fig. 1B, the response pattern of intestinal bacteria communities in the control group was closest to that in the nZVI-TCEP coexposure group, indicating that the impact of nZVI-TCEP coexposure on the intestinal bacteria communities was lower than that in the individual exposure groups. The nZVI exposure group located between the nZVI-TCEP coexposure group and the TCEP exposure group, and the TCEP exposure group was far from the control group, implying that the disrupting effect of TCEP on intestinal bacteria communities was stronger than that of nZVI. For epidermal bacteria (Fig. 1C), the combined exposure group exhibited the most significant impact, which was consistent with our previous finding that nZVI and TCEP induced synergistic toxicity in earthworms [19]. This result implied that the response mode of epidermal bacteria was more accordant with host toxicity than that of intestinal microorganisms. Previous studies also proved that the disturbances of environmental pollutants to earthworm epidermal microbiota were complex and variable, depending on the specific abilities of microorganisms. For instance, the relative abundance of *Sorangium* and *Fluviicola* significantly declined after exposure to 5,000 mg/kg nZVI, which is well known for its cellulose-dissolving properties [25] and nitrate nitrogen utilization [26]. To establish the relationship between the microbial response and host toxicity, we further investigated the correlation between the abundance of key species of intestinal/epidermal bacteria and the host physiochemical endpoints.

3.2. Epidermal microorganisms exhibited high correlations with joint toxicity in earthworms

At the genus level, 29 bacterial genera in the intestine showed significant changes after exposure to nZVI, TCEP, or nZVI-TCEP co-exposure. Among the top 15 genera, 5 Proteobacteria and 3 Firmicutes were identified (Fig. 2A). Existing research suggests that *Tumebacillus* can augment the degradation of sulfamethoxazole (SMX) [27] and *Halocella* is an anaerobic and halophilic bacterium with cellulose decomposition capabilities [28]. *Luteimonas* is known for its robust resistance to pollutants and the metabolic capability of various substrates [29]. The increase in these microbes implied the adaptation of earthworm intestinal bacteria to nZVI and TCEP. Meanwhile, among the epidermal microbes, 357 genera exhibited significant alterations at the genus level. Fig. 2B shows that among the top 15 known genera, *Microbacterium*, *Agromyces*, *Mesorhizobium*, *Ensifer*, and *Kaistia* had relatively higher abundances. Importantly, the mean proportions of these genera

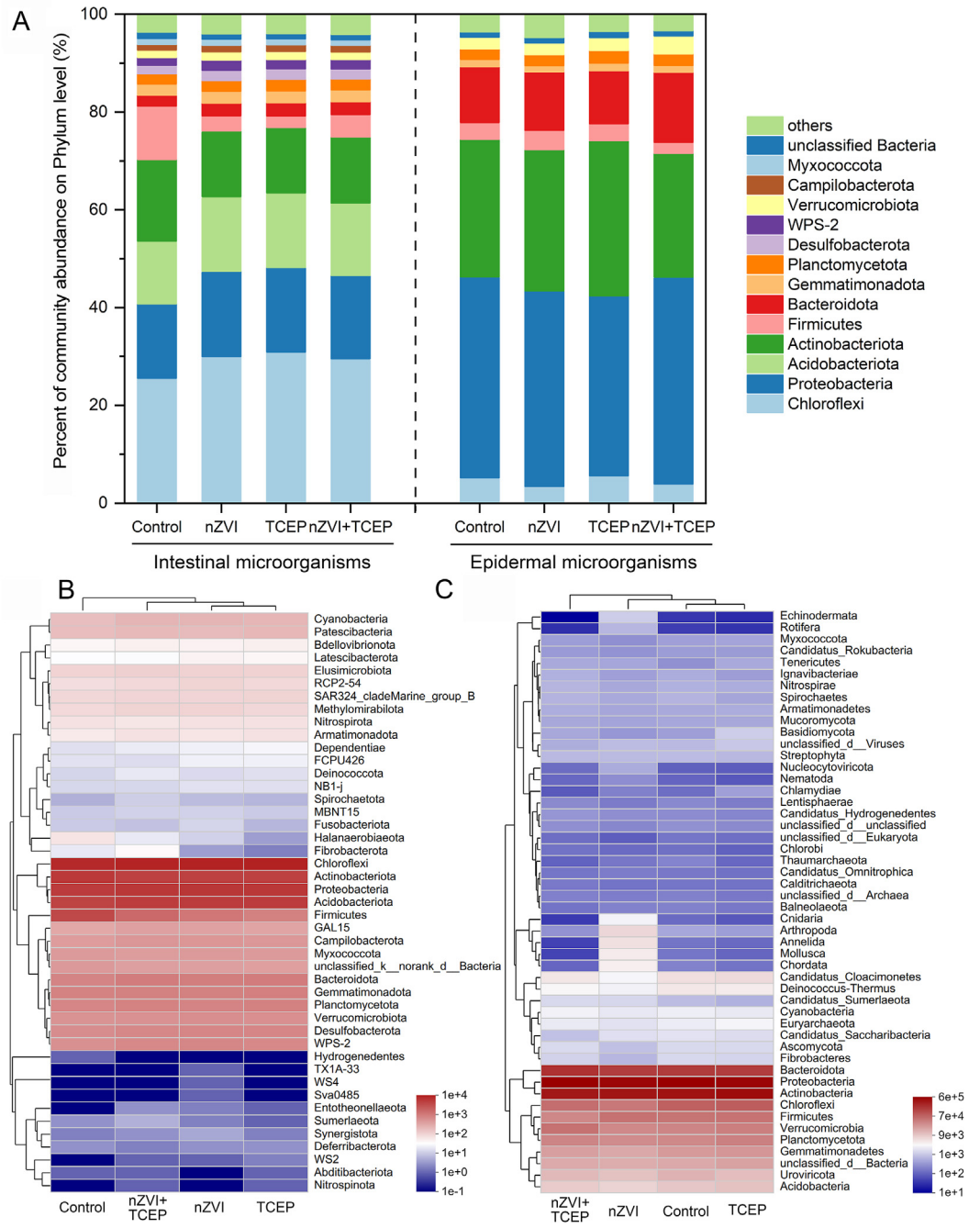

Fig. 1. The intestinal and epidermal bacterial communities at the phylum level after exposure to nZVI, TCEP, and nZVI-TCEP. (A) Histogram analyses of species compositions in the earthworm gut/on the earthworm epidermis; (B) Cluster analyses of earthworm intestinal bacterial communities; (C) Cluster analyses of earthworm epidermal bacterial communities.

all exhibited certain increases after exposure to nZVI, TCEP, and nZVI-TCEP, indicating that these epidermal microorganisms positively responded to the contaminations.

According to the Pearson correlation analysis between the physiochemical indices of earthworms and the top 15 intestinal microorganisms (Fig. 2C), there were nine pairs of highly positive correlations and nine pairs of highly negative correlations, which occupied merely 15% of the total points. The two principal microorganisms were *Tumebacillus* and *f_Bacillaceae*. Concurrently, between epidermal bacteria and the physiochemical indices of earthworms (Fig. 2D), there were 23 pairs of highly positive correlations and 31 pairs of highly negative correlations, accounting for as much as 45% of the total points. The eight key microorganisms included *Microbacterium*, *Mesorhizobium*, *f_Verrucomicrobiaceae*, *Ensifer*, *Kaistia*, *c_Verrucomicrobiae*, *Brevundimonas*, and *o_Verrucomicrobiales*. These results, for the first time, demonstrated the correlations between symbiotic bacteria and host toxicity under a typical soil contamination scenario and indicated that the response of the epidermal bacterial community was much closer to host toxicity than that of the intestinal bacterial community.

3.3. Epidermal microorganisms aggravated toxicity in earthworms by robbing metal elements and nutrients

The inner link between epidermal microbial community and host toxicity lies in the specific abilities of microorganisms. Among the eight key bacterial genera identified in the present study, *Brevundimonas*, *Microbacterium*, *Mesorhizobium*, and *Ensifer* are well-known heterotrophic

microorganisms whose specific functions are associated with elemental utilization, nutrient uptake, and pollutant transformation (Fig. 3A). In detail, according to the literature investigation (references and details are provided in Table S1), *Brevundimonas* sp., known for their metal tolerance, have been reported as plant growth-promoting rhizobacterial strains with functions such as elemental utilization and sludge remediation [30,31]. *Microbacterium* sp. are specifically good at the utilization of metal elements (Ca, Fe, Cu, Zn, Ni, Mn, etc.) as well as the hydrolysis of carbohydrates, amino acids, and even organic pollutants such as organophosphorus (OP) pesticides, polycyclic aromatic hydrocarbon (PAH), and aflatoxin B1 (AFB1) [32–34]. *Mesorhizobium* sp. consistently demonstrates functions mostly relevant to heavy metal resistance and usage [35,36]. *Ensifer* sp. is similar to *Brevundimonas* sp., whose functions are also involved in utilizing a wide range of elements and carbohydrates as sources for growth [37,38]. *Kaistia* sp. is famous for its resistance and degradation abilities to organic pollutants such as phenol and 4-chlorophenol [39,40]. In this study, it was noted that TCEP concentrations in earthworms showed no significant increases under the coexposure of nZVI and TCEP (Fig. S7), therefore excluding the possibility of synergistic toxicity via pollutant transformation or bioaccumulation. Thus, we further investigated the possibility of element and nutrient uptake as toxicological mechanisms of epidermal microorganism-related host toxicity at the genetic and gene expression levels.

Major and trace elements are crucial for the survival and metabolism of both heterotrophic microorganisms and their hosts [41]. Element uptake by epidermal microorganisms from the host could be conducted through several mechanisms, such as ion exchange, chelation, and reduction processes [36,42], mainly controlled by metal-responsive proteins [43–45]. As shown in Fig. 3B and Table S3, the abundance of the *Fur* gene could be induced by both nZVI and TCEP exposure, which significantly increased by 4.72-fold ($P < 0.01$) under the coexposure condition. Similarly, the *ScaC* gene was upregulated by nZVI (1.49-fold, $P < 0.05$) and TCEP (2.80-fold, $P < 0.01$), as well as nZVI-TCEP coexposure (4.14-fold, $P < 0.01$). The protein products of the *Fur* gene and *ScaC* gene are involved in the uptake of Fe^{2+} [46], indicating that the epidermal microbial community tended to enhance Fe element storage abilities after coexposure to nZVI and TCEP. Although the *MntR* gene exhibited no significant changes, the *Fur* gene-encoded protein can also bind Mn^{2+} to form Mn-Fur complex [47,48]. Such overlapped Mn uptake function might also result in the decrease of Mn content in the host earthworms. Different from *Fur* paralogs, *CueR* gene encodes a copper efflux regulon and is involved in the negative regulation of Cu storage [49]. In our study, the *CueR* gene was significantly decreased after exposure to nZVI (0.68-fold, $P < 0.01$) and nZVI-TCEP (0.56-fold, $P < 0.01$), indicating that the Cu storage was also enhanced [50,51]. Overall, these findings suggested epidermal microbial communities

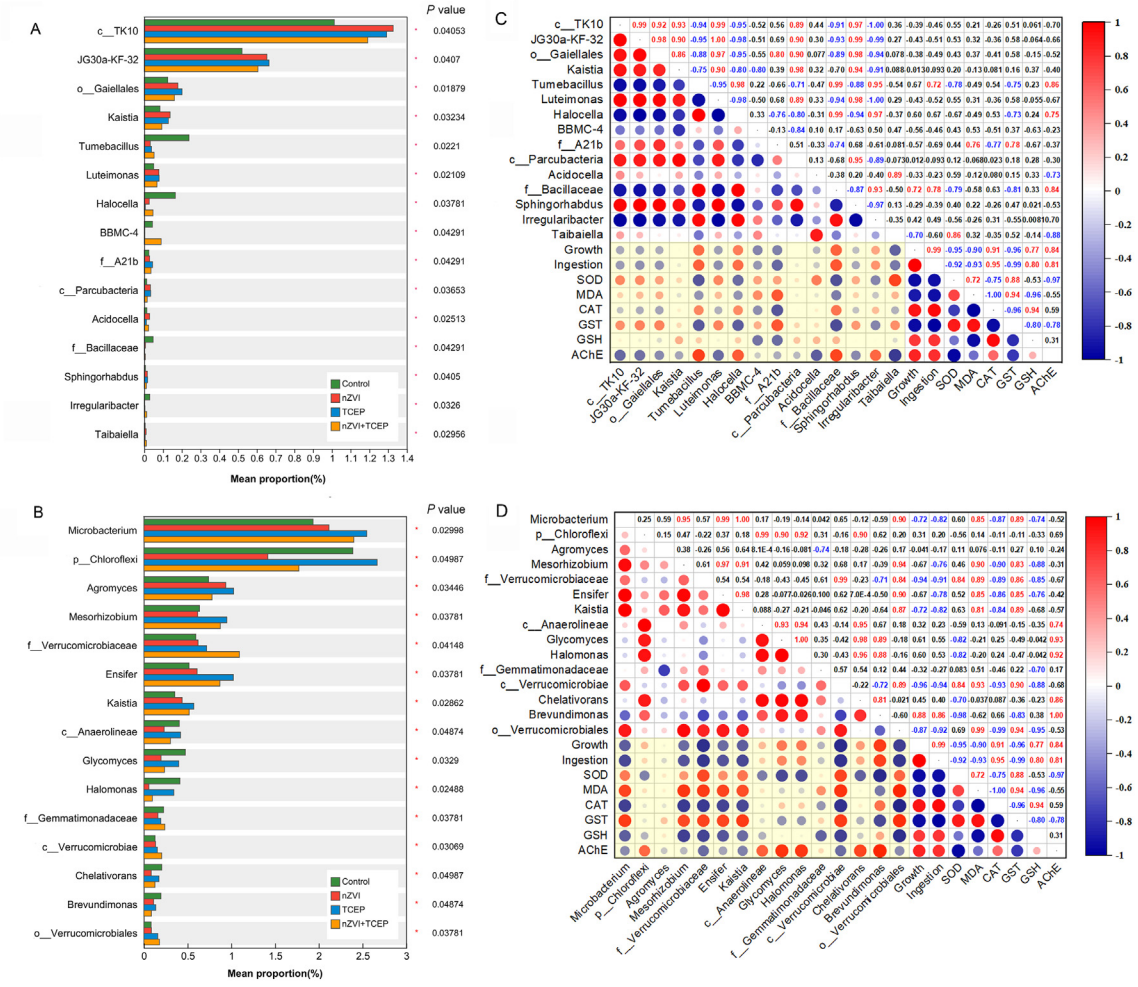

Fig. 2. Major bacterial communities and their relationship with the host toxicological endpoints after exposure to nZVI, TCEP, and nZVI-TCEP. (A) Histogram of multispecies difference test at the genus level of earthworm intestinal bacterial communities; (B) Histogram of multispecies difference test at the genus level of earthworm epidermal bacterial communities, *P* values present significant differences between genera in multiple samples; (C) Pearson correlation analyses between physiological and biochemical indices and different genera of intestinal bacteria of earthworms at the genus level; (D) Pearson correlation analyses between physiological and biochemical indices and different genera of intestinal bacteria of earthworms at the genus level; the data of physiochemical indices in earthworms is provided in Fig. S2; the yellow area points to the available points of correlation analysis physiological and biochemical indices and bacterial genera.

tended to strengthen their metal elements uptake and storage when facing nZVI, TCEP, and especially their coexposure.

Previous evidence showed that symbiotic microorganisms could loot metal elements from hosts and affect the homeostasis of host elements, thus influencing the pollution tolerance of the host via specific functional proteins. For example, many pathogenic/nonpathogenic Gram-negative and Gram-positive bacteria can acquire iron by using host iron compounds such as heme and transferrin, which may further regulate host health [12]. To further confirm the coupling relationship between microbial element uptake functions and host toxicity, six major elements (Na, K, Ca, P, Mg, and Fe) (Fig. S1) and three trace elements (Zn, Mn, and Cu) (Fig. 4A–C) in the host earthworms were investigated. It was found that Ca, P, and Mg contents in earthworms remained steady, while Na and K displayed a slight increase (Fig. S8), potentially linked to muscle tissue atrophy and an elevated osmotic pressure of intracellular fluid [52]. Notably, the metal elements, including Zn, Cu, Mn, and Fe, in the host earthworms all showed declines after nZVI and TCEP coexposure (Fig. 4B and S3, Table S4). In detail, Zn contents slightly decreased from 0.26 to 0.23 mg/g body weight (bw) after exposure to 5000 μg/kg TCEP, while under the coexposure condition, the value was as low as 0.18 mg/g bw ($P < 0.01$). Such Zn deficiency may cause immune organ shrinkage and lymphocyte reduction in animals [53,54]. The Mn contents in earthworms were sensitive to TCEP exposure, with values decreasing from 0.37 to 0.26, 0.20, and 0.18 mg/g bw after exposure to 50, 500, and 5,000 μg/kg TCEP and 0.25, 0.21, and 0.19 mg/g bw after exposure to 50, 500, and 5,000 μg/kg TCEP combined with 5,000 mg/kg nZVI, respectively. The Cu content also decreased from 0.30 to approximately 0.20 mg/g bw after exposure to 5,000 μg/kg TCEP and high-dose nZVI-TCEP coexposure, and the Fe content significantly decreased from 467 to 271 and 74 mg/g bw after coexposure to 500, 5,000 μg/kg TCEP and 5,000 mg/kg nZVI. These decreases of Zn, Mn, Cu, and Fe contents in the host earthworms were highly consistent with the significantly altered abundance of *Fur*, *ScaC*, and *CueR* in the epidermal microbial communities along with their multiple metal ion uptake functions [47,48]. Moreover, Zn, Cu, Mn, and Fe are known as cofactors for various cellular functions, such as energy metabolism and antioxidant enzymes [50,55,56]. For example, Zn-, Mn- and Cu-superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion and is a metalloenzyme ubiquitous to living organisms [56], and the decreased metal element contents were consistent with the suppressed antioxidative abilities in the host reported in our previous study [19]. These findings indicate that heterotrophic epidermal microorganisms tended to upregulate their uptake abilities of metal elements from the host, which may be responsible for the weakened stress tolerance of earthworms.

It is also noted that *Brevundimonas*, *Microbacterium*, and *Ensifer* have specific capabilities related to the uptake of saccharides and amino acids [31,38,57]. These substances, usually provided by the host, are essential nutrients and energy sources for the survival of heterotrophic bacteria [43–45]. As shown in Fig. 3B, the *Bgl* and *LacZ* genes involved in the uptake

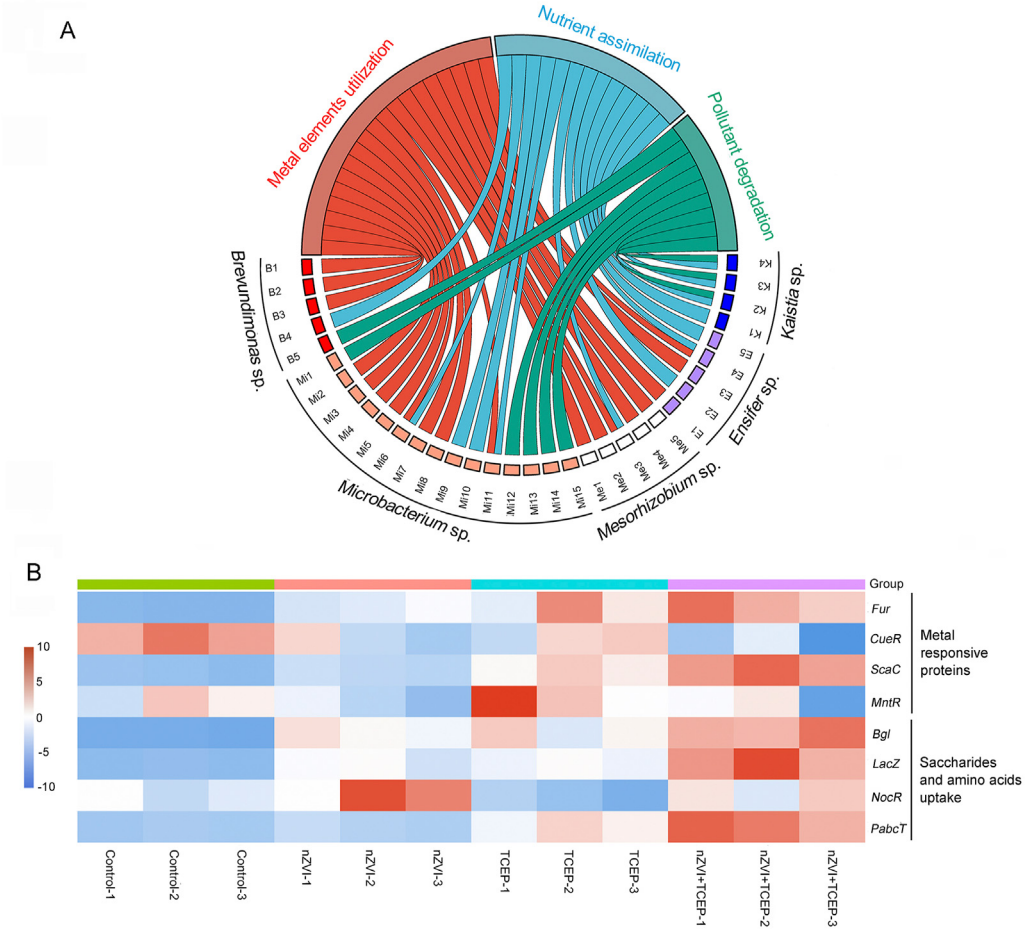

Fig. 3. Reported functions of key species and the relative abundance of related genes after exposure to nZVI, TCEP, and nZVI-TCEP. (A) A chord map of reported category of microbial functions summarized from literature (Table S1); (B) Gene expressions related to metal-responsive proteins (*Fur*, *CueR*, *ScaC*, and *MntR*) and saccharides/amino acids uptake (*Bgl*, *LacZ*, *NocR*, and *PabcT*). *Fur,* Ferric uptake regulator; *CueR,* Cu(I)-responsive transcriptional regulator; *ScaC,* scaffoldin anchoring protein C; *MntR,* Mn^{2+} transporter; *Bgl,* beta-glucosidase; *LacZ,* beta-galactosidase; *NocR,* nucleoid occlusion protein regulatory protein; *PabcT,* Peptide ABC transporter.

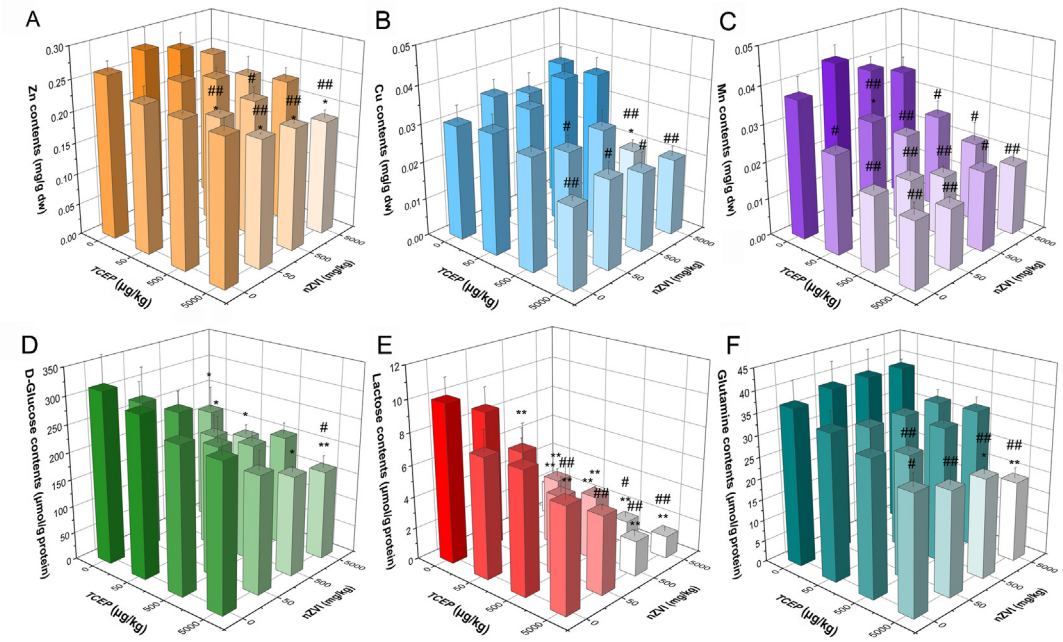

Fig. 4. Contents of metal elements and nutrients in earthworms after exposure to nZVI, TCEP, and nZVI-TCEP. (A) Zn, (B) Mn, (C) Cu, (D) D-glucose, (E) lactose, and (F) glutamine. * and # represent the significance among different nZVI and TCEP treatments (*,#$P < 0.05$, **,##$P < 0.01$).

and utilization of D-glucose and lactose were upregulated by 2.84 to 3.73-fold ($P < 0.01$) after individual exposure to nZVI and TCEP. Moreover, *Bgl*, *LacZ*, and *PabcT* all positively responded to the coexposure of nZVI and TCEP, peaking at 4.11 to 7.86-fold ($P < 0.01$). Meanwhile, the corresponding contents of D-glucose, lactose, and glutamine in earthworms showed significant decreases ($P < 0.01$) in a synergistic manner after exposure to nZVI, TCEP, and their combination (Fig. 4D–F and Table S4). In detail, D-glucose contents were sensitive to nZVI but not TCEP, which decreased from 314 to 272, 234, and 211 μmol/g protein after exposure to 50, 500, and 5,000 mg/kg nZVI ($P < 0.05$), respectively. Under the coexposure condition, these values were as low as 183, 203, and 162 μmol/g protein ($P < 0.01$). Lactose contents in earthworms were also sensitive to nZVI exposure, with the value significantly decreasing from 9.97 to 4.90 and 2.08 mg/g bw after exposure to 500 and 5,000 mg/kg nZVI ($P < 0.01$) and 1.61, 1.42, and 1.40 mg/g bw after exposure to 50, 500, and 5,000 μg/kg TCEP combined with 5,000 mg/kg nZVI ($P < 0.01$). In contrast, glutamine contents were sensitive to TCEP exposure, decreasing from 37 to 32 and 28 μmol/g protein after exposure to 500 and 5,000 μg/kg TCEP and 24–19 μmol/g protein under nZVI-TCEP coexposure, respectively. The decreases in nutrients as energy sources were coincident with the upregulated uptake abilities in the key microorganisms, which may be related to the deterioration of host vulnerability [58–61] and thus induce synergistic toxicities under nZVI and TCEP co-contamination. Taken together, it might be possible that key epidermal microorganisms may avail themselves of the opportunity to obtain metal elements and nutrients from earthworms when facing multiple contaminants and thus contribute to the malnutrition of the host at the physiochemical level.

4. Conclusions

This study compared the distinct responses of intestinal and epidermal microorganism communities in earthworms under a typical co-contamination scenario. It was found that intestinal and epidermal microorganisms were mostly composed of heterotrophic anaerobic bacteria sharing similar phyla, but the species and functions were different at the genus level. Notably, the community changes of epidermal microbes exhibited higher correlations with host toxicity than that of intestinal microbes. Facing the co-exposure of nZVI and TCEP, a shift in the microbial community occurred, with heterotrophic epidermal microorganisms that possess special functions of metal elements and nutrient utilization becoming dominant. This shift was accompanied by a substantial increase in the abundance of metal and nutrient uptake genes such as *Fur*, *ScaC*, *Bgl*, and *LacZ*. These changes might be responsible for the deficiency of corresponding substances in host earthworms. The combined pollution scenario simulated in this study is a representative scenario, and the intricate interactions between the epidermal microorganisms and the host are worthy of further investigation.

Author contributions

J.H.: conceptualization, data curation, experiment, writing–original draft, funding acquisition. M.R.Y.: experiment, data curation, formal analysis, writing–original draft. X.Y.W. and Y.Q.L.: writing–review & editing. Q.Q.C.: data curation, formal analysis. J.Y.Z.: resources. D.H.L.: writing–review & editing, supervision, funding acquisition.

Declaration of competing interests

The authors declare that there are no conflicts of interest in the present experiment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2023.11.001.

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