



Heavy-metal Pollution Affects *Daphnia* Fitness by Altering Diversity of the Gut Microbiota

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Abstract

The role of gut microbiota in shaping host fitness is already well established. However, it remains unclear to what extent the gut microbiota influences host fitness in the presence of environmental stressors. Here, we tested the hypothesis that responses of water flea *Daphnia* to the heavy metal nickel are mediated by gut microbiota. Germ-free *D. magna* exhibited somewhat lower fitness than did those with gut microbiota transplant. Among germ-free *Daphnia*, those that were exposed to heavy metals did not differ in fitness from unexposed *Daphnia*. In contrast, when incubated with their donors' gut microbiota, initially germ-free *D. magna* continuously exposed to nickel for 21 days showed a significantly lower survival rate than those not exposed to nickel. We detected a reduced set of microbes in the formerly germ-free *Daphnia* in the presence of nickel. Transcriptomic analysis of *Daphnia* showed that expression/regulation of genes related to oxygen transport, chitin metabolism, and detoxification changed in response to the reduced gut microbiomes acquired in the presence of nickel. Our findings show that the toxic effects of heavy metal led to a reduced diversity of gut microbiota in *Daphnia* and can thus affect host fitness.

Keywords Heavy metal · Germ-free *Daphnia* · Fitness · Gut microbiota · Diversity · Transcriptomics

Introduction

Many previous studies have shown that the gut microbiota plays a critical role in host health and fitness (e.g., [1–3]). In particular, the gut microbiota benefits the host by providing essential nutrients, promoting digestion, detoxifying harmful compounds, and enhancing the ability of the immune system to resist pathogens [4–6]. The gut

microbiota is also important for maintaining host fitness when facing environmental stressors, such as pollution and climate change [7].

Although the gut microbiota was traditionally regarded as stable throughout the life-span of an animal [8, 9], it is now known to be a plastic entity: it is typically developed in early life stages and then moves into a stable microbiome at adult stages, capable of being reconfigured in response to environmental challenges [10–12]. These fluctuations of gut microbiota composition assist the host to rapidly cope with changing environments [12]. For example, cold exposure leads to a substantial shift of the gut microbiota composition in mice, and the transplantation of the cold microbiota to germ-free mice increases insulin sensitivity of the host, improving tolerance of cold conditions [11]. Similarly, the microbiomes of freshwater zooplankton are highly flexible and can be influenced by changes in food or temperature [13]. Reduced microbiota diversity in rats was observed in response to exposure to the heavy metals chromium and cobalt [14]. However, it remains unclear to what extent the gut microbiomes influence host responses against environmental stressors, and the

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genetic mechanisms brought into play by the host in response to the altered gut microbiota resulting from the presence of environmental stressors.

Water fleas of the genus *Daphnia* (Crustacea: Cladocera) are excellent model organisms to address these questions. They are key components in freshwater ecosystems [15] and are highly responsive to a wide range of environmental stressors, such as pollution, predation, and presence of cyanobacteria [16]. For example, *Daphnia* can change body size to avoid size-selective predation, in response to threats from visually hunting (e.g., fish) or gap-limited (e.g., small invertebrates) predators [17, 18]. *Daphnia* gut microbiota plays a crucial role in its survival and fitness [3]; it was found that the longevity of germ-free *Daphnia* was shorter than that of *Daphnia* with gut microbiota [19], and germ-free *Daphnia* were smaller and less fecund than *Daphnia* with microbiota [3]. Previous studies had also shown that the alterations of *Daphnia* gut microbiota and the role of gut microbiota could help them to cope with different environmental stressors [20–22], such as toxic *Microcystis* and temperature [23], fish predation [24], and microplastics [25]. Additionally, we can easily culture *Daphnia* in the laboratory because of its clonal reproduction [15] and easily manipulate its gut microbiota [3, 26], providing a unique opportunity to investigate the interactions between host and gut microbiota when encountering environmental stressors [27]. Here, we used the germ-free *Daphnia* system to study the role of the gut microbiota in the host's responses to an environmental stressor, the heavy metal nickel (Ni). Heavy-metal pollution is common in aquatic ecosystems and represents a critical environmental concern globally [28, 29], and the concentrations of nickel in freshwater range from 5 to 100 µg/L [30] and even reach up to 200 µg/L in seriously polluted groundwater and tap water [31]. Nickel is one of the well-known toxicological hazards for many aquatic organisms [28, 32, 33]. For example, exposure to nickel led to a significant reduction of growth and reproduction in *D. magna* [34]. In this study, we first tested the hypothesis that the tolerance of *Daphnia* to nickel is mediated by the gut microbiota. Specifically, we investigated whether the life history responses of germ-free *Daphnia* to nickel exposure depend on the presence of gut microbiota. To address this, we compared the survival rates of germ-free *Daphnia* in the presence and absence of nickel, both without and with inoculation of donor gut microbiota. Next, we investigated the changes in gut microbiota of the germ-free *Daphnia* when inoculated with their donors' gut microbiota in the presence of nickel. Our hypothesis was that we should find a reduced set of microbes when exposed to nickel. Finally, we looked at transcriptomic responses of host *Daphnia* to the altered diversity of gut microbiota. We expected to detect particular changes of gene expression and regulation in host *Daphnia* in response to the altered diversity of gut microbiota.

Methods

Study Species

Daphnia magna clone M2, which was originally isolated from “Yulong” pond (36°08' N, 118°01' E) in 2015, was used for the present study. Species identification was confirmed by sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 gene (GenBank accession numbers OP777907). Stock cultures of the *D. magna* clone were maintained in the laboratory in COMBO medium [35], at 20 °C under a 16:8 h light to dark cycle and fed three times per week with unicellular algae, *Ankistrodesmus falcatus* (at a dose of 1 mg C/L). Here, the target dry weight concentration (mg DW/L) = Target C (mg/L)/*f*, where carbon content factor *f* = 0.45; therefore, 2.22 mg of algal dry weight was added per liter of culture medium to reach the target carbon concentration of 1 mg C/L.

Cultivation of Axenic *Ankistrodesmus Falcatus*

Axenic cultures of *A. falcatus* were grown by inoculating a small number of cells into a sterile Erlenmeyer flask containing 200 mL autoclaved COMBO medium. Cultures were grown for 5 days on a shaking plate at 20 °C under a regime of 16:8 h light to dark. Cells were then collected, washed twice, and resuspended in the filtered COMBO medium. Harvested cells were stored at 4 °C for use. The axenic nature of the *A. falcatus* cultures (algal cells and their culture media) was confirmed on LB-medium agar plates. Further confirmation was obtained by extracting total DNA from the *A. falcatus* cultures using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, USA) based on the manufacturer's protocol. The copy number of bacterial 16S rRNA genes (<50 copies of 16S rRNA genes/1 mL *A. falcatus* culture) was under the detection limit of the quantitative polymerase chain reaction (qPCR).

Generation of Germ-Free *Daphnia*

Germ-free *Daphnia* were generated based on a previously published protocol [19]. Parthenogenetic eggs (second brood) were dissected from adult females by using a stereomicroscope and then collected in a Petri dish containing sterile COMBO medium. We only included deposited eggs with an intact external membrane. Germ-free *Daphnia* were obtained by disinfecting these parthenogenetic eggs by exposing them for 30 min to a 0.25% solution of glutaraldehyde (A600875, BBI) [19]. The eggs were subsequently washed three times with sterile COMBO medium and then

transferred to six-well sterile plates containing 8 mL of sterile COMBO medium per well. Finally, they were incubated at 20 °C and a 16:8 h light to dark cycle for 48 h under sterile conditions, and the resulting germ-free juveniles were subsequently used for the experiment. To confirm the removal of bacteria from daphniids, DNA was extracted from both the juveniles (less than 48 h old) and subsequent germ-free adults (10 and 21 days old) by applying the PowerSoil DNA Isolation Kit, and qPCR of bacterial 16S rRNA genes was performed based on a previous protocol for *D. magna* [19]. The copy number of bacterial 16S rRNA genes (< 50 copies of 16S rRNA genes/daphniid) of *Daphnia* (both juveniles and adults) was under the detection limit by qPCR (Table S1). Also, the axenic nature of *Daphnia* was confirmed by plating on LB-agar medium.

Experimental Setup

In this study, 600 germ-free *D. magna* juveniles (hatched within the previous 48 h) were obtained by incubating ~2000 disinfected eggs, which were dissected from 500 adult females (second brood) that were transferred from stock cultures (see above). These germ-free *D. magna* juveniles were randomly divided into three experimental sets: life history parameters, gut bacterial community, and transcriptome analysis. All these experiments were performed under sterile conditions: the jars (150 mL) were closed with a 0.2- μ m membrane that allowed for air exchange but prevented bacterial contamination, the germ-free *Daphnia* were fed with axenic *A. falcatus* daily (at a dose of 1 mg C/L), and the sterile COMBO media were refreshed every second day.

Life History Parameters

A total of 120 germ-free *D. magna* juveniles were individually placed into 150-mL jars with 80 mL COMBO medium and incubated at 20 °C in a 16:8 h light to dark cycle. Then, they were randomly divided into four treatments: “Bac-Suppl” (sterile COMBO medium supplemented with donor gut microbiota (see below)), “Bac-Suppl + Ni” (sterile COMBO medium containing 100 μ g L⁻¹ NiCl₂ supplemented with donor gut microbiota), “Bac-Free” (sterile COMBO medium only), and “Bac-Free + Ni” (sterile COMBO medium containing 100 μ g L⁻¹ NiCl₂”; see Fig. 1). Prior to the experiment, we ran independent life history tests for a range of concentrations from a low (20 μ g L⁻¹ NiCl₂) to high (160 μ g L⁻¹ NiCl₂) concentration, and the concentration of 100 μ g L⁻¹ NiCl₂ was deemed the best to test the response of *Daphnia* to nickel (data not shown). To obtain “donor gut microbiota” for these experiments, twenty adult *D. magna* (10 days old) were randomly selected and then placed in sterile COMBO medium for 24 h to allow elimination of food particles from the gut.

Their guts were subsequently extracted with dissection needles under a stereomicroscope, placed together in 1.5-mL Eppendorf tubes containing 200 μ L of sterile COMBO, and crushed with a pestle. Thirty such replicates were prepared every second day. These crushed guts were mixed/homogenized and then randomly inoculated into jars containing the germ-free *Daphnia* every second day during the experiment. Fresh donor individuals were used each time. However, this procedure lacks proper control because our study did not measure donors’ gut microbiota, which was transferred to the germ-free neonates. Survival and reproductive output of each individual were monitored daily for 21 days. Any offspring (as observed daily) were removed immediately after recording. Significant differences in the survival curves between the “Bac-Suppl” and “Bac-Suppl + Ni” treatments, between “Bac-Free” and “Bac-Free + Ni” treatments, or between “Bac-Suppl + Ni” and “Bac-Free + Ni” were calculated separately by log-rank (Mantel-Cox) tests performed in GraphPad Prism version 8 (GraphPad, San Diego, CA, USA). To test if age at first reproduction and number of first-clutch neonates significantly differed among the four treatments (see above), pairwise comparisons were performed by applying one-way ANOVA based on Tukey’s multiple comparisons test in GraphPad.

Gut Bacterial-community Analysis

We compared the gut microbiota composition of germ-free *D. magna* in the “Bac-Suppl” and “Bac-Suppl + Ni” groups, see above (Fig. 1). The guts from 30 *D. magna* at day 10 in each replicate (each treatment was done in triplicate) were extracted with dissection needles under a stereomicroscope and then pooled. Total DNA was extracted from these 30 pooled *D. magna* guts per replicate using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, USA), according to the manufacturer’s protocol. Then, the purity and concentration of DNA were determined by 1% agarose gel electrophoresis. Sample DNA was diluted to 1 ng/ μ L with sterile water. The V4 hypervariable region of the 16S rRNA gene was amplified by applying the primers (515F and 806R) [36], with barcodes as tags on the 5’ ends of the primers to identify the different treatments. PCR was carried out in 30 μ L with 15 μ L of Phusion High-Fidelity PCR Master Mix (New England Biolabs), a final concentration of 0.2 μ M forward and reverse primers, and approximately 10 ng of template DNA. 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 and final extension at 72 °C for 5 min. Six sequencing libraries were generated using an Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA), with index codes added following the manufacturer’s recommendations. The Library quality was assessed on a

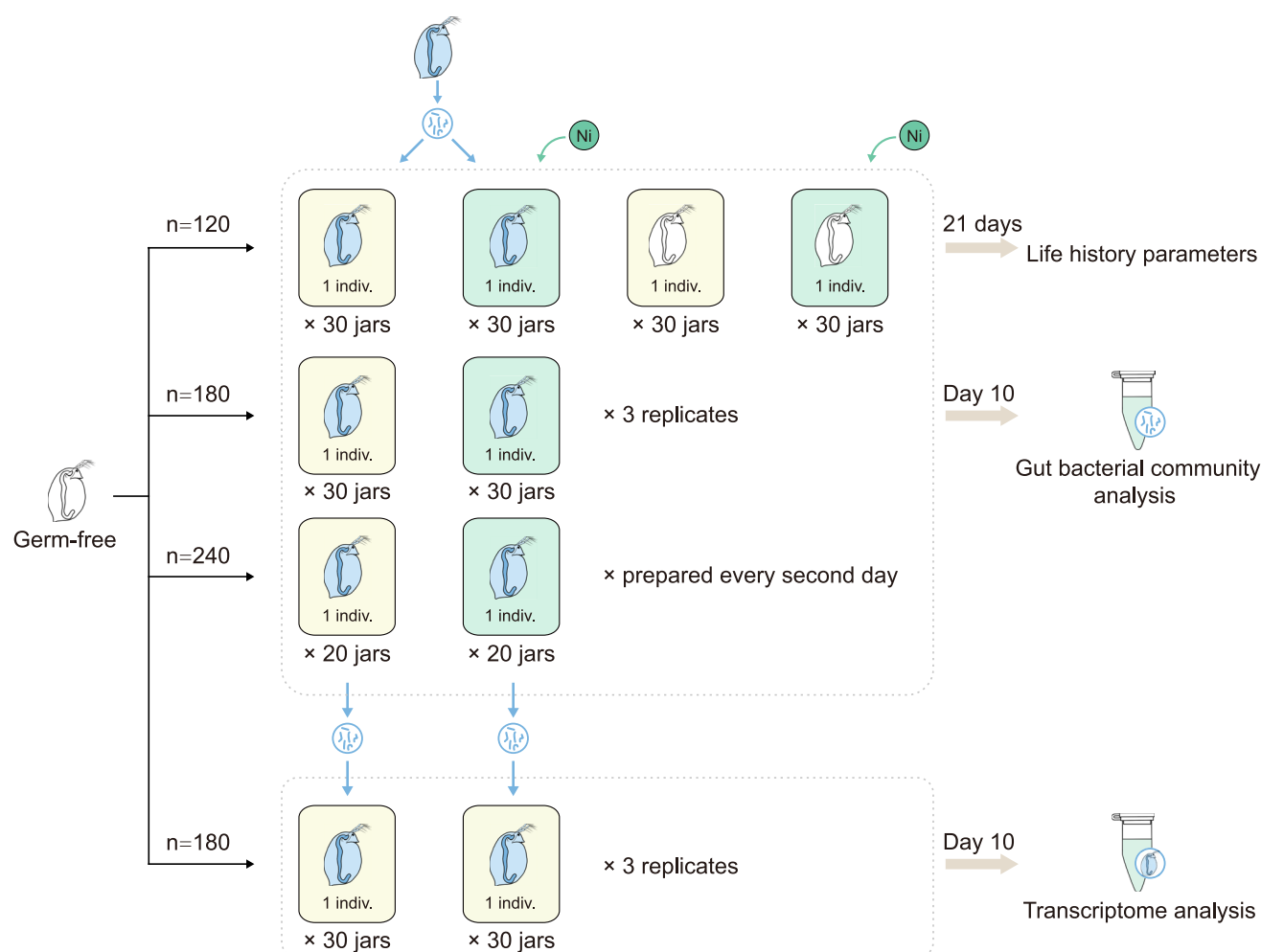


Fig. 1 Experimental design: 600 germ-free *Daphnia magna* juveniles (born within the previous 48 h) were randomly divided into three experimental sets (i.e., life history parameters, gut bacterial-community analysis, and transcriptome analysis). For the survival-rate analysis, 120 germ-free *D. magna* juveniles were randomly chosen and divided into four treatments: “Bac-Suppl” (sterile COMBO medium supplemented with donor gut microbiota), “Bac-Suppl+Ni” (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2 supplemented with donor gut microbiota), “Bac-Free” (sterile COMBO medium only), and “Bac-Free+Ni” (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2). Survival and reproductive output of each individual were monitored daily for 21 days. For the gut bacterial community analysis, we compared the gut microbiota of germ-free *D. magna* under

two different treatments: “Bac-Suppl” and “Bac-Suppl+Ni”. The guts from 30 *Daphnia* at Day 10 from the treatment “Bac-Suppl+Ni” (or “Bac-Suppl”) were extracted with dissection needles under a stereomicroscope and then pooled. For the transcriptome analysis, 30 germ-free *D. magna* (on Day 10) that were inoculated with different donor gut microbiotas (from *Daphnia* raised without the presence of Ni, or from *Daphnia* raised with the presence of Ni) were randomly collected and pooled into RNeasy lysis solution for the subsequent RNA-seq analyses. The microbiota was extracted from the guts of 20 adult *Daphnia* in the treatment of “Bac-Suppl+Ni” or those from the treatment of “Bac-Suppl.” For the gut bacterial community analysis and transcriptome analysis, each treatment was performed on three biological replicates

Qubit 2.0 fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. The Libraries were sequenced on an Illumina NovaSeq 6000 platform, and 250-bp paired-end reads were generated.

Paired-end reads were assigned to each replicate from the two different treatments (i.e., “Bac-Suppl” and “Bac-Suppl+Ni”) based on their unique barcodes and truncated by cutting off the barcode and primer sequences. These

paired-end reads were then merged by using FLASH V 1.2.7 [37]. High-quality clean reads were obtained by quality filtering on the raw reads in QIIME V 1.9.1 (<http://qiime.org/>) [38]. The clean reads were compared with the reference Silva database (<http://www.arb-silva.de/>) in VSEARCH V 2.9.0 (<https://github.com/torognes/vsearch/>) [39] to find chimeric sequences. These chimeric sequences were then removed to obtain the effective reads. Sequence

analysis was performed by Uparse V 7.0.1001 (<http://drive5.com/uparse/>) [40]. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic unit (OTUs). A representative sequence from each OTU was screened for further taxonomic annotation in the Silva138 SSU rRNA Database using Mothur V 1.33.3 (<https://mothur.org/>) [41]. We measured alpha diversity within the different microbial communities by computing observed-species, Chao1, Shannon index, and abundance-based coverage estimator (ACE) within each replicate from the two different treatments (i.e., “Bac-Suppl” and “Bac-Suppl + Ni”) in QIIME. Then, we calculated the statistical significance of differences in these indices between the treatments by applying a *t*-test. To measure beta diversity within the different microbial communities, we calculated the weighted UniFrac distances between replicates from each of the two treatments (i.e., “Bac-Suppl” and “Bac-Suppl + Ni”) in QIIME, and then principal coordinate analysis (PCoA) was performed in R. Finally, we applied the linear discriminant effect size (LEfSe) analysis in LEfSe V 1.0 [42] to detect differentially abundant taxa between the two treatments.

Transcriptome Analysis

A total of 180 germ-free *D. magna* juveniles were randomly divided into two treatments, each consisting of three biological replicates. One treatment was inoculated with gut microbiota extracted from the guts of 20 adult *Daphnia* (10-days-old) that had been exposed to nickel (the “Bac-Suppl + Ni” treatment) every second day. Another treatment was inoculated with gut microbiota extracted from *Daphnia* that had not been exposed to nickel (the “Bac-Suppl” treatment) every second day (Fig. 1). The preparation of gut microbiota was as above. Then, a total of 30 animals at day 10 for each replicate were collected and pooled into RNAlater solution for the subsequent RNA-seq analyses.

Total RNA was extracted from the 30 pooled animals in each replicate using the RNeasy Mini Kit (Qiagen, Valencia, CA) and purified using the RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The quality and quantity of the RNA were estimated by using a NanoDrop 2000c Spectrophotometer (Thermo, USA) and an RNA Nano Chip assay on an Agilent Bioanalyzer (RIN > 7.5 for all samples). Then, 10 μ g of total RNA from each replicate was used for RNA-seq library preparation, resulting in a total of six libraries. Libraries were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY) and sequenced using the Illumina NovaSeq 6000 platform.

For each replicate, the quality and adapter presence of RNA-seq reads were initially processed using FastQC [43]. RNA-seq reads with a PHRED score below 25 were discarded and adapter sequences were trimmed using Trim-Galore v 0.6.5 ([https://github.com/FelixKrueger/TrimG](https://github.com/FelixKrueger/TrimGalore)

alore) to generate clean reads. The removal of low-quality reads and adapter contamination was once again verified in FastQC. The STAR aligner [44] was applied to align clean reads to the *D. magna* genome [45]. All gene counts were summarized using featureCounts in subread v 2.0.1 [46]. Any genes for which the count was below 1 in all treatments was removed. Afterwards, the gene counts were normalized among all treatments using DESeq2 [47], and significantly differentially expressed genes (DEGs; at least absolute value of $\log_2\text{FoldChange} > 1$ and adjusted *P* value < 0.01) between *Daphnia* inoculated with gut microbiota from the “Bac-Suppl + Ni” and “Bac-Suppl” treatments were identified using the negative binomial distribution estimated in DESeq2. A heatmap was generated using R to visualize the correlation of DESeq2 normalized gene-expression values among all independent biological samples. Before performing function enrichment analysis on DEGs, the genome was annotated to Gene Ontology (GO) terms by combining NCBI’s non-redundant protein database and InterPro annotations applying BLAST2GO [48]. Additionally, pathway assignments were performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via online KEGG Automatic Annotation Server (KAAS). Then, Fisher’s exact test was applied to determine whether the DEGs were selectively enriched in KEGG pathways with corrected *P* values ≤ 0.05 . In addition, similar enrichment analysis for GO terms on the DEGs was performed. From the GO annotation outputs, GO terms were classified into three domains: cell components, molecular functions, and biological processes.

Results

Life History Parameters

Initially germ-free *D. magna* continuously exposed to nickel for 21 days (i.e., the “Bac-Free + Ni” treatment) exhibited survival patterns similar to those of controls (i.e., “Bac-Free” treatment; log rank test, $P = 0.2507$, $X^2 = 1.320$; Fig. 2A). However, when incubated with their donors’ gut microbiota, survival of initially germ-free *D. magna* exposed to nickel (i.e., the “Bac-Suppl + Ni” treatment) was significantly lower than in controls (i.e., the “Bac-Suppl” treatment; log rank test, $P = 0.0016$, $X^2 = 9.993$; Fig. 2A). The survival rate of initially germ-free *D. magna* exposed to nickel was significantly higher when incubated with donor gut microbiota (i.e., the “Bac-Suppl + Ni” treatment) compared to those not incubated with donor gut microbiota (i.e., the “Bac-Free + Ni” treatment; $P = 0.0248$, $X^2 = 5.037$; Fig. 2A). Initially germ-free *D. magna* with or without exposure to nickel (i.e., from the “Bac-Free” or “Bac-Free + Ni” treatments) did not differ significantly in the life history parameters

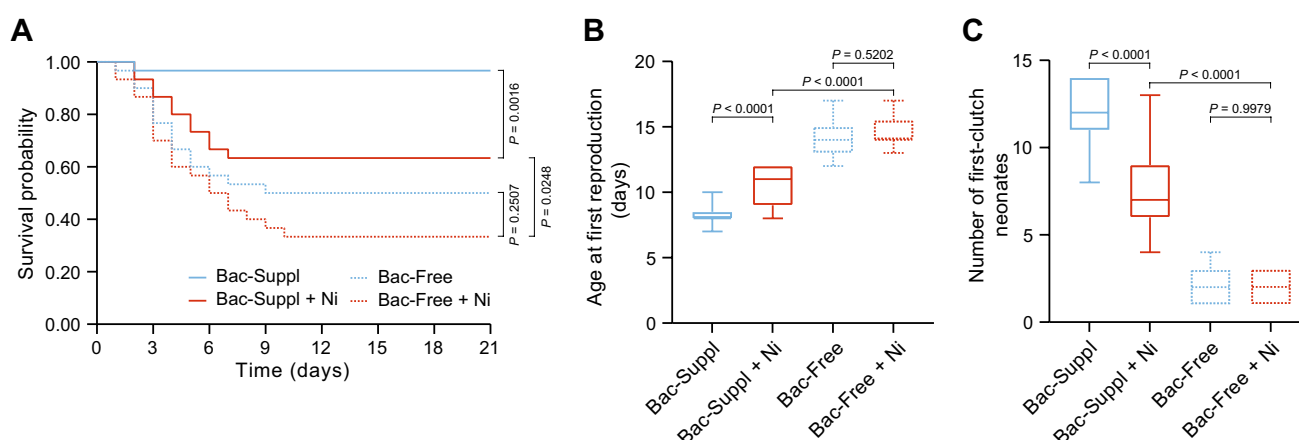


Fig. 2 Life history traits of germ-free *Daphnia magna* under four different treatments. **A** Survival curves of germ-free *D. magna* under four different treatments. Colored lines indicate the different treatments: the blue solid line represents the “Bac-Suppl” group (sterile COMBO medium supplemented with donor gut microbiota), the red solid line represents the “Bac-Suppl + Ni” group (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2 supplemented with donor gut microbiota), the blue dotted line indicates the “Bac-Free” group (sterile COMBO medium only), and the red dotted line indicates the

“Bac-Free + Ni” group (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2). Sample size was 30 (1 individual $\times$ 30 biological replicates) for each treatment. Pairwise significant differences in the survival curves were calculated by log-rank (Mantel-Cox) tests. Box-and-whisker plot of **B** the age at first reproduction (days) and **C** the number of first-clutch neonates of germ-free *D. magna* under four different treatments. Statistical significance was assessed using one-way ANOVA based on Tukey’s multiple comparisons test

measured. They reproduced later and produced fewer offspring in the first clutch than those incubated with donor gut microbiota (i.e., the “Bac-Suppl” or “Bac-Suppl + Ni” treatments) (all comparisons, $P < 0.0001$; Fig. 2 B and C). When incubated with donor gut microbiota, initially germ-free *D. magna* (from the “Bac-Suppl” treatment) reproduced earlier and produced more offspring in the first clutch than those continuous exposure of nickel ($P < 0.0001$; Fig. 2 B and C).

Gut Bacterial Community

After filtering, an average of 64,884 reads per replicate were obtained (minimum = 61,379 reads, maximum = 69,069 reads). When incubated with their donors’ gut microbiota, initially germ-free *D. magna* continuously exposed to nickel for 10 days (i.e., the “Bac-Suppl + Ni” treatment) showed a significant difference in gut microbiota community composition when compared with controls (i.e., the “Bac-Suppl” treatment; Fig. 3 A). Although the gut microbiota in both treatments were dominated by the phyla Proteobacteria and Bacteroidota, a clear distinction between the treatments could be seen in the relative abundances of the dominant phyla. Specifically, Proteobacteria and Bacteroidota comprised $67.1 \pm 8.0\%$ (mean $\pm$ standard deviation) and $29.9 \pm 6.9\%$ of sequence reads in the “Bac-Suppl + Ni” treatment, and $74.4 \pm 1.7\%$ and $11.6 \pm 1.6\%$ in the “Bac-Suppl” treatment, respectively (Fig. 3 A). The quantification of beta diversity, visualized in a PCoA based on weighted UniFrac distances, revealed that most of the variation in the

gut microbiota composition was explained by the two treatments (Fig. 3 B). Interestingly, the phyla Fusobacteriota and Spirochaetota were represented (albeit in low abundance) in the “Bac-Suppl” treatment, but not in the “Bac-Suppl + Ni” treatment. Values for alpha diversity were significantly lower in the “Bac-Suppl + Ni” group than in the “Bac-Suppl” group; observed species (524.00 ± 66.43 vs 324.00 ± 54.29 ; $P < 0.05$), Shannon (4.38 ± 0.21 vs 2.97 ± 0.16 ; $P < 0.001$), Chao1 (562.81 ± 71.63 vs 349.07 ± 56.04 ; $P < 0.05$), and ACE (564.81 ± 72.72 vs 356.02 ± 58.33 ; $P < 0.05$), respectively (Fig. 3 C).

LefSe analysis revealed a significant increase of the relative abundance of certain bacterial taxa [41] in the “Bac-Suppl + Ni” treatment relative to the “Bac-Suppl” treatment. This applied in particular to *Herbaspirillum huttiense* (belonging to the family Oxalobacteraceae) and to the phylum Bacteroidota, class Bacteroidia. However, in the “Bac-Suppl + Ni” treatment, there was a significantly lower representation of the class Actinobacteria (phylum Actinobacteriota), the family Rhizobiaceae (order Rhizobiales, class Alphaproteobacteria), the genus *Lactobacillus* (family Lactobacillaceae, order Lactobacillales, class Bacilli, and phylum Firmicutes), the genus *Flavobacterium* (family Flavobacteriaceae, order Flavobacteriales), and of the family Prevotellaceae ($P < 0.05$, Wilcoxon rank-sum test; LDA > 4.0 ; Fig. 4 A). These different taxonomic distributions between treatments are further verified in Fig. 4 B. For example, the phylum Bacteroidota is over-represented in the “Bac-Suppl + Ni” treatment, as are the

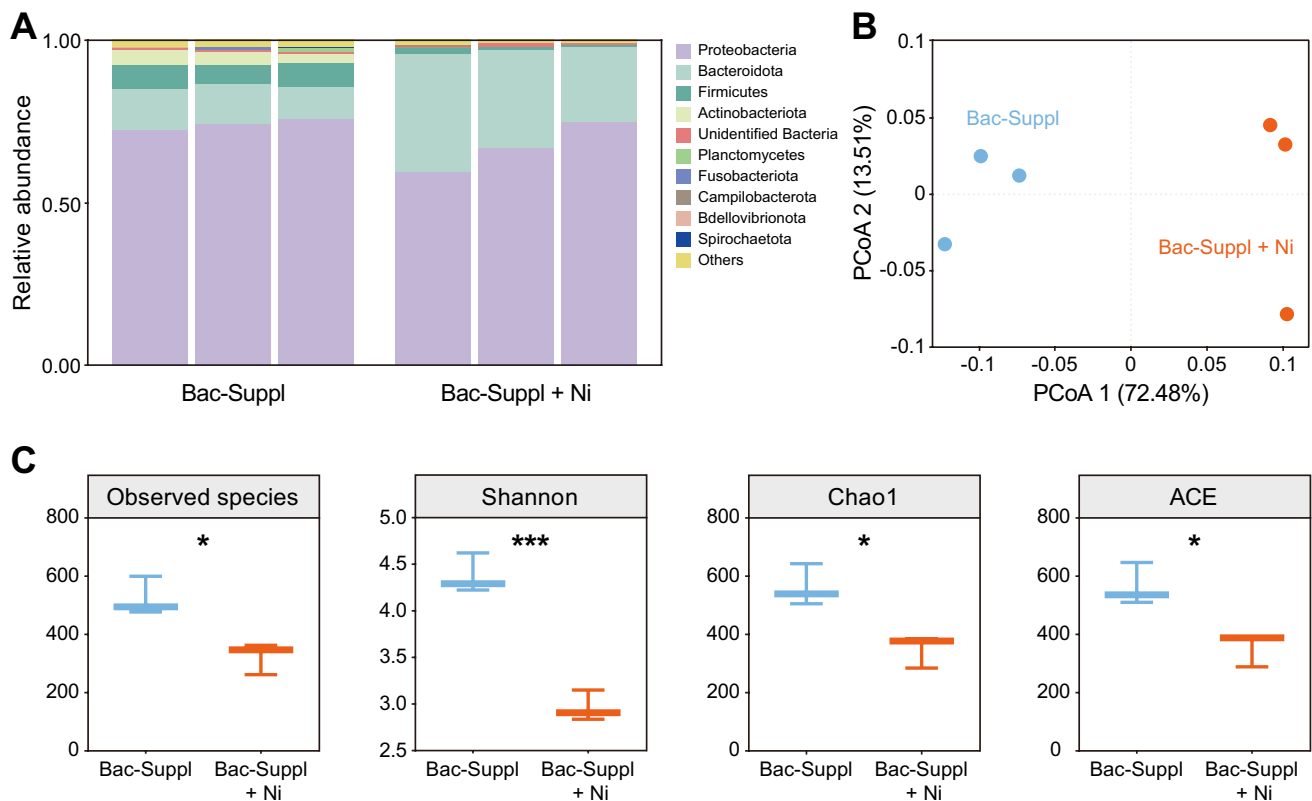


Fig. 3 The composition of gut microbiota of initially germ-free *Daphnia magna* under two different treatments: “Bac-Suppl” (sterile COMBO medium supplemented with donor gut microbiota) and “Bac-Suppl + Ni” (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2 supplemented with donor gut microbiota). **A** Relative abundance of *D. magna* gut microbiota at the phylum level in the “Bac-Suppl” and “Bac-Suppl + Ni” groups for each of three replicates.

Colors indicate different bacterial phyla. **B** Principal coordinate analysis (PCoA) of *D. magna* gut microbiota using weighted UniFrac distances. The blue dots represent three pools of samples from the “Bac-Suppl” treatment and the orange dots represent three pools of samples from the “Bac-Suppl + Ni” treatment. **C** Multiple indices (Observed species, Shannon, Chao1, and ACE) for alpha diversity estimation (* $P < 0.05$, *** $P < 0.001$) in the two treatments

phyla Actinobacteriota and Firmicutes in the “Bac-Suppl” treatment (Fig. 4B).

Transcriptomic Responses

After trimming, an average of 22,273,867 reads per replicate were obtained, about 88.15% of which were mapped to the *D. magna* genome (Table S2). We detected expression of 14,352 genes, out of 15,338 predicted genes in the *D. magna* genome. A clear separation was observed between the *Daphnia* inoculated with gut microbiota extracted from the “Bac-suppl + Ni” and those from the “Bac-suppl” treatment, as shown in the correlation analysis (Fig. 5A). *Daphnia* inoculated with gut microbiota extracted from the “Bac-suppl + Ni” treatment exhibited 558 upregulated and 1180 downregulated DEGs relative to those inoculated with microbiota from “Bac-suppl” treatment (Fig. 5B). The upregulated DEGs were enriched in four KEGG pathways, two of them

associated with “glycosphingolipid biosynthesis” (Fig. 5C). Additionally, the upregulated DEGs were overrepresented in 13 GO terms, 6 of them associated with “detoxification” (alpha-1,3-fucosyltransferase activity, galactosyltransferase activity, fucosyltransferase activity, toxic substance binding, fucosylation, and protein glycosylation; Fig. 5D). The downregulated DEGs were enriched in five pathways, two of them associated with “hedgehog signaling pathway” (Hedgehog signaling pathway-fly and Hedgehog signaling pathway; Fig. 5C). These downregulated DEGs were overrepresented in 23 GO terms: five of them associated with “oxygen transport” (heme binding, oxygen binding, oxygen carrier activity, iron ion binding and hemolysis in other organism) and another five associated with “chitin metabolism” (chitin-based extracellular matrix, structural constituent of cuticle, structural constituent of pupal chitin-based cuticle, structural constituent of chitin-based larval cuticle, and chitin-based cuticle development; Fig. 5D).

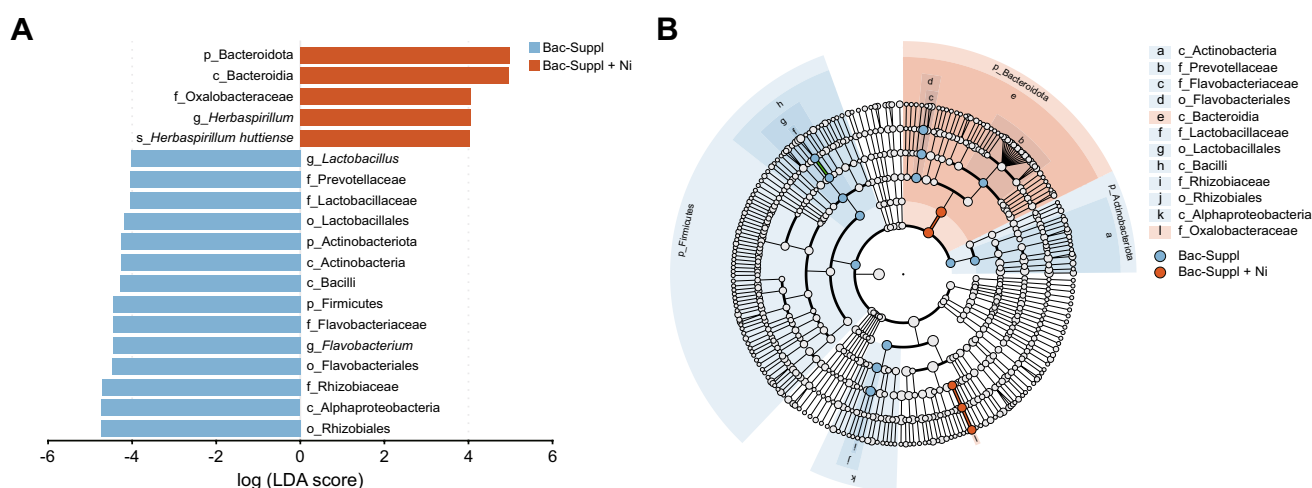


Fig. 4 LefSe analysis identifying taxonomic differences in the gut microbiota of initially germ-free *Daphnia magna* under two different treatments: “Bac-Suppl” (sterile COMBO medium supplemented with donor gut microbiota) and “Bac-Suppl + Ni” (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2 supplemented with donor gut microbiota). Key phylotypes of differentially abundant taxa were identified using linear discriminant analysis (LDA) combined with the effect size (LEfSe) algorithm. **A** Histograms of LDA scores in the “Bac-Suppl” and “Bac-Suppl + Ni” treatments are shown, with a cutoff value of LDA score ($\log_{10}$) above 4.0. Taxa enriched in the “Bac-Suppl” and “Bac-Suppl + Ni” treatments are indicated with a negative and positive LDA score, respectively. The LDA score shows

the effect size and ranking of each differentially abundant taxon. **B** Cladogram derived from LEfSe analysis of differentially abundant gut microbial taxa. Colored regions/branches indicate differences in the microbial taxa between the two treatments. Regions in blue indicate taxa that were relatively enriched in the “Bac-Suppl” treatment, while regions in orange indicate taxa that were relatively enriched in the “Bac-Suppl + Ni” treatment. The central point denotes the root of the tree of bacteria and each successive ring moving outwards from this represents the next lowest taxonomic level from phylum to species. The diameter of each circle at nodes of the tree is proportional to that taxon's relative abundance

Discussion

It is well known that the gut microbiota plays a key role in host health and that perturbing it can result in detrimental effects (e.g., [49, 50]). However, no studies have shown how environmental stressors can hamper the ability of the host to obtain their donors' gut microbiota and how this process might affect host fitness. Interestingly, our results showed that heavy-metal exposure had no significant impact on the fitness of germ-free *Daphnia magna* without gut microbiota transplantation. In contrast, when incubated with their donors' gut microbiota in the continuous presence of nickel for 21 days, initially germ-free *D. magna* showed a significantly lower fitness than did those not exposed to nickel. These findings strongly suggested that the toxic effects of nickel led to a reduced diversity of gut microbiota in *Daphnia* and consequentially affected their fitness.

Many previous studies have shown that metal pollution can reduce the diversity of host gut microbiomes (e.g., [51–53]). For example, exposure to zinc oxide nanoparticles profoundly affects the diversity and composition of the gut microbiota of *Hydra magnipapillata* [54]. Mercury exposure might cause xenobiotic-mediated dysbiosis of the gut microbiome and thus affect host fitness in both *D. magna* [20] and the copepod *Tigriopus japonicus* [55]. Here, from another perspective, we were the first to show

that heavy-metal pollution can also hamper the ability of germ-free hosts to successfully obtain microbiota. The presence of nickel might interfere with the ability of *Daphnia* to recruit gut microbiota. This effect might also be produced directly via the toxicity of nickel for bacteria in the water. Future studies are required to explore how the heavy metals affect the formation of host gut microbiota. Together with numerous previous studies (e.g., [52, 56]), we showed that, at the phylum level, exposure to heavy metal decreased the abundance of Firmicutes and Proteobacteria and increased the abundance of Bacteroidetes in the host. Several specific taxa of these phyla, such as *Bacillus* (Firmicutes), *Pseudomonas* (Proteobacteria), and *Bacteroides* (Bacteroidetes), are known to play roles in metal resistance [57], nutrient metabolism [58], and immune modulation [59], respectively. These changes in the composition of the gut microbiota at a functional level could alter the metabolic profile of hosts (e.g., [51, 60]). Notably, microbes from distinct phyla might play a similar function and thus serve as a stabilizing mechanism to buffer the host against environmental stresses [61].

Our results showed that representatives of the phylum Proteobacteria were less represented in the gut microbiome in *Daphnia* when treated with nickel. In agreement with our finding, a previous study showed that heavy metal Cd exposure significantly decreased the abundance of Proteobacteria from intestinal microbiota in the freshwater

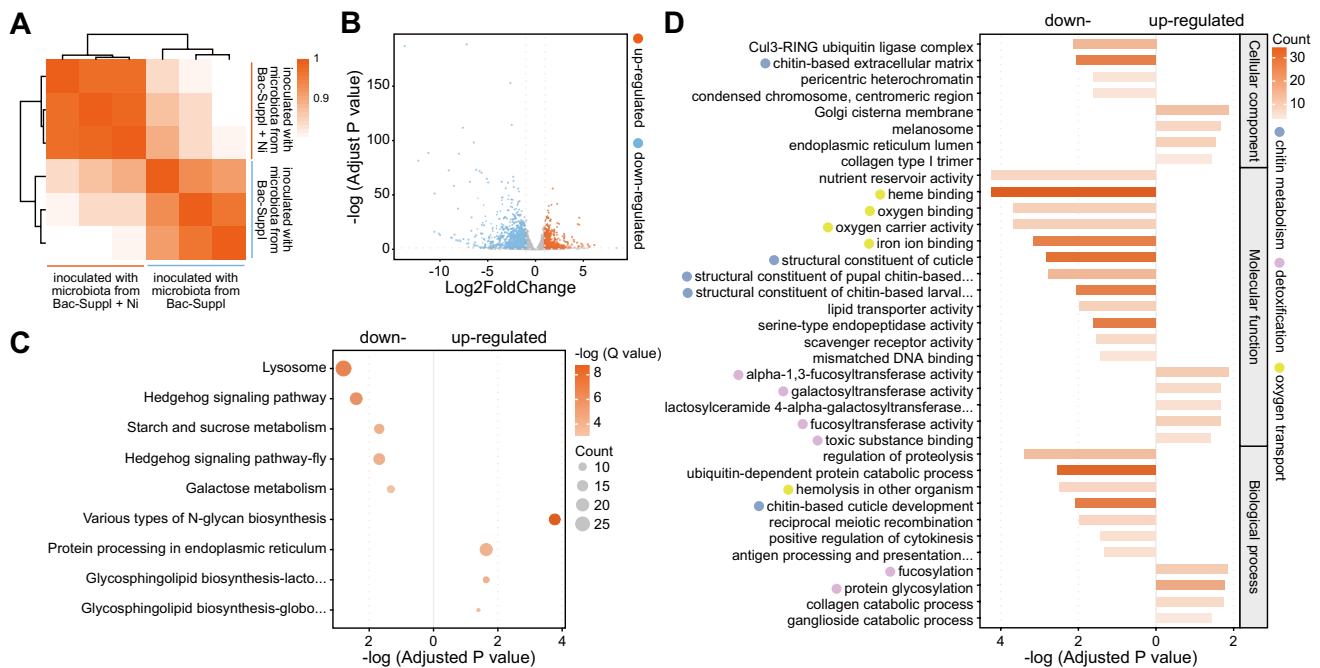


Fig. 5 The gene-expression signatures of two groups of germ-free *Daphnia magna* inoculated with gut microbiotas of different origins. One microbiota was extracted from the guts of 20 adult *Daphnia* in the "Bac-Suppl" treatment (sterile COMBO medium supplemented with donor gut microbiota) and another from the "Bac-Suppl + Ni" treatment (sterile COMBO medium containing $100 \mu\text{g L}^{-1}$ NiCl_2 supplemented with donor gut microbiota; Fig. 1). **A** Heatmap of the correlation of highly expressed genes (top 1000) among *Daphnia* inoculated with gut microbiota from the "Bac-Suppl" and "Bac-Suppl + Ni" treatments. **B** Volcano plots displaying differential gene expression in *Daphnia* inoculated with gut microbiotas from the

"Bac-Suppl + Ni" treatment, relative to the "Bac-Suppl" treatment. Each point represents an individual gene transcript. Orange and blue points represent significantly up- and downregulated transcripts in *Daphnia* inoculated with microbiota from the "Bac-Suppl + Ni" treatment as compared to those from the "Bac-Suppl" treatment, respectively. **C** KEGG enrichment pathways and **D** GO enrichment terms of *D. magna* DEGs that were up- or downregulated in response to microbiota from the "Bac-Suppl + Ni" treatment, compared to those from the "Bac-Suppl" treatment. The following functional categories are marked by solid circles: chitin metabolism- (blue), detoxification- (purple), and oxygen transport- (yellow) related GO terms

crayfish *Procambarus clarkii* [62]. The reduction of the abundance of Proteobacteria could potentially harm intestinal integrity and thus affect metabolic and immune functions [62]. Another study showed that exposure to mercury significantly decreased the abundance of Proteobacteria and altered the expression of immune-related genes in the red swamp crayfish *Procambarus clarkii* [63]. Here, we observed a downregulation of genes involved with chitin metabolism in *Daphnia* inoculated with microbiota from the "Bac-Suppl + Ni" treatment, when compared with those inoculated with microbiota from the "Bac-Suppl" treatment. In arthropods, molting, gut development, and features of the immune system are highly dependent on the ability to process and shape or remodel chitinous structures [64, 65]. In particular, chitin and chitin metabolism are central features of the arthropod innate immune system [66–68]. The peritrophic matrix, a chitinous layer in the midgut of arthropods, provides both structural and immune defense against pathogens and parasites (e.g., [69, 70]). Recently, downregulation of genes related to chitin metabolism was observed in *Daphnia* after exposure to parasites, suggesting an important role

for the repair or restructuring of the peritrophic matrix in the host's response to the parasite [71]. Previous studies also showed the interactions between the peritrophic matrix and bacteria, affecting the ability of bacteria and their toxins to reach the gut epithelia [72, 73]. For example, *Bacillus thuringiensis* produces a chitin-binding protein to enable adhesion to the peritrophic matrix and thus successfully infect the gut surface of the host [74]. In this study, the downregulation of genes involved in chitin metabolism in host *Daphnia* inoculated with microbiota from the "Bac-Suppl + Ni" treatment suggested a possible link between a particular microbiome and the host immune system [5].

Our results also suggest that exposure to nickel could impede the ability of germ-free *Daphnia* to acquire members of the genus *Lactobacillus*. Many *Lactobacillus* strains, used as traditional probiotics, have heavy-metal binding and other beneficial properties that are useful for heavy-metal detoxification (reviewed in [51]). For example, many *Lactobacillus* strains have been found to contribute to the detoxification of chemical substances in honeybees *Apis mellifera* [75]. Also, *L. rhamnosus* might be useful for reducing toxic

organophosphate pesticide exposure via passive binding in *Drosophila melanogaster* [76]. Another probiotic strain *L. reuteri* P16, which was isolated from the gut contents of *Cyprinus carpio*, exhibited in vitro probiotic properties and could protect against lead exposure-induced toxicities in *C. carpio* [77]. Interestingly, our results showed that the genes associated with six detoxification-related GO terms, especially those encoding glycosyltransferase (GT), were upregulated in host *Daphnia* in response to the microbiota from the “Bac-Suppl + Ni” treatment. Members of the GT family of enzymes transfer saccharide units from an activated nucleotide sugar to nucleophilic glycosyl acceptors to catalyze the glyco-conjugation of various biomolecules, and this process might help the organism to excrete hazardous material (reviewed in [78]). GTs participate in numerous biological processes associated with survival and growth in arthropods by regulating various metabolic and physiological events; for example, they detoxify heavy metals and other xenobiotic compounds (e.g., [79, 80]). For instance, exposure to cadmium can lead to hyper-expression of the genes encoding the detoxifying enzyme UDP-glucuronosyltransferase (UGT) in *Daphnia* [81]. Our results suggested that reduced representation of *Lactobacillus* in the gut microbiome might influence the expression of genes encoding GTs in the host. However, direct evidence for such a causal link requires future investigations.

It is already known that members of the genus *Lactobacillus* can rapidly respond to oxidative stress and exhibit substantial antioxidant activity, promoting the production of antioxidant enzymes to help remove reactive oxygen species in the host intestine [82]. For instance, a pseudo-catalase has been discovered in *L. plantarum* [83], and a heme-dependent catalase has been identified in *L. sakei* [84]. These enzymes could provide protection against oxidative stress [82]. Here, we found that inoculating microbiota from adults in the “Bac-Suppl + Ni” group resulted in the downregulation of genes involved in five GO terms associated with “oxygen transport” in host *Daphnia*. Two of them in particular (GO “heme binding” and “hemolysis in other organism”) were associated with hemoglobin. The changes of the hemoglobin suite in both quantity and quality could be linked to their physiological performances [85]. Arthropods can respond to changing oxygen levels by increasing or decreasing the rate of synthesis of extracellular hemoglobin and thus change the oxygen transport capacity to meet their needs [86]. Specifically, many aquatic crustaceans increase their hemoglobin levels in response to hypoxia [87]. For example, previous studies had already clearly demonstrated that *Daphnia* can change hemoglobin concentrations and their subunit components in response to hypoxia stress (e.g., [88–90]). Another study had shown that exposure to nickel resulted in a downregulation of transcription of genes that were involved in oxygen transport

and heme metabolism [91]. Therefore, we assume nickel exposure directly reduces the abundance of *Lactobacillus* in the gut of host *Daphnia*, thereby inducing oxidative stress due to a lack of antioxidant protection. The oxidative stress triggers the downregulation of oxygen transport genes, suggesting a potential decrease in the oxygen transport capacity in *Daphnia*.

In conclusion, our data showed that heavy-metal pollution hampers the ability of germ-free *Daphnia* to acquire donor gut microbiota, and this process might in turn affect host fitness. Further, we have applied transcriptomics to ascertain the molecular basis underlying the effects on the host caused by the altered gut microbiomes. We found an upregulation of genes related to detoxification occurs in *Daphnia* in response to gut microbiota with reduced diversity, alongside a downregulation of genes related to oxygen transport and chitin metabolism. These findings suggest a potential trade-off mechanism within the microbiota, prioritizing detoxification in the presence of nickel, at the expense of other functions such as immune response-related genes. Overall, our study shows that the gut microbiota in *Daphnia* could be altered by the toxic effects of heavy metal, thus reducing the diversity of gut microbiota and affecting the host’s health and fitness.

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Author Contributions MY designed the study, WY and ZD carried out experiments and analyzed data. WY, ZD, DB, WH and MY interpreted the data. MY wrote the manuscript with the help of WY and ZD. All authors read and approved the final version.

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Declarations

Competing interests The authors declare no competing interests.

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