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Effects of common artificial sweeteners at environmentally relevant concentrations on soil springtails and their gut microbiota

Xiang-Long Lin [a], Fei Guo [b], Matthias C. Rillig [c], Chun Chen [e], Gui-Lan Duan [a,d,e,*], Yong-Guan Zhu [a,d,f]

[a] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
[b] State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100000, China
[c] Institut Für Biologie, Freie Universität Berlin, Berlin 14195, Germany
[d] University of Chinese Academy of Sciences, Beijing 100049, China
[e] State Key Laboratory of Crop Stress Biology for Arid Areas, Shaanxi Key Laboratory of Agricultural and Environmental Microbiology, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China
[f] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

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ABSTRACT

Artificial sweeteners (AS) are extensively utilized as sugar substitutes and have been recognized as emerging environmental contaminants. While the effect of AS on aquatic organisms has garnered recent attention, their effects on soil invertebrates and gut microbial communities remain unclear. To address this knowledge gap, we exposed springtails (*Folsomia candida*) to both single and combined treatments of four typical AS (sucralose [SUC], saccharin [SAC], cyclamate [CYC], and acesulfame [ACE]) at environmentally relevant concentrations of 0.01, 0.1 and 1 mg kg^{-1} in soil. Following the first-generational exposure, the reproduction of juveniles showed a significant increase under all the AS treatments of 0.1 mg kg^{-1}. The transcriptomic analysis revealed significant enrichment of several Kyoto Encyclopedia of Gene and Genome pathways (e.g., glycolysis/gluconeogenesis, pentose and glucuronate interconversions, amino sugar, and nucleotide sugar metabolism, ribosome, and lysosome) in springtails under all AS treatments. Analysis of gut bacterial microbiota indicated that three AS (SUC, CYC, and ACE) significantly decreased alpha diversity, and all AS treatments increased the abundance of the genus *Achromobacter*. After the sixth-generational exposure to CYC, weight increased, but reproduction was inhibited. The pathways that changed significantly (e.g., extracellular matrix-receptor interaction, amino sugar and nucleotide sugar metabolism, lysosome) were generally similar to those altered in first-generational exposure, but with opposite regulation directions. Furthermore, the effect on the alpha diversity of gut microbiota was contrary to that after first-generational exposure, and more noticeable disturbances in microbiota composition were observed. These findings underscore the ecological risk of AS in soils and improve our understanding of the toxicity effects of AS on living organisms.

1. Introduction

Artificial sweeteners (AS) are highly sweet and water-soluble, providing little to no calories. As a result, AS are extensively utilized as sugar substitutes in the global food and beverage industry, as well as in personal care products and animal feeds (Buerge et al., 2011; Saputra et al., 2021). As emerging contaminants, AS can enter agricultural soils through various anthropogenic activities, including wastewater irrigation and the application of organic fertilizers (Gan et al., 2014). For instance, Gan et al. observed that sucralose (SUC), saccharin (SAC), cyclamate (CYC), and acesulfame (ACE) were common AS in the topsoil, with residual concentrations ranging from 0.11 to 34.7 μg kg^{-1} for SUC, 0.11 to 34.7 μg kg^{-1} for SAC, 0.01 to 1280 μg kg^{-1} for CYC, and 0.08 to 569 μg kg^{-1} for ACE (Gan et al., 2014). Moreover, Buerge et al. (2011) revealed that CYC, SAC, ACE, and SUC were degraded in six types of soils under the aerobic conditions, with half-lives of 0.4–6 d, 3–12 d, 3–49 d, and 8–124 d, respectively. The degradation of sweeteners in soils appeared to be biologically mediated, since in a sterile control

* Corresponding author at: State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.
E-mail address: duangl@rcees.ac.cn (G.-L. Duan).

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experiment, no (acesulfame, saccharin, and sucralose) or a considerably slower (cyclamate) dissipation was observed.

Soil invertebrates, such as earthworms, springtails, and nematodes, are abundant organisms in soil ecosystems and play a crucial role in maintaining soil structure and function. Conducting toxicity tests on soil invertebrates at various levels provides a fundamental basis for assessing the ecological risk of contamination. Molecular-level endpoints, including gene expression, protein profiles, and metabolites, offer higher sensitivity compared to traditional individual-level endpoints. These molecular responses serve as early warning signs of exposure and provide valuable insights into potential toxicity mechanisms (He et al., 2020; Scanlan et al., 2015; Zhu et al., 2019). Furthermore, symbiotic microorganisms in the animal gut engage in interactions and co-evolution with hosts. As a widely accepted concept, the gut microbiome is recognized to regulate host physiology and health directly and indirectly. Consequently, imbalances in the gut microbial communities of invertebrates can serve as indicators, explaining the toxic effects of environmental pollution on the host (Wang et al., 2019; Yu et al., 2020; Zhu et al., 2018).

The toxicity of contaminants to living organisms is closely linked to both concentration and exposure time (He et al., 2020). Many previous studies assessing the toxicity of emerging contaminants to soil invertebrates have been conducted at high concentrations, significantly exceeding those observed in field soils. Additionally, invertebrates are often exposed to emerging contaminants throughout their entire life cycle or even for several successive generations under realistic soil conditions (Guimarães et al., 2019). Currently, there is minimal research reporting information about the toxicity of emerging contaminants to invertebrates after multi-generational exposure.

Thus far, several studies have unveiled the eco-toxicity of typical AS to aquatic invertebrates, including crustaceans and fish (Praveena et al., 2019; Saputra et al., 2021). For instance, SUC can cause oxidative damage in carp *Cyprinus carpio* and behavioral changes in the water flea *Daphnia magna* (Praveena et al., 2019; Saucedo Vence et al., 2017). However, the effects of AS on soil invertebrates and their gut microbial communities are largely unknown. Parthenogenetic *Folsomia candida* is a soil-dwelling springtail (Collembola) and is frequently used as a model organism for toxicity tests (ISO, 2014; Zhu et al., 2018). Utilizing the springtail, this study aimed to elucidate: (1) the effects of single and combined treatments of prevalent AS (SUC, SAC, CYC, and ACE) on springtails at environmentally relevant concentrations; (2) the responses of springtails after multi-generational exposure to AS. Various endpoints at the individual level (growth, survival, and reproduction), molecular level (gene expression), and gut microbiota were assessed. The findings of this study will enhance the understanding of the toxicity of AS and furnish crucial data for the risk assessment of AS in soils.

2. Materials and methods

2.1. Test chemicals, soil, and organisms

Granular or powdery SUC, SAC, CYC, and ACE (purity $\geq$ 98 %, CAS numbers are 56038–13–2, 81–07–2, 135–05–9, and 33665–90–6, respectively) were procured from J&K Scientific Ltd (Beijing, China).

Uncontaminated fluvo-aquic soils were collected from topsoil (0–10 cm) from a park in Beijing. The soil was sieved through a mesh of 2 mm after air-drying at room temperature. The physicochemical properties of soil were as follows: pH 7.74, water holding capacity 45.5 %, clay 16.2 %, organic matter 1.89 %, and cation exchange capacity 12.6 cmol kg^{-1}.

The springtails (*Folsomia candida)* were initially acquired from Aarhus University in Denmark. The test organisms were cultured in Petri dishes with a moist substrate mixture of plaster and charcoal (supplemented with dried baker's yeast). The culture was maintained in a climate chamber under standardized conditions of 20 $\pm$ 1 °C, 75 % relative humidity, and 16 h light/8h dark cycles), adhering to the standard test guideline (ISO, 2014).

2.2. Toxicity measurement of different AS after exposure for one generation

For assessing responses at the individual level, the experimental procedure followed the standard guidelines (ISO, 2014). The control and AS-contaminated soils were prepared by introducing deionized water and an AS-aqueous solution into the test soils, respectively. In both single and combined treatments, the environmentally relevant concentration of each AS was set at 0.01, 0.1 and 1 mg kg^{-1}. The soil water content was adjusted to 60 % of the maximum water-holding capacity. Ten synchronized springtail juveniles (10–12 days old) were introduced into 120 mL glass containers with vented lids, each containing 30 g of either control or single/combined AS-treated soils (four replicates for each treatment). The bottle lids were securely tightened, and the exposure lasted for 28 days in a climate chamber under the same conditions as described in Section 2.1. A few grains of dried yeast and deionized water were added three times each week. After the exposure period, the glass containers were emptied into glass beakers. The floating springtails were photographed after adding tap water and gently stirring. The number of adults and juveniles was counted using Image J software. Subsequently, the adults were collected and washed, and residual water was removed from dry Petri dishes. The fresh weight was measured using an XP6 electronic microbalance (Mettler Toledo, Switzerland).

For assessing responses at the molecular level, 30 synchronized juveniles (10–12 days old) were placed into 200 mL glass containers with 90 g of soils (three replicates for each treatment). The concentration of each AS was 0.1 mg kg^{-1}, because the individual level endpoint of springtails was affected in single and combined AS-treated groups at this concentration (see the Section 3.1 in detail). Following a 28-day exposure period (under the same conditions as described above), the adults were collected, snap-frozen in liquid nitrogen, and stored at −80 °C. After total RNA extraction from the springtails, RNA sequencing was conducted. Raw reads were filtered to obtain clean reads for subsequent bioinformatics analysis. The details are presented in Supporting Information.

To assess effects at gut microbiota level, 30 synchronized juveniles (10–12 days old) were introduced into 200 mL glass containers with 90 g of soils (each AS concentration of 0.1 mg kg^{-1}, three replicates for each treatment). Following a 28-day exposure period, the adults were promptly collected and euthanized with chloroform. According to the study by Zhu et al. (2018), the adults were immersed in a 2 % sodium hypochlorite solution for 10 s to eliminate surface microbiota. Following rinsing with sterilized water, dissection was carried out using sterile forceps under aseptic conditions. The extracted guts were transferred into a 2 mL centrifuge tube containing 1 mL sterile phosphate buffer solution and stored in a −80 °C refrigerator. After DNA extraction from the gut samples, the hypervariable V4-V5 region of the bacterial 16S rRNA gene was amplified, and high-throughput sequencing was performed (details presented in the Supporting Information). Subsequently, the raw data were analyzed using QIIME 1, and high-quality reads (effective reads) were obtained.

2.3. Toxicity measurements for CYC-treated soil after multi-generational exposure

According to the methods described by Guimarães et al. (2019), the responses of springtails to AS after multi-generational exposure were explored. CYC was randomly selected as test AS and the experiment procedure was presented as flowchart as Fig. S1. In detail, after the control and CYC-contaminated (concentration of 0.1 mg kg^{-1}) soils were prepared, the first-generational (F1) exposure of 28 d was performed (details were consistent with those described in Section 2.2). Then, 10 of the largest F1 juveniles were chosen and transferred to new glass containers with 30 g of newly prepared control or CYC-treated soils to initiate the second-generational exposure (F2). This exposure process

was continuously conducted until the end of the sixth-generational exposure (F6). After the exposure of each generation, the number and weight of springtails adults were measured.

For assessing responses at the molecular and gut microbiota levels, the continuous five generations exposure (F1–F5) were conducted following the method described above. At the end of F5, 30 of the biggest juveniles were exposed to newly prepared control or CYC-treated soils for 28 d to initiate the sixth-generational exposure. Then, the adults were collected for RNA extraction or gut sampling, respectively (details were consistent with those described in Section 2.2).

2.4. Data processing and statistical analysis

Statistical difference comparisons were performed using Student's *t*-tests and one-way analysis of variance (ANOVA). A *p*-value of <0.05 was considered statistically significant.

The expression of each gene was computed using fragments per kilobase of transcript per million mapped reads (FPKM). Principal component analysis (PCA) was performed to compare gene expression differences between treatments or replicates within each group. Differentially expressed genes (DEGs) were identified when the false discovery rate (FDR) was <0.05 in the DESeq2 package. An additional fold-change cutoff was not selected because even slight changes in gene expression can have significant physiological effects. Functional annotation and enrichment analysis of DEGs were performed using the Kyoto Encyclopedia of Gene and Genome (KEGG) databases, with the criterion of significant enrichment adjusted at $p < 0.05$. A Venn diagram was constructed to represent the shared and unique DEGs among different treatments.

Clust clustering was used to obtain the operational taxonomic units (OTUs at a 97 % similarity level. Species annotation of representative OTUs was conducted based on the SILVA 16S rRNA gene database. Ace and Shannon indices were obtained to analyze the alpha diversity of the gut bacterial community. The beta diversity among different treatments was compared using the Adonis test and nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis distance matrix. The vegan package (version 2.3-0) in the R programming language was used to analyze the similarities (Anosim) of gut microbial communities between different treatments.

3. Results

3.1. Effects of AS on individual level endpoints of springtails

3.1.1. Effects of different AS on springtails after first-generational exposure

Following a single generation of exposure to both single and combined AS for 28 days, there were no significant differences observed in the survival rates and body fresh weight of adult springtails at all the concentrations compared to the control group (data not shown). However, the number of juveniles significantly ($p < 0.05$) increased in SUC and combined treatment groups at the concentration of 0.01 mg kg^{-1} (Fig. 1A). In all the AS treatments, the reproduction significantly ($p < 0.05$) increased by 10.3–25.9 % compared with that in the control at the concentration of 0.1 mg kg^{-1} (Fig. 1A).

3.1.2. Effects of CYC on springtails after multi-generational exposure

For the CYC treatment of 0.1 mg kg^{-1}, after multi-generational exposure (F2–F6), the survival rates were not significantly different from that of the control (data not shown). The fresh weights of adults were significantly increased ($p < 0.05$) after the fifth- and sixth-generational exposure (Fig. 1B). Nevertheless, juvenile production significantly decreased ($p < 0.05$) after the sixth generational exposure (Fig. 1C).

3.2. Effects of AS on the transcriptome profiles of springtails

3.2.1. Effects of different AS on transcriptome after first-generational exposure

PCA showed that the gene expression between control and single or combined AS-treated groups exhibited distinct separation along the PC1 and PC2 axis (Fig. 2A), indicating that AS exposure affected the transcriptome profiles of springtails. The relative cluster between T_COM and T_SAC was observed (Fig. 2A). Compared with the control group, 9831, 9433, 9108, and 10,464 DEGs were identified in T_SAC, T_SUC, T_CYC, and T_ACE groups, respectively. Furthermore, the T_COM caused fewer DEGs (8421) (Fig. 2B). The numbers of downregulated DEGs were slightly more than those of upregulated DEGs in all the groups except in the T_CYC group (Fig. 2B). In total, 4862 shared DEGs were found among four single treatments, and 4143 DEGs were found among all the treatment groups (Fig. 2C). The clustering analysis of shared DEGs between all the groups is shown in Fig. 2D. Results showed that the gene expression profiles were different among treatments. Furthermore, compared with the control, the T_ACE group showed the most distinct difference (Fig. 2D). Additionally, the regulation direction of most

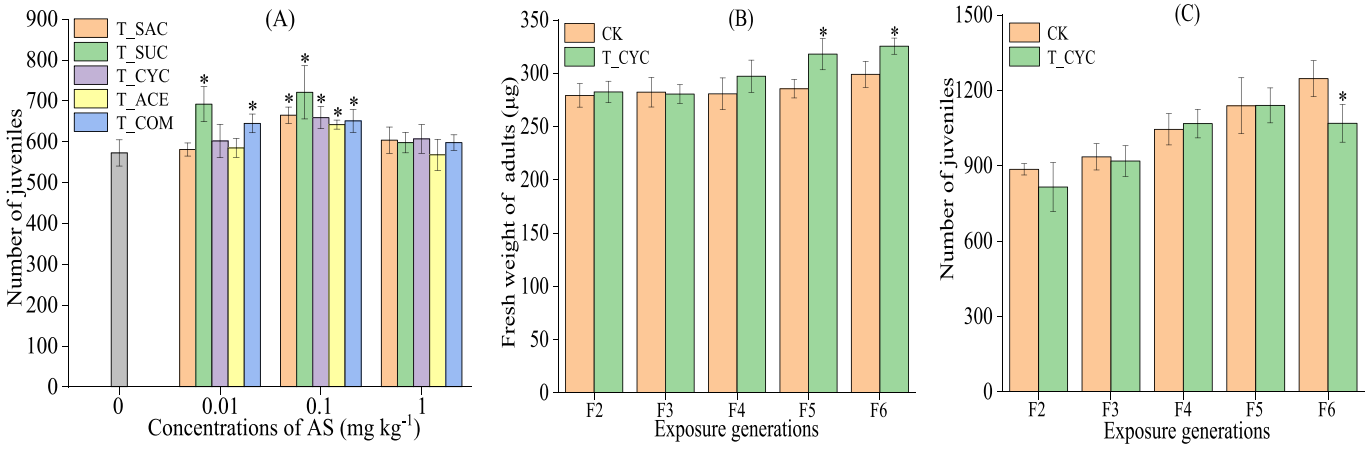

Fig. 1. The reproduction (A) of springtails *Folsomia candida* after 28 days of first-generational exposure to single and combined AS-treated soils. The body weight (B) and reproduction (C) after multi-generational exposure (F2–F6) to CYC treatment of 0.1 mg kg^{-1}. T_SAC, T_SUC, T_CYC, T_ACE, and T_COM represent saccharin, sucralose, cyclamate, acesulfame, and combined AS treatments, respectively (the same abbreviation in the following figures). F2–F6 represented the second to sixth generations. Values represent the mean ± SE (n = 4). The asterisk indicates significant differences ($p < 0.05$) compared with control.

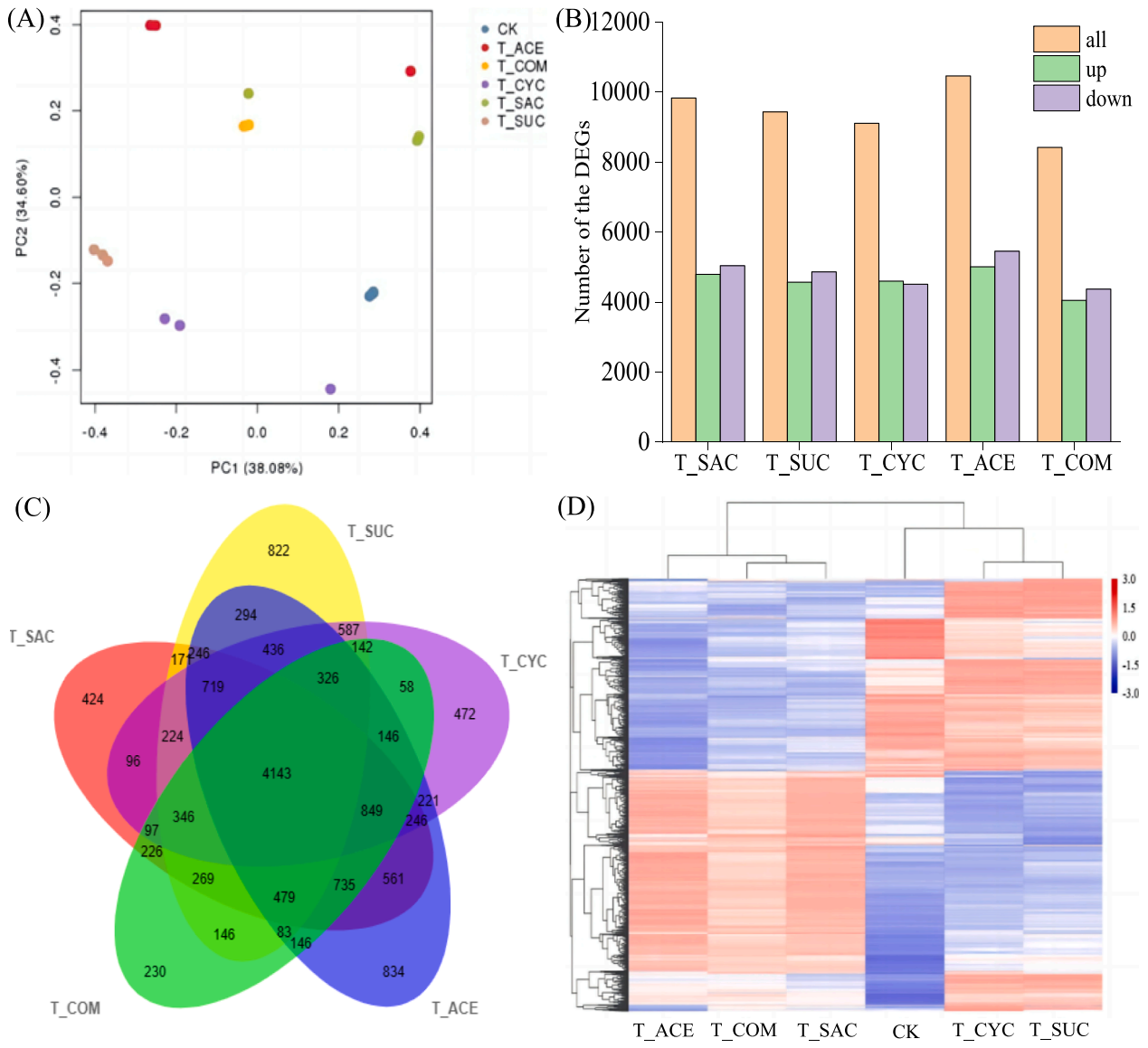

Fig. 2. Principal component analysis (PCA) of normalized expression value of the gene in springtails *Folsomia candida* after 28 days of exposure to control and AS-treated soils (A). Numbers (B) and Venn diagram (C) of differentially expressed genes (DEGs) in each group. Hierarchical clustering analysis of shared DEGs between all the groups (D).

shared DEGs in the T_SUC and T_CYC groups was contrary to that in the other treatment groups (Fig. 2D).

The enrichment analysis of DEGs showed 23, 18, 24, and 26 significantly enriched KEGG pathways in the T_SAC, T_SUC, T_CYC, and T_ACE groups, respectively. Among these pathways, glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, ribosome, and glycolysis/gluconeogenesis were the most significantly enriched pathways, respectively (Fig. 3A and B). Among the top ten significantly enriched pathways, four KEGG pathways (glycolysis/gluconeogenesis, amino sugar, nucleotide sugar metabolism, ribosome, pentose, and glucuronate interconversions) were shared between all the single AS-treatment groups (Fig. 3A and B). Furthermore, fatty acid degradation, lysosome, and glutathione metabolism were also shared pathways (Table S1). In the combined treatment group (T_COM), glycolysis/gluconeogenesis was the most significantly enriched pathway (Fig. 3C) among 27 significantly enriched KEGG pathways. The aforementioned shared pathways between single AS groups were also observed in the combined group (Fig. 3; Table S1).

3.2.2. *Effects of CYC on transcriptome after multi-generational exposure*

After the sixth generation of exposure (F6), PCA showed that control (CK_F6) and CYC-treated (T_CYC_F6) groups exhibited distinct separation (Fig. 4A). Compared with the control group, 7287 DEGs were identified, and most of these DEGs were downregulated (Fig. 4B). In total, 4167 DEGs were shared between the sixth-generational and the aforementioned first-generational exposure treatments (Fig. 4C). Cluster analysis further revealed that the majority of shared DEGs exhibited contrary regulation directions (Fig. 4D).

Six significantly enriched KEGG pathways were found in the T_CYC_F6 group, and extracellular matrix (ECM)-receptor interaction was the most significantly enriched pathway (Fig. 4E). Except for longevity regulating pathway-multiple species, the other pathways (e.g., amino sugar and nucleotide sugar metabolism, lysosome, retinol metabolism) were also observed in the first generation of exposure (Fig. 3B and Fig. 4E).

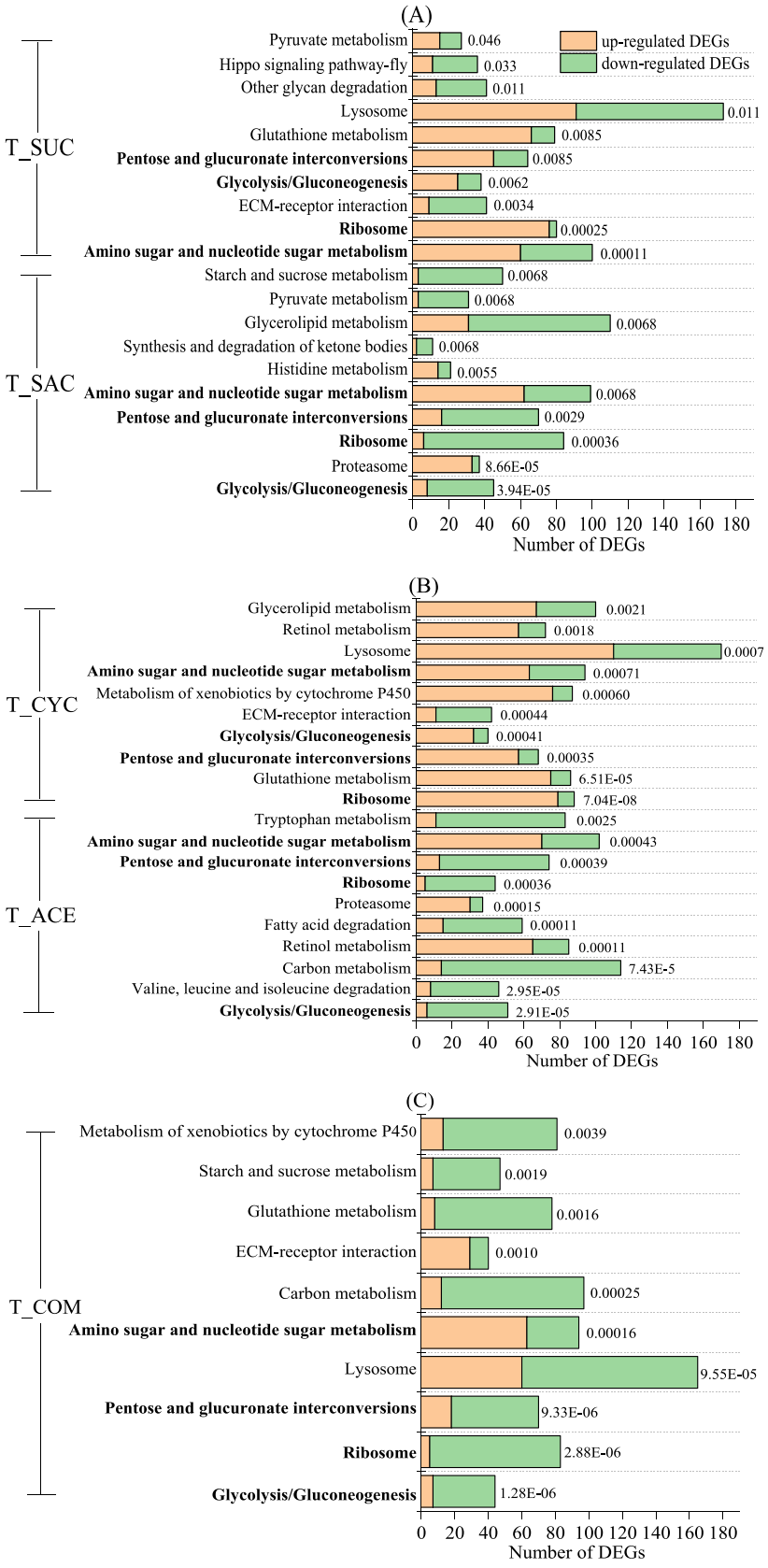

Fig. 3. The significantly enriched top ten KEGG pathways in springtails in the single (A and B) and combined (C) AS-treated groups. Adjusted p-values are shown above the bar graph. The shared pathways between all the groups are in bold.

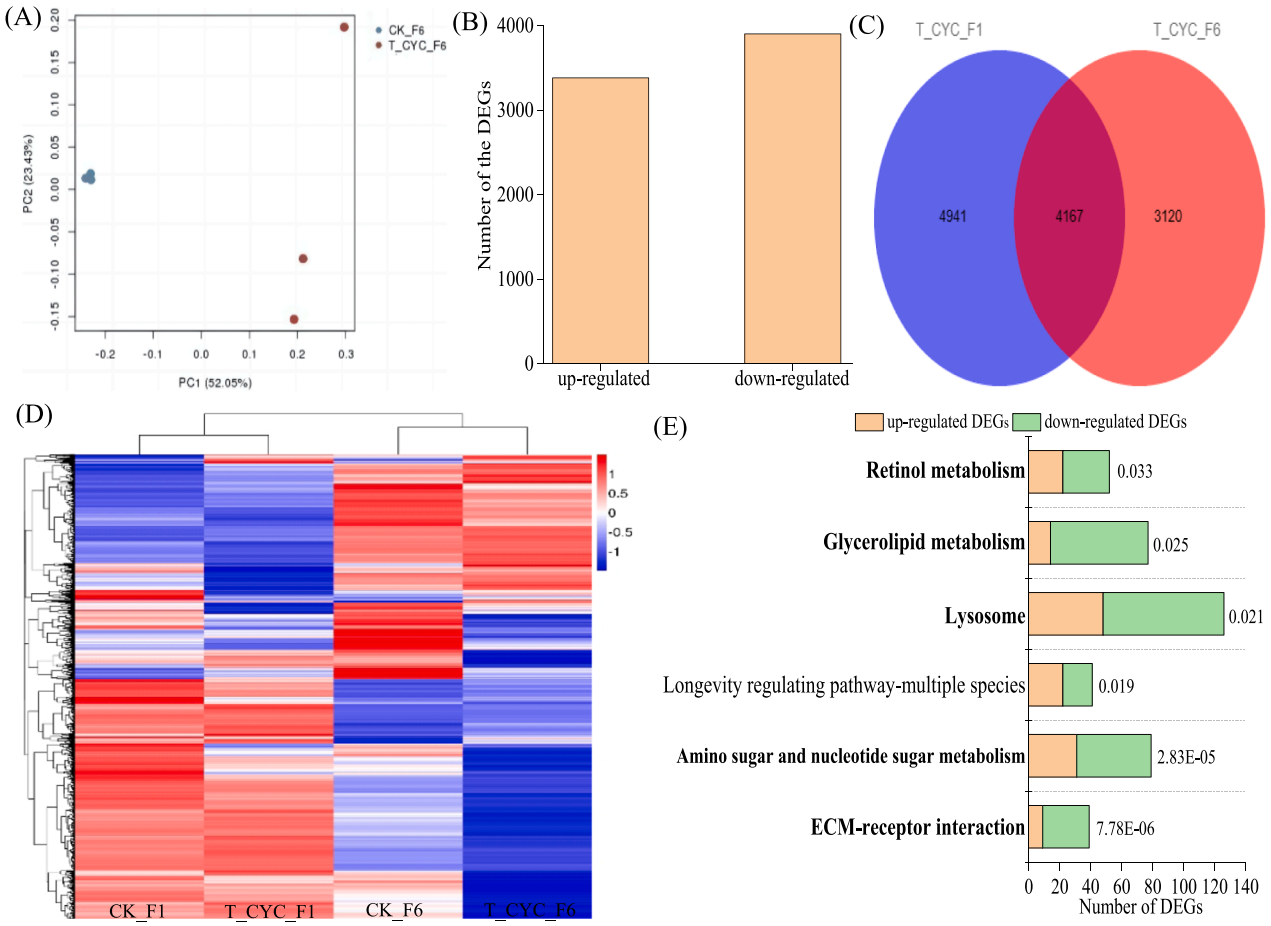

Fig. 4. PCA of normalized expression value of all the genes in springtails after the sixth-generation of exposure in cyclamate (CYC)-treated group (T_CYC_F6) (A). Numbers of differentially expressed genes (DEGs) after the sixth-generation of exposure (B) and Venn diagram of DEGs after the first- and sixth-generational exposure (C). Hierarchical cluster analysis of shared DEGs between the first- and sixth-generational exposure treatments (D). All the significantly enriched (adjusted p-values were shown above the bar graph) KEGG pathways after the sixth generation of exposure, and the shared pathways between the first and sixth generation of exposure are in bold (E).

3.3. Effects of AS on gut bacterial microbiota of springtails

3.3.1. Effects of different AS on gut microbiota after first-generational exposure

For the alpha diversity of springtail gut microbiota, the Ace and Shannon indices significantly decreased in the T_SUC, T_CYC, and T_ACE groups compared with those in the control group (Fig. 5A and B). For the beta diversity of gut microbiota, NMDS analysis (stress = 0.116) revealed different degrees of separation between control and single or combined AS exposure treatments, indicating that the AS exposure induced a shift in the gut microbial community (Fig. 5C). The Anosim test further showed a significant difference ($p = 0.001$) in microbiota composition between different AS treatments (Fig. S2).

At the phylum level, Proteobacteria, Actinobacteriota, Bacteroidetes, and Firmicutes were the dominant phyla in the control and AS-treated groups, and the relative abundances of these dominant phyla in the gut of four single AS-treated groups were similar to that of the control (Fig. 5D). The relative abundance of Patescibacteria significantly ($p < 0.05$) decreased in the T_ACE compared with that in the control. The relative abundance of Proteobacteria significantly ($p < 0.05$) decreased in the combined groups, whereas Bacteroidetes significantly ($p < 0.05$) increased (Fig. 5D). At the genus level, *Achromobacter*, one of the dominant genera, was significantly enriched ($p < 0.05$) by 76.5–161.8 % in single and combined AS treatment groups compared with that in the control (Fig. 5E).

3.3.2. Effects of CYC on gut microbiota after multi-generational exposure

After the sixth-generational exposure (F6), the alpha diversity (Ace and Shannon indexes) of the springtail gut microbiota significantly increased in the CYC-treated groups (T_CYC_F6) compared with that in the control group (CK_F6) (Fig. 6A and B). For the beta diversity, the NMDS analysis of gut microbiota (stress = 0.009) revealed a degree of separation between CK_F6 and T_CYC_F6 (Fig. 6C).

At the phylum level, Proteobacteria, Actinobacteriota, and Bacteroidetes were the dominant phyla in the CK_F6 and T_CYC_F6 groups (Fig. 6D). The relative abundance of Firmicutes was significantly ($p < 0.05$) increased by 301.3 % in the T_CYC_F6 compared with that in the CK_F6 (Fig. 6D). At the genus level, *Achromobacter*, the most dominant genus, was significantly enriched in the T_CYC_F6 (Fig. 6E). Relative abundances of other dominant genera, such as *Arthrobacter* and *Pseudomonas*, were also significantly ($p < 0.05$) changed (Fig. 6E).

4. Discussion

4.1. The responses of springtails to different AS exhibit different extent

There is a growing body of evidence suggesting that various emerging pollutants may be associated with accelerated development rates and/or increased offspring production in organisms, albeit with potential fitness costs and physiological disruptions, even at low concentrations (Campos et al., 2013; Yu et al., 2020). This represents an additional mechanism through which natural organisms may be

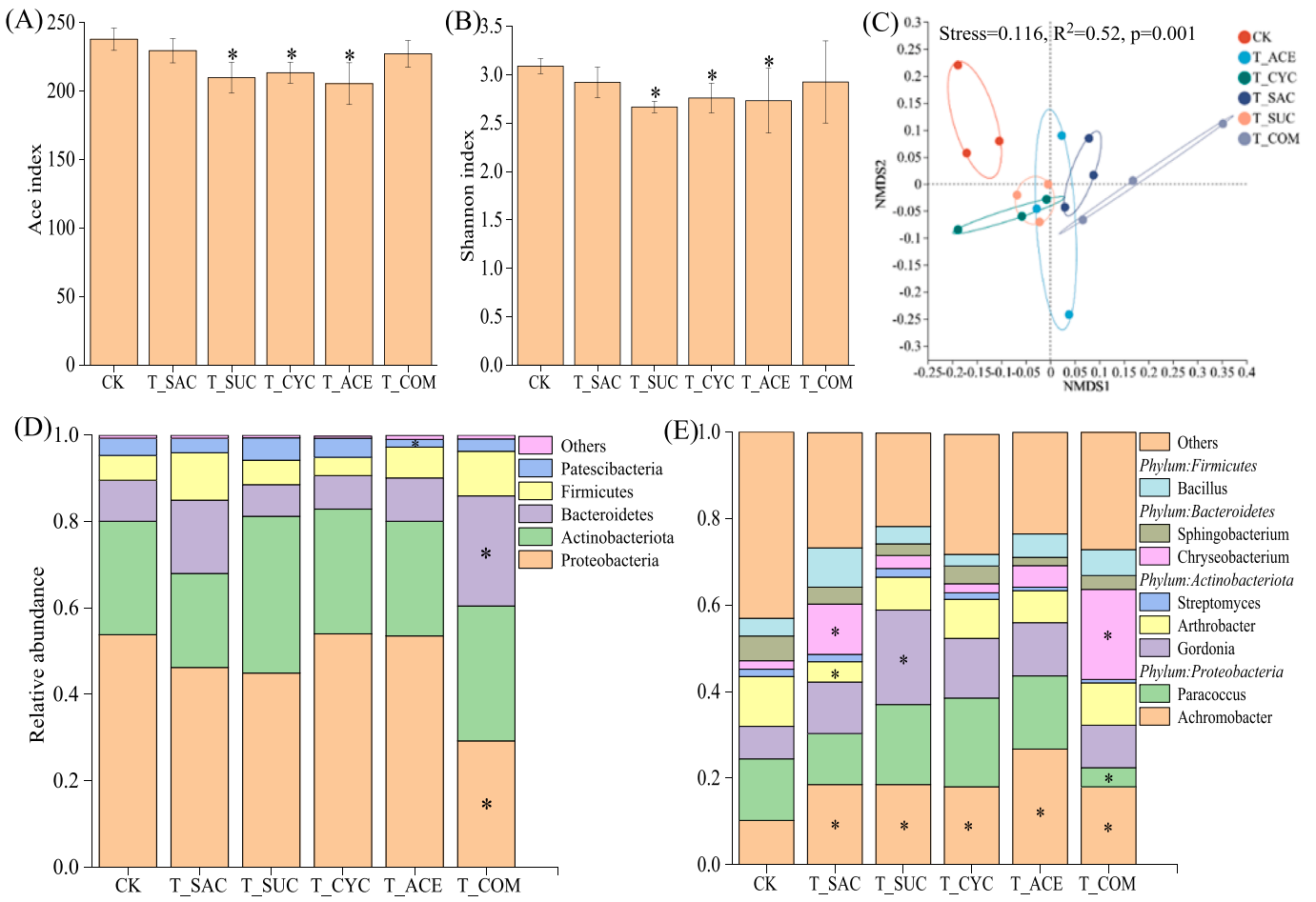

Fig. 5. Changes in Ace (A) and Shannon (B) indexes of springtail gut microbiota in single and combined AS-treated groups after 28 days of exposure. NMDS plot of gut microbiota based on Bray − Curtis distances (C). Composition of gut microbiota at phylum (D) and genus (E) level. The asterisk indicates significant differences ($p < 0.05$) from the control.

potentially modulated or influenced under the stress of pollutants. In the context of this study, the reproduction of springtails significantly increased after 28 days of exposure to four typical AS (in both single and combined treatments) at environmentally relevant concentrations in soil. These findings indicate that by acting as non-nutritive sugar substitutes, AS could function as a physiological disruptor.

A comparative transcriptome analysis was conducted on springtails exposed to different typical AS. Each single and combined AS treatment led to noticeable transcriptional changes in genes, predominantly showing downregulation. Despite the distinct chemical structures and intrinsic properties of different AS, the results indicated that the majority of significantly enriched KEGG pathways (such as glycolysis/gluconeogenesis, pentose and glucuronate interconversions, amino sugar and nucleotide sugar metabolism, ribosome, and lysosome) in springtails were commonly shared among the four single AS-treated groups. Moreover, these pathways were also significantly affected in the combined group.

Glycolysis, a component of carbohydrate metabolism occurring in the cytoplasm without oxygen, is the process that converts glucose into pyruvate—an essential organic acid. Pyruvate plays a crucial role in mitochondrial ATP generation, serving as one of the key intermediates in the citric acid cycle (Gray et al., 2014). Pentose and glucuronate interconversions, another aspect of carbohydrate metabolism, intersect with the glycolysis/gluconeogenesis pathway, playing a crucial role in sustaining energy supply. Additionally, various pathways, such as pyruvate metabolism and starch and sucrose metabolism, linked to carbohydrate metabolism, were notably affected by both single and combined AS exposure (Fig. 3). Hence, our findings suggest that AS exposure has the potential to disrupt the energy metabolism and nutritional status of springtails by influencing the mentioned pathways. Notably, energy metabolism is crucial for maintaining physiological homeostasis and supporting growth, and disturbances in energy-related metabolism represent a common response in living organisms facing pollution-induced stress (He et al., 2020; Scanlan et al., 2015; Yu et al., 2020).

Amino sugars are derivatives of sugar, in which at least one of the hydroxyl groups is replaced by an amino functionality. The majority of amino sugars are crucial components of complex biomacromolecules, including chitin, glycoproteins, and polysaccharides (Skarbek and Milewska, 2016). Nucleotide sugars are activated sugar donors and the main precursors of glycan synthesis (Bar-Peled and O'Neill, 2011). The metabolism of amino sugar and nucleotide sugar is closely associated with the growth and development of living organisms. Zhu et al. reported that this pathway is significantly upregulated in earthworms when exposed to TiO_2 nanoparticles. With this strategy, earthworms maintain normal growth and reproduction (Zhu et al., 2020). The upregulation of this pathway is also associated with the mechanism of rice tolerance to the combined pollutions of herbicide quinclorac and polystyrene nanoplastics (Lu et al., 2023). Our results indicated that the pathways of amino sugar and nucleotide sugar were significantly upregulated under AS treatments. Therefore, the growth and reproduction of springtails were either increased or not affected due to AS exposure.

The ribosome, a complex organelle or ribonucleoprotein particle

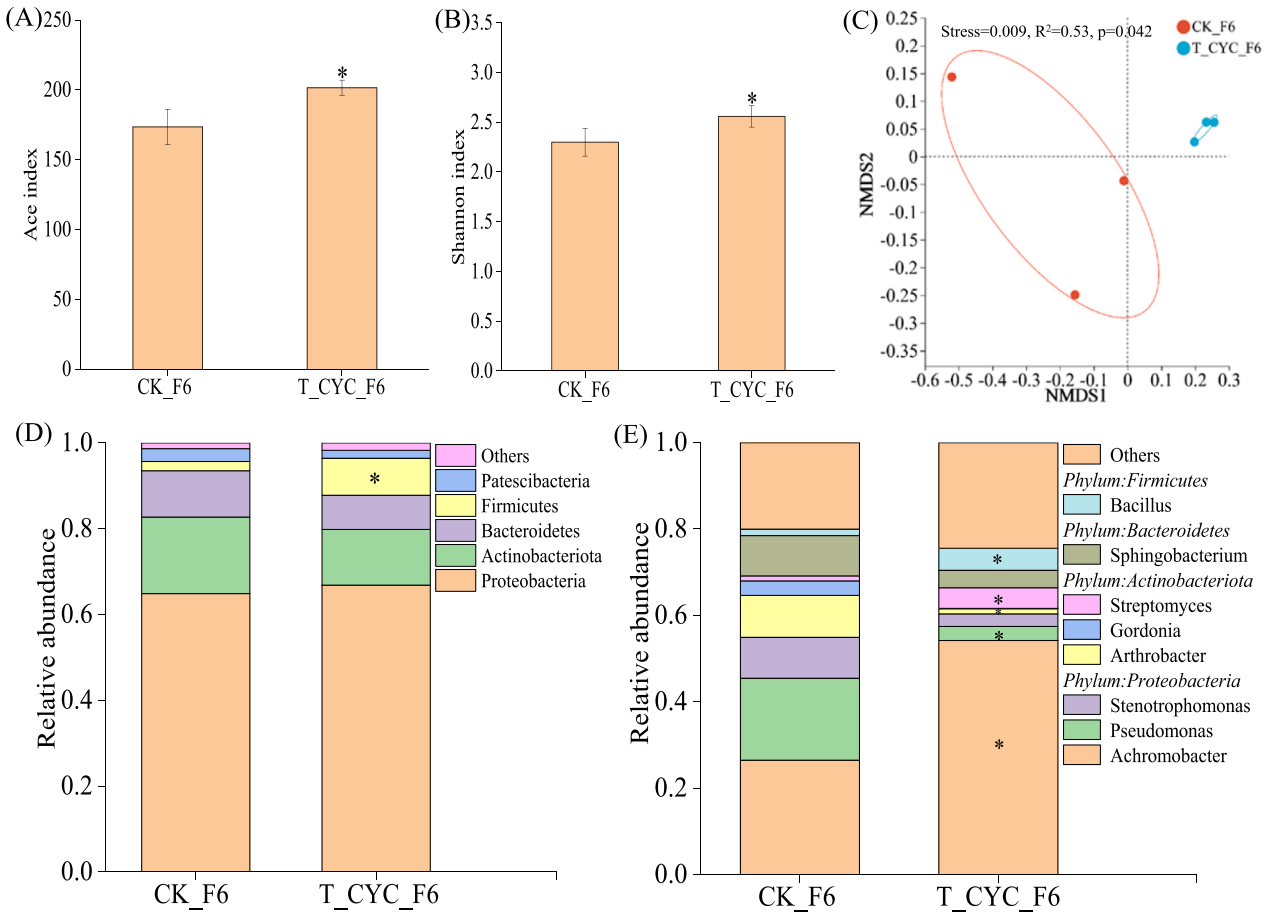

Fig. 6. Changes in Ace (A) and Shannon (B) indices of gut microbiota in springtails after the sixth-generation of exposure in cyclamate (CYC)-treated group (T_CYC_F6). Nonmetric multidimensional scaling (NMDS) plot of gut microbiota based on Bray − Curtis distances (C). Composition of gut microbiota at phylum (D) and genus (E) levels. The asterisk indicates significant differences ($p < 0.05$) from the control.

lacking a membrane coating, consists primarily of ribosomal RNA and various ribosomal proteins. Serving as the crucial nano-factories for protein synthesis, ribosomes are indispensable for the growth and development of living organisms through the process of translation (Liu et al., 2020). The lysosomes are subcellular membrane-delimited organelles widely present in eukaryotic cells, serving multiple functions such as maintaining intracellular homeostasis, facilitating cell signal transduction, and participating in macromolecule degradation (Liu et al., 2020; Yu et al., 2015). Liu et al. (2020) reported that exposure to trifloxystrobin and trifloxystrobin acid significantly decreased the weight of earthworms and also significantly affected ribosome and lysosome pathways. Li et al. (2015) reported that exposure to tris(1,3-dichloro-2-propyl) phosphate significantly changed the expression of genes involved in ribosome in *Daphnia magna*, which was responsible for the developmental and reproductive toxicity effects. In this study, the significantly influenced ribosome and lysosome pathways in springtails suggest their sensitivity to AS exposure. The disrupted functions of these organelles are likely associated with the observed effects of AS at the individual level.

Moreover, evaluating the responses of gut microbiota contributes to a comprehensive understanding of pollutant toxicity to organisms. The diversity and composition of the gut microbiome in springtails were significantly affected by AS treatment, with combined AS exposure exerting more pronounced effects on the dominant phyla (Proteobacteria and Bacteroidetes) compared to single exposures. Alterations in Proteobacteria are considered indicative of gut microbial dysbiosis and inflammation in response to pollution stress (Wang et al., 2019; Yu et al., 2020). Bacteroidetes is a highly diverse bacterial phylum, and its members possess the capability to degrade complex biopolymers, dietary polymers, and host-derived carbohydrates (Wang et al., 2019; Thomas et al., 2011). Therefore, the significant increase in Bacteroidetes in the springtail gut could associated with their ability to degrade AS. Similarly, Bian et al. (2017) reported that AS consumption can induce a higher abundance of Bacteroidetes in mice. Notably, the significant increases in the genus *Achromobacter* (belonging to the phylum Proteobacteria) were observed in all the single and combined AS treatment groups. This suggests its sensitivity and potential role as an indicator of AS stress. *Achromobacter* is associated with the degradation and metabolism of xenobiotic compounds. Similar enrichments have been observed in the gut microbiota of springtails exposed to the antiepileptic drug carbamazepine and in earthworms exposed to triclosan (Ma et al., 2017; Wang et al., 2020). Moreover, this genus was further found to significantly correlate with several DEGs shared in all the AS treatment groups and involved in glycolysis/gluconeogenesis and amino sugar and nucleotide sugar metabolism pathways (Table 1). These indicated that AS likely influenced key physiological processes of springtails by regulating the abundance of this genus, and the potential mechanism remained to be further elucidated. For example, mucin is highly glycosylated protein secreted by many types of epithelial cells that mediate interactions between cells and their external environment and have important implications for the growth and development of organisms (Zhao et al., 2020). The positive correlation between *Achromobacter* and DEGs encoding mucin likely contributed to the abnormal increase of springtails reproduction.

Note: only the DEGs that significantly correlated with *Achromobacter* ($p < 0.05$) were listed; the unannotated DEGs were not listed in the

Table 1
The relationship between gut genus Achromobacter and differentially expressed genes (DEGs) shared between all the different AS-treated groups in the springtails.

Gene name	Description	Involved KEGG pathways	r
LOC110842641	probable phosphoglycerate kinase	Glycolysis/ Gluconeogenesis; Carbon metabolism; Biosynthesis of amino acids	−0.47
LOC110846370	aldose reductase-related protein 2	Glycolysis/ Gluconeogenesis; Pentose and glucuronate interconversions; Glycerolipid metabolism	−0.78
LOC110847495	glyceraldehyde-3-phosphate dehydrogenase	Glycolysis/ Gluconeogenesis; Carbon metabolism; Biosynthesis of amino acids	0.32
LOC110854455	farnesoate epoxidase isoform X1	Insect hormone biosynthesis	0.57
LOC110842129	galactokinase	Amino sugar and nucleotide sugar metabolism; Galactose metabolism	0.74
LOC110843956	methyltransferase-like protein 7A	Amino sugar and nucleotide sugar metabolism	0.62
LOC110844324	GDP-L-fucose synthase	Amino sugar and nucleotide sugar metabolism; Fructose and mannose metabolism	0.38
LOC110846208	NADH-cytochrome b5 reductase 2	Amino sugar and nucleotide sugar metabolism	0.55
LOC110848339	mucin-5AC isoform X1	Amino sugar and nucleotide sugar metabolism	0.65
LOC110846331	integumentary mucin C.1	Amino sugar and nucleotide sugar metabolism	0.79
LOC110845108	probable chitinase 10 isoform X1	Amino sugar and nucleotide sugar metabolism	0.67
LOC110848073	probable chitinase 10	Amino sugar and nucleotide sugar metabolism	0.53

table.

4.2. *Effects of multi-generational exposure on CYC toxicity*

Exposure time is a crucial factor affecting the extent of toxicity of pollutants. The long-term exposure tests are, therefore, more ecologically relevant and contribute to a highly accurate assessment of toxicity (Campiche et al., 2007; Guimarães et al., 2019). In this study, we observed different individual-level responses in springtails after one- and multi-generational exposure to CYC. During the first-generational exposure to CYC, the weight of springtails did not show a significant change, but reproduction was significantly stimulated. In the case of multi-generational exposure, neither weight nor reproduction showed significant changes until the fifth-generational exposure, and reproduction decreased after the sixth-generational exposure. This finding underscores the importance of considering multi-generational exposure in toxicity tests, as it suggests that the potential effects of contaminants may be underestimated when only standard toxicity tests are employed.

After the sixth-generational exposure, the majority of significantly enriched KEGG pathways in springtails (e.g., ECM-receptor interaction, amino sugar and nucleotide sugar metabolism, lysosome, glycerolipid metabolism, and retinol metabolism) were found to be consistently affected, similar to the pathways enriched after the first-generational exposure to CYC. This suggests that these physiological processes were continually influenced by CYC across generations. The amino sugar and nucleotide sugar metabolism, as well as lysosome pathways, played indicative roles in responding to AS stress, as they were shared between single and combined AS-treated groups. However, it is important to note that most of the differentially expressed genes (DEGs) involved in these pathways, except ECM-receptor interaction, were upregulated after the first-generational exposure, while they were downregulated after the sixth-generational exposure. This contrasting regulation direction might contribute to the different phenotypic responses observed in springtails between the first and sixth-generational exposure groups.

The ECM-receptor interaction pathway was found to be the most significantly enriched in springtails after the sixth-generational exposure. The extracellular matrix (ECM) consists of a three-dimensional network of extracellular macromolecules synthesized by resident cells and secreted outside the cell through exocytosis. Interactions between cells and the ECM play a crucial role in intercellular information transmission, cell adhesion, and the regulation of cell functions (Bonnans et al., 2014; Theocharis et al., 2016). Zhu et al. (2021) demonstrated that the co-exposure of TiO_2 nanoparticles and tris(1,3-dichloro-2-propyl) phosphate significantly affected the growth of earthworms, with the disturbed ECM-receptor interaction identified as one of the potential mechanisms. In our study, the majority of differentially expressed genes (DEGs) involved in this pathway were downregulated. Therefore, prolonged exposure to CYC might lead to abnormal changes in the growth and reproduction of springtails by inhibiting this pathway.

Moreover, the longevity-regulating pathway-multiple species, associated with organismal aging, was the only pathway significantly enriched in springtails after the sixth-generational exposure to CYC compared to the first-generational exposure. This distinction was not observed in the other single and combined AS-treated groups after the first-generational exposure, further indicating specific effects of long-term exposure. Aging is a complex process resulting from cumulative molecular, cellular, and organ damage, leading to functional loss, increased vulnerability to disease, and eventual death. The longevity and aging processes are influenced by both genetic and environmental factors. Wang et al. found that the combined exposure to high temperature and abamectin significantly regulated this pathway in the pest *Liriomyza trifolii* (Wang et al., 2021). The number of upregulated and downregulated DEGs (e.g., encoding heat shock protein, catalase, alpha-crystallin chain) (Table S2) involved in this pathway were similar. This indicated that long-term exposure to CYC can disrupt the normal aging process and long-term exposure can be harmful to springtails.

Regarding the responses of gut microbiota, the effect of CYC on alpha diversity after multi-generational exposure was contrary to that after the first-generational exposure, aligning with the varied phenotypic responses of springtails. Furthermore, multi-generational exposure led to more pronounced disturbances in microbiota composition at both the phylum and genus levels compared to single-generational exposure. This underscores a potential long-term threat to soil springtails, emphasizing the importance of considering long-term exposure in the toxicity assessment of pollutants.

Following multi-generational exposure, there was a significant increase in the relative abundance of Firmicutes and the Firmicutes/Bacteroidetes (F/B) ratio in the gut of springtails (Fig. S3). Notably, Firmicutes and Bacteroidetes in the gut play an important role in the growth and physiology metabolism of animals (Wang et al., 2019). Previous study on human microbiota have shown that the F/B ratio is significantly higher in obese individuals than in lean individuals because an increase in the F/B ratio is associated with increased energy production through colonic fermentation and short-chain fatty acids (Turnbaugh et al., 2006). Yu et al. reported that a low concentration of sulfamethoxazole exerts an obesogenic effect on the fruit fly *Drosophila melanogaster*, which is accompanied by a significant increase in the F/B ratio in the gut (Yu et al., 2020). However, high concentrations of pollutants, such as norfloxacin, oxytetracycline, and arsenic, caused a decline in the body weight of soil springtails and earthworms and a significant decrease in the F/B ratio of the gut (Wang et al., 2019; Zhu et al., 2018). Therefore, prolonged exposure to low concentrations of CYC could potentially induce obesity in springtails by altering the gut F/B ratio. Furthermore, the significant increase in the genus *Achromobacter* persisted after multi-generational exposure, similar to the results observed in single and combined AS-treated groups after first-generational exposure. This emphasizes the continued effect on this

genus with long-term exposure and underscores its role as an indicator of gut microbial dysbiosis. Moreover, the increase amplitude of this genus abundance compared to control after the sixth-generational exposure (104.7 %) was much higher than that after the first-generational exposure (76.5 %). The more significant disturbance of this genus after long-term exposure may be a reflection of worse health status of springtails.

Altogether, our findings underscore the importance of considering the effects of AS on soil-dwelling invertebrates, such as springtails, which play important roles in vital soil ecosystem processes. Even at environmentally relevant concentrations, various AS showed comparable effects at both molecular and intestinal microbiota levels. Given the growing use of AS across various industries, the increasing occurrence of these substances in soil environments warrants sustained attention to their potential influence on soil-dwelling organisms in the years to come.

CRediT authorship contribution statement

Xiang-Long Lin: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fei Guo:** . **Matthias C. Rillig:** Writing – review & editing, Conceptualization. **Chun Chen:** Writing – review & editing. **Gui-Lan Duan:** . **Yong-Guan Zhu:** 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

The data that has been used is confidential.

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Appendix A. Supplementary data

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