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Effects of subchronic oral toxic metal exposure on the intestinal microbiota of mice

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ABSTRACT

Oral exposure to toxic metals such as cadmium (Cd), lead (Pb), copper (Cu) and aluminum (Al) can induce various adverse health effects in humans and animals. However, the effects of these metals on the gut microbiota have received limited attention. The present study demonstrated that long-term toxic metal exposure altered the intestinal microbiota of mice in a metal-specific and time-dependent manner. Subchronic oral Cu exposure for eight weeks caused a profound decline in gut microbial diversity in mice, whereas no significant changes were observed in groups treated with other metals. Cd exposure significantly increased the relative abundances of organisms from the genera *Alistipes* and *Odoribacter* and caused marked decreases in *Mollicutes* and unclassified *Ruminococcaceae*. Pb exposure significantly decreased the abundances of eight genera: unclassified and uncultured *Ruminococcaceae*, unclassified *Lachnospiraceae*, *Ruminiclostridium_9*, *Rikenellaceae_RC9_gut_group*, *Oscillibacter*, *Anaerotruncus* and *Lachnoclostridium*. Cu exposure affected abundances of the genera *Alistipes*, *Bacteroides*, *Ruminococcaceae_UCG-014*, *Allobaculum*, *Mollicutes_RF9_norank*, *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_unclassified* and *Turicibacter*. Al exposure increased the abundance of *Odoribacter* and decreased that of *Anaerotruncus*. Exposure to any metal for eight weeks significantly decreased the abundance of *Akkermansia*. These results provide a new understanding regarding the role of toxic metals in the pathogenesis of intestinal and systemic disorders in the host within the gut microbiota framework.

1. Introduction

In recent years, industrial progress has been accompanied by frequent metal pollution incidents, leading to significant public health concerns. Cadmium (Cd) and lead (Pb) are non-essential heavy metals that can adversely affect the liver, kidney, brain, blood and reproductive system [1,2]. Copper (Cu) and aluminum (Al) have also been reported to be toxic to humans and animals at doses exceeding safe limits [3,4]. The general public is exposed to these metals through ambient air, drinking water, food, industrial materials and consumer products [2,5]. Following oral ingestion, the metals are absorbed in the gut and thus accumulate in the host. Accordingly, the intestinal tract is the first organ susceptible to toxic metals [2]. Oral Cd, Pb, Cu and Al exposure can induce inflammation, epithelial cell death and tight junctions dysfunction in the intestines, leading to disruption of the intestinal barrier and increased metal absorption [6–9].

Although the underlying the toxic effects of these metals have been well addressed in different organs (e.g., liver, kidney, gut and brain), their effects on the gut microbiota have received much less attention. The gut microbiota is considered a "forgotten organ" of the host, although it provides beneficial functions such as the fermentation of unused energy substrates, regulation of the immune system, and prevention of pathogenic bacterial growth [10–12]. Many studies have shown that Cd, Pb, Cu and Al are toxic to various intestinal microorganisms because they disrupt metal metabolism and induce oxidative stress in the bacterial cell [13–15]. Taking the adverse effects of these metals on intestines into consideration as well, the gut microbiota, which lives in symbiosis with intestinal epithelial cells, could be affected by oral exposure to toxic metals. In mice, exposure to Cd has been reported to induce sharp decreases in the populations of representative intestinal microbial species and a reduction in the abundance of total intestinal bacteria [16,17]. High levels of dietary Pb and Cu exposure were also found to induce gut microbiota dysbiosis in rats,

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piglets and humans [18–20]. Furthermore, the gut microbiota likely plays an important role in regulating the bioavailability and toxicity of these metals. Notably, a recent study revealed that germ-free mice are more susceptible to Cd and Pb, compared to conventional mice [21].

To the best of our knowledge, the effects of toxic metal exposure on the gut microbiota are not currently well understood. Several related studies have used culture-based and polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis technologies to evaluate the effects of Cd, Pb and Cu on the gut microbiota of animals [16–19]. However, these investigations could have been improved by microbiome analyses based on Illumina high-throughput platforms, which provide more extensive and detailed information about the structure and diversity of the gut microbiota [22]. Moreover, those previous reports focused only on acute metal exposure and occasionally used relatively high metal doses. Further studies are needed to assess the effects of long-term exposure to environmentally relevant concentrations of metal on the gut microbiota. We also noticed that no previous studies investigated the relationship between Al exposure and the gut microbiota. Given the status of this metal as one of the most abundant elements in the earth's crust and its wide usage in daily life [23], the effect of oral Al exposure on intestinal ecology should be investigated.

Therefore, in this study, we evaluated the effects of subchronic oral exposure to Cd, Pb, Cu and Al on the gut microbiota of mice through a microbiome analysis with the aim to further understand the toxic mechanisms of these metals in the host.

2. Materials and methods

2.1. Chemicals

All of the analytical laboratory chemicals and reagents were purchased from Sinopharm Chemical Reagent Company (Shanghai, China).

2.2. Animals and experimental design

Adult male C57black/6 mice (6 weeks of age on arrival) were obtained from the Shanghai Laboratory Animal Centre (Shanghai, China) for use in these experiments. Mice were kept in cages in a temperature- and humidity-controlled room equipped to maintain a 12-h light/dark cycle. The mice were fed standard commercial chow, and water was provided *ad libitum*. All of the study protocols were approved by the Ethics Committee of Jiangnan University, China (JN No. 20150721-1030-51-2). All of the procedures were performed in accordance with the European Community guidelines (Directive 2010/63/EU) for the care and use of experimental animals. All of the applicable institutional and/or national guidelines for the care and use of animals were followed.

The mice were divided randomly into five groups of 10 mice each. Group 1 served as the toxic metal-free control, and the mice in this group received distilled drinking water with no added Cd, Pb Cu and Al. Group 2 was the Cd-treated group, and the mice received 100 mg/L $CdCl_2$ in their drinking water. Group 3 was the Pb-treated group, and the mice received 1.83 g/L $(CH_3COO)_2Pb{\cdot}3H_2O$ in their drinking water. Group 4 was the Cu-treated group, and the mice received 1.00 g/L $CuSO_4{\cdot}5H_2O$ in their drinking water. Group 5 was the Al-treated group, and the mice received 1.80 g/L $AlCl_3{\cdot}6H_2O$ in their drinking water. The mice were exposed to these metals for 8 weeks. Based on previous studies and our preliminary experiments, the metal doses provided in drinking water were selected to mimic environmentally relevant chronic toxic metal exposure according to the respective "lowest observed adverse effect" levels [24–29]. Our preliminary experiment showed that the consumption of $CH_3COONa{\cdot}3H_2O$, NaCl or Na_2SO_4 in drinking water did not significantly change the gut microbiota of the mice; therefore, the effects of chloride, acetate and sulfate could be excluded from the present study.

At the beginning of the experiment (before metal exposure, indicated as T0) and after 4 (T1) and 8 weeks (T2) of metal exposure, each mouse was transferred into a fresh, sterilized cage and fecal samples were collected from the cages within 1 h. The samples were immediately stored at 4 °C or at −80 °C for long-term storage.

2.3. DNA extraction and sequence data processing

Microbial genomic DNA was extracted from the fecal samples using the E.Z.N.A.® DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The V4-V5 region of 16S rRNA was amplified by PCR from microbial genomic DNA using barcoded fusion primers. The PCR conditions were 95 °C for 3 min; 35 cycles at 95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s; and a final extension at 72 °C for 10 min. Amplicons were separated on 2% agarose gels, extracted and purified using AxyPrep DNA Gel Extraction Kits (Axygen Biosciences, Union City, CA, USA) per the manufacturer's protocols. Purified amplicons were quantified using QuantiFluor™ -ST (Promega, Madison, WI, USA) and subsequently pooled in equimolar amounts and paired-end sequenced (2×250) on an Illumina MiSeq platform using standard protocols.

2.4. Data processing and analysis

Several raw fastq files were obtained after sequencing; these were de-multiplexed and quality-filtered using QIIME (version 1.17) with the following three criteria. First, all 300-base pair (bp) reads were quality-scored; any reads with scores <20 were discarded, as were truncated reads shorter than 50 bp. Second, barcodes were matched, and ambiguous and/or unmatched contained reads were removed. Third, only sequences with overlaps longer than 10 bp were assembled according to their overlap sequences. Reads that could not be assembled were discarded. Operational Taxonomic Units (OTUs) were clustered using UPARSE (version 7.1 http://drive5.com/uparse/) with a cutoff of 97% similarity, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed using RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU115)16S rRNA database, using a confidence threshold of 70%. Core OTUs were selected according to a relative abundance standard >0.05%. The sequence data reported in this study have been deposited in the NCBI BioProject database (PRJNA356482).

The alpha and beta diversities of the sequence were calculated using the QIIME pipeline (version 1.17). Sequencing depth and biodiversity richness were accessed using the Chao1, Observed_otus and Shannon-Wiener indexes. The principal coordinate analysis (PCoA) was derived using the Bray-Curtis distance, and jackknifed hierarchical clustering was performed using an unweighted-pair group method with arithmetic mean (UPGMA).

2.5. Statistics

SPSS (version 22.0; SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Comparisons between the relative abundances of grouped samples were calculated pair-wise using the Mann–Whitney algorithm. Significance was defined as a P value <0.05.

3. Results

3.1. Sequencing coverage and estimation of bacterial diversity

After quality control, a dataset comprising 5,723,310 valid 16S rRNA reads was obtained through a Miseq sequencing analysis of the 150 samples. Each sample was covered by an average of 38,155 reads, and the number of OTUs varied between 11,549 and 39,034. The rarefaction curves of all samples plateaued with increased sequencing depth (Fig. S1 online), indicating that the analysis had already covered most of the microbial diversity.

3.2. Time-dependent alterations in the gut microbiota of mice after oral metal exposure

Time-dependent changes in the microbiota were observed in both the Pb- and Cu- treated groups (Fig. 1). It is noteworthy that significant alterations occurred in the former group within the first 4 weeks, whereas those in the latter occurred during the last 4 weeks. During the experimental period, microbial diversity was not markedly affected after Cd, Pb and Al treatments for either 4 or 8 weeks (Table S1 online). In contrast, Cu exposure for 8 weeks induced a significant decrease ($P < 0.05$) in gut microbiota diversity, characterized by reduced Chao1, observed OTU, and Shannon-Wiener index values (Fig. 2).

Regarding phylum-level changes in the microbiota, significant disturbances in the populations of primary phyla, such as *Proteobacteria* or *Actinobacteria*, were observed in metal-treated groups (Fig. 3a and Table S2 online). Alterations in the microbiota of Cd- and Pb-exposed mice were more drastic in the first 4 weeks, compared to the latter 4 weeks. In contrast, the main phylum-level changes associated with Cu exposure occurred during the latter 4-week period. Al treatment had completely different effects on microbial phyla between the two exposure periods.

The genus-level changes in the microbiota during the 8-week experiment period were shown in Fig. 3b and Table 1. We observed complex microbial up-regulation and down-regulation during the first 4-week period. However, during the latter period, we mainly observed downtrends in microbial abundance, with significant decreases in 2, 6, 8 and 5 core OTUs in the Cd-, Pb-, Cu- and Al-treated groups, respectively ($P < 0.05$).

3.3. Metal-specific alterations in the gut microbiota of mice after subchronic oral exposure

A PCoA based on the Bray-Curtis distances was performed to compare gut microbiota among different metal-treated groups after 8 weeks of exposure (Fig. 4a). In each group, the microbiota tended to cluster and separate from the control group microbiota. Among the four metal-treated groups, Cu exposure induced the most significant change in gut microbiota, which was further confirmed by a UPGMA cluster analysis (Fig. 4b).

At the phylum level, the mouse microbiota was dominated by *Bacteroidetes* (63.70%–72.62%) and *Firmicutes* (25.10%–31.96%) after an 8-week metal exposure (Fig. 5 and Table S3 online). Compared with the metal-free control group, all metal treatments significantly decreased the abundances of some prevalent phyla, and no marked increases were observed in certain phyla. To be specific, significant decreases were observed in the relative abundances of *Tenericutes* and *Verrucomicrobia*: from 1.30% and 0.12% in the control group to 0.47% and 0.00% in the Cd-treated group, respectively (Mann–Whitney *U* test, $P = 0.007$ and 0.000, respectively). The relative abundances of *Proteobacteria* and *Verrucomicrobia* decreased significantly from 2.41% and 0.12% to 0.78% and 0.01% after Pb exposure, respectively (Mann-Whitney *U* test, $P = 0.035$ and 0.009, respectively). Cu treatment led to significant decreases in the relative abundances of three phyla, *Tenericutes, Actinobacteria* and *Verrucomicrobia*, whereas Al exposure led to decreases in *Actinobacteria* and *Verrucomicrobia*.

Regarding genus level of microbiota at the time point of the 8th week, *Bacteroidales_S24-7_group_norank* and *Lachnospiraceae_NK4A136_group* were the dominant genera in all analyzed mice

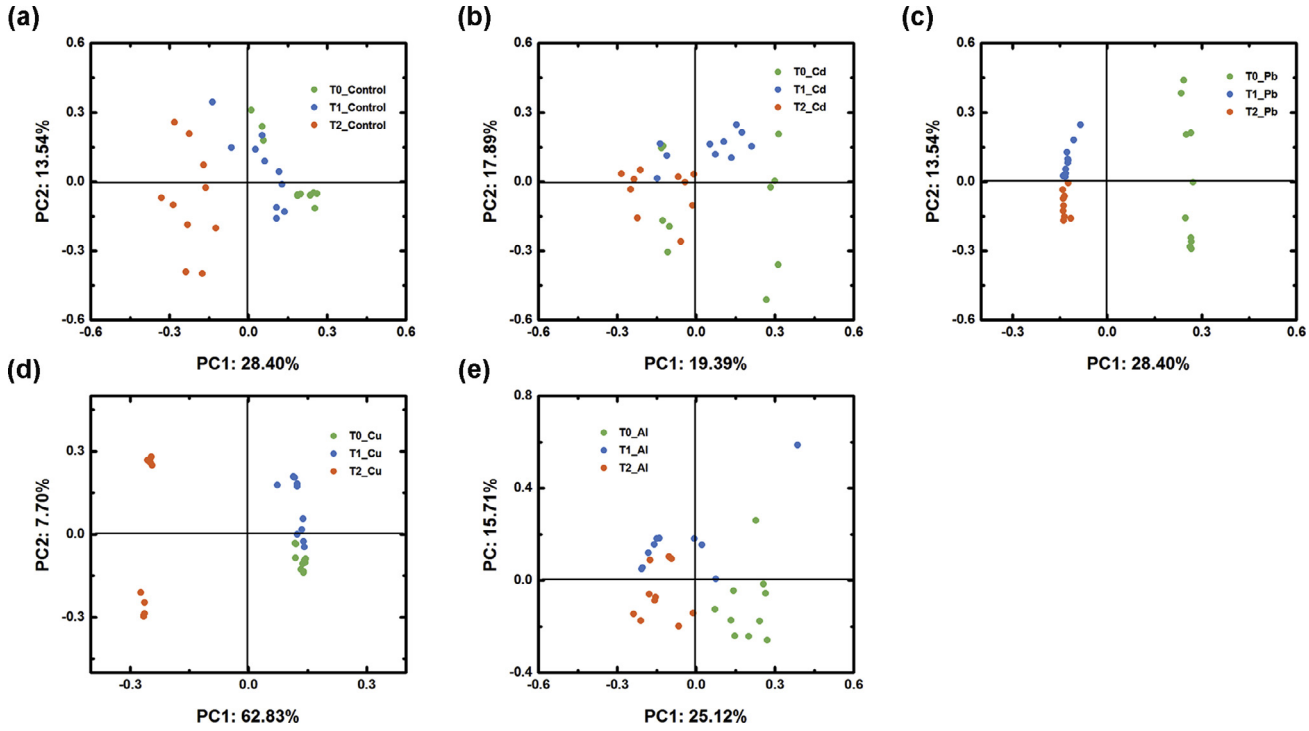

Fig. 1. Principal coordinate analysis (PCoA) of the fecal bacterial compositions of mice during the experimental period. (a) PCoA of the Control group at T0 (baseline), T1 (4-week exposure) and T2 (8-week exposure). (b) PCoA of the Cd-treated group at T0, T1 and T2. (c) PCoA of the Pb-treated group at T0, T1 and T2. (d) PCoA of the Cu-treated group at T0, T1 and T2. (e) PCoA of the Al-treated group at T0, T1 and T2.

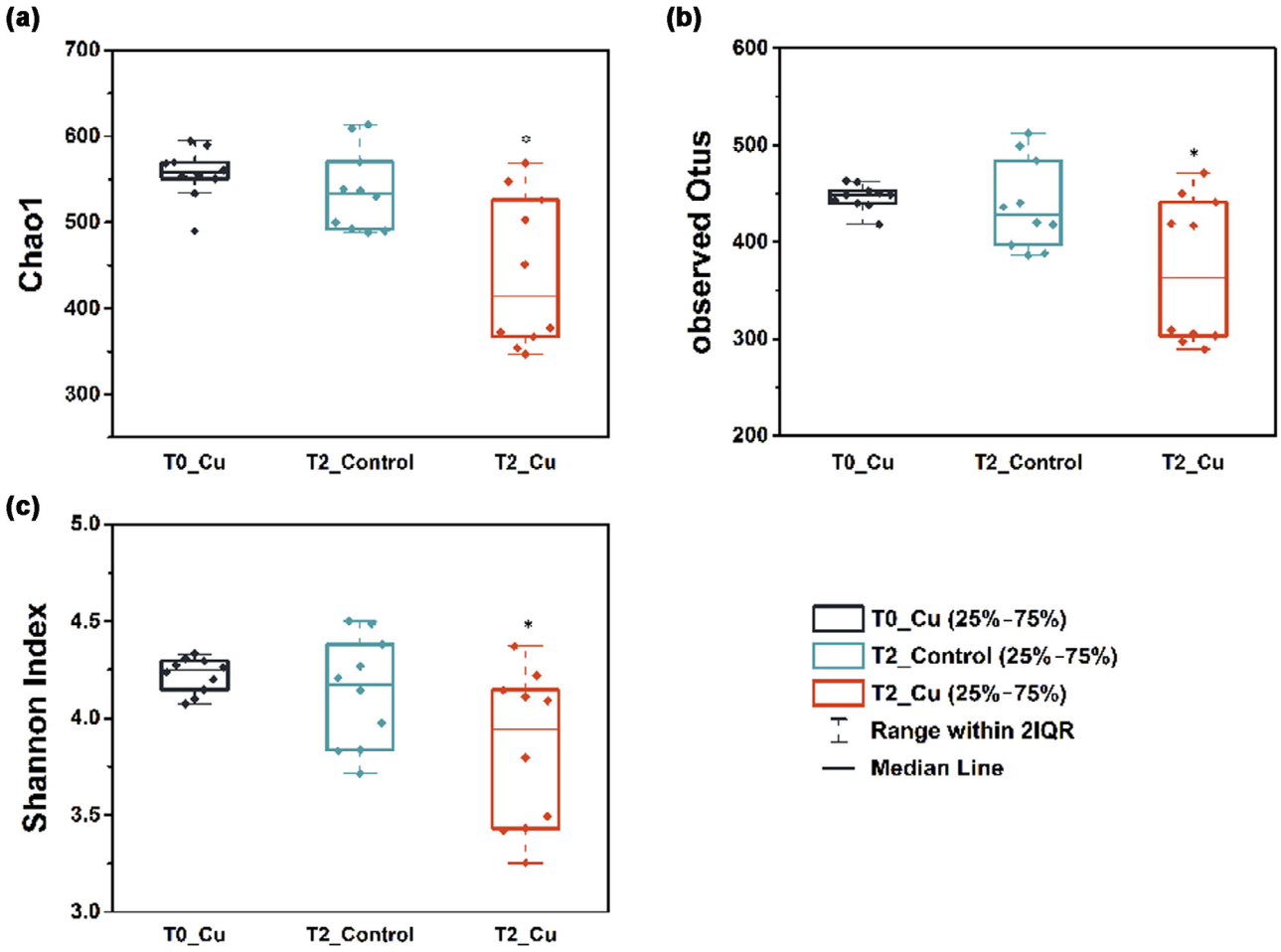

Fig. 2. Biodiversity and richness of sequenced bacterial communities in the gut microbiota of Cu-treated mice. (a) Chao1 comparisons among Cu-treated mice at baseline (T0_Cu) and control and Cu-treated mice after 8 weeks (T2_control and T2_Cu, respectively). (b) Comparisons of observed operational units (OTUs) among T0_Cu, T2_control and T2_Cu mice. (c) Shannon–Wiener index comparisons among T0_Cu, T2_control and T2_Cu mice. Asterisks indicates significant differences ($P < 0.05$) in comparisons of T0_Cu vs. T2_Cu and T2_control vs. T2_Cu.

gut microbiota datasets, and their relative abundances were not significantly affected by metal exposure. On average, the third-most dominant genus was *Alistipes*, the abundance of which was increased by Cd and Cu treatment ($P < 0.05$). Other significantly altered ($P < 0.05$) core OTUs in the various metal-treated groups are shown in Fig. 6: Cd exposure significantly increased the relative abundances of organisms from the geneus *Odoribacter* and caused marked decreases in *Mollicutes* and unclassified *Ruminococcaceae*. Pb exposure significantly decreased the abundances of eight genera: unclassified and uncultured *Ruminococcaceae*, unclassified *Lachnospiraceae*, *Ruminiclostridium_9*, *Rikenellaceae_RC9_gut_group*, *Oscillibacter*, *Anaerotruncus* and *Lachnoclostridium*. Cu exposure affected abundances of the genera *Bacteroides*, *Ruminococcaceae_UCG-014*, *Allobaculum*, *Mollicutes_RF9_norank*, *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_unclassified* and *Turicibacter*. Al exposure increased the abundance of *Odoribacter* and decreased that of *Anaerotruncus*. We also noticed a marked decrease in the abundance of *Akkermansia* after exposure to any metal.

4. Discussion and conclusion

Although numerous studies have focused on the toxic effects of Cd, Pb, Cu and Al on various host organs, such as the liver, kidneys and reproductive system [1–4], the effects of these metals on intestinal microflora have not been thoroughly studied. The present study demonstrated that 8-week toxic metal exposure altered the intestinal microbiota of mice in a metal-specific and time-dependent manner.

During the 8-week exposure period, continuous changes in the intestinal microbiota were observed in each metal exposure group. We further observed that different metals had varying effects on gut microbiota during this period (Fig. 3), and observed different clustering patterns among the four metal-treated groups at the end of the 8-week treatment (Fig. 4). Compared with the control group, subchronic oral Cu exposure caused a profound decline in gut microbial diversity, whereas no significant changes were observed in other metal-treated mice (Fig. 2 and Table S1 online). Consistent with our results, previous studies revealed that Pb and Cd exposure at environmentally-relevant low doses could alter the abundance of certain bacterial strains in the gut of mice, but may not induce significant effects on the global microbial diversity [16,17,19]. It is also noteworthy that very few studies can confirm the relationship between Al exposure and the gut microbiota. We will carry on a further study to investigate the dose-related effects of different metals on gut microbial diversity. However, at the end of the experiment, all metal treatments were found to have significantly decreased the population of the *Verrucomicrobia* phylum and to have respectively reduced the abundances of other prevalent phyla (Fig. 5 and Table S3 online). Previous reports of axenic mice models demonstrated that the gut microbiota plays a role in limiting intestinal heavy metal absorption [21]. Disruptions in

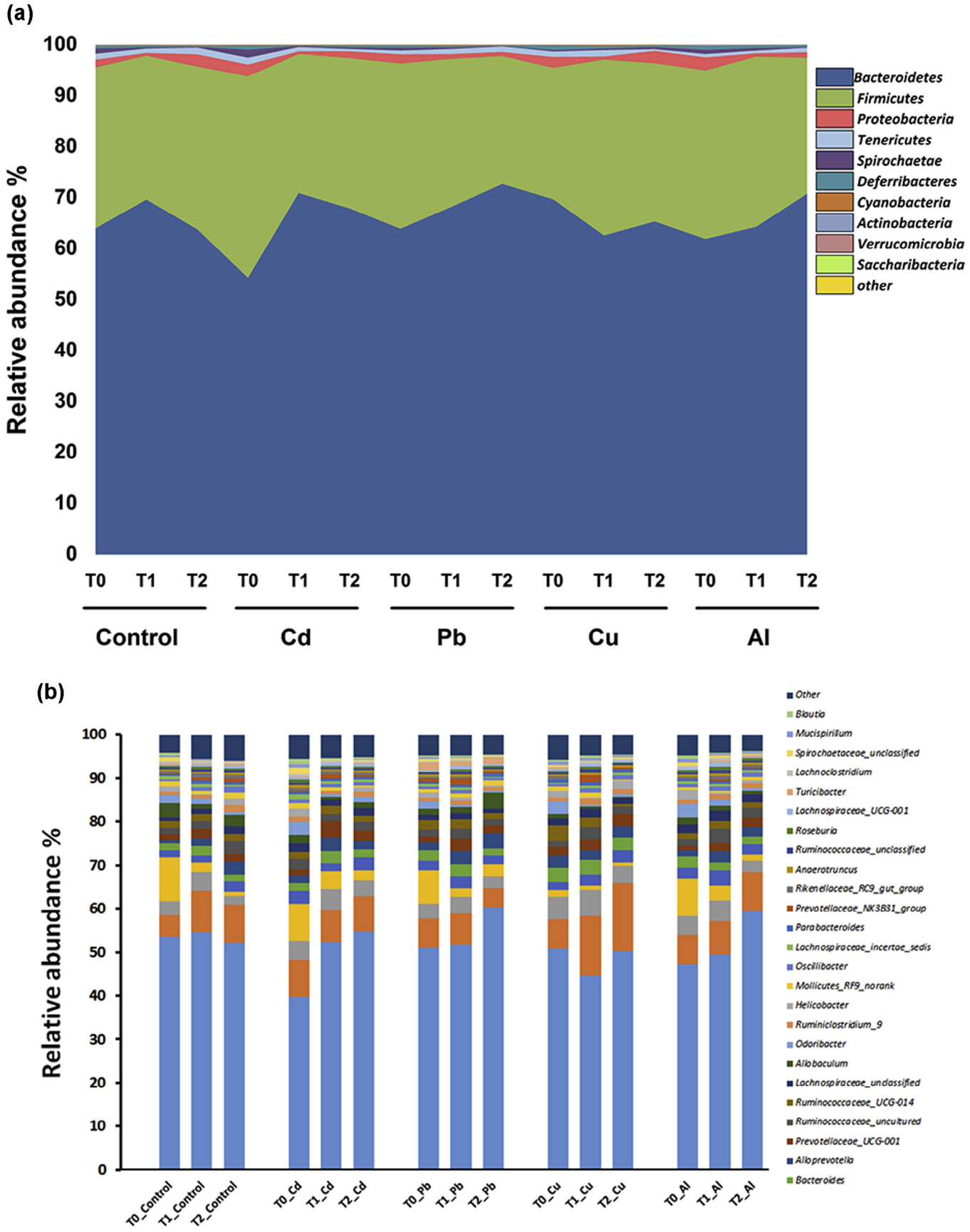

Fig. 3. Changes in the relative abundances of the most abundant phyla (a) and genera (b) in mice during the experimental period.

Table 1
Genus-level changes in the intestinal microbiota of mice during the experimental period.

Group	Comparison[a]	Implicated microbial genera	
		Relatively increased	Relatively decreased
Control	T1 vs. T0	*Alloprevotella* ↑ *Lachnoclostridium*↑	*Lactobacillus* ↓ *Allobaculum* ↓ *Helicobacter* ↓ *Spirochaetaceae_unclassified* ↓ *Blautia* ↓
	T2 vs. T1	*Ruminiclostridium_9* ↑ *Helicobacter* ↑ *Mollicutes_RF9_norank* ↑ *Oscillibacter* ↑	*Lachnospiraceae_incertae_sedis*↓
Cd	T1 vs. T0	*Bacteroidales_S24-7_group_norank* ↑ *Bacteroides* ↑ *Prevotellaceae_UCG-001* ↑ *Prevotellaceae_NK3B31_group* ↑	*Allobaculum* ↓ *Odoribacter* ↓ *Helicobacter* ↓
	T2 vs. T1	*Lactobacillus* ↑ *Allobaculum* ↑ *Parabacteroides* ↑	*Ruminiclostridium_9* ↓ *Helicobacter* ↓
Pb	T1 vs. T0	*Prevotellaceae_UCG-001* ↑ *Parabacteroides* ↑ *Rikenellaceae_RC9_gut_group* ↑	*Lactobacillus* ↓ *Odoribacter* ↓
	T2 vs. T1		*Bacteroides* ↓ *Lachnospiraceae_incertae_sedis* ↓ *Prevotellaceae_NK3B31_group* ↓ *Rikenellaceae_RC9_gut_group* ↓ *Ruminococcaceae_unclassified* ↓ *Spirochaetaceae_unclassified* ↓
Cu	T1 vs. T0	*Lachnospiraceae_NK4A136_group* ↑ *Ruminiclostridium_9* ↑ *Lachnospiraceae_incertae_sedis* ↑ *Prevotellaceae_NK3B31_group* ↑ *Anaerotruncus* ↑ *Ruminococcaceae_unclassified* ↑	*Ruminococcaceae_UCG-014* ↓ *Odoribacter* ↓ *Rikenellaceae_RC9_gut_group* ↓
	T2 vs. T1		*Ruminococcaceae_UCG-014* ↓ *Lachnospiraceae_unclassified* ↓ *Allobaculum* ↓ *Helicobacter* ↓ *Mollicutes_RF9_norank* ↓ *Prevotellaceae_NK3B31_group* ↓ *Ruminococcaceae_unclassified* ↓ *Turicibacter* ↓
Al	T1 vs. T0	*Prevotellaceae_UCG-001* ↑ *Prevotellaceae_NK3B31_group* ↑ *Ruminococcaceae_unclassified* ↑	*Lactobacillus* ↓ *Odoribacter* ↓ *Helicobacter* ↓ *Blautia* ↓
	T2 vs. T1		*Alistipes* ↓ *Odoribacter* ↓ *Ruminococcaceae_unclassified* ↓ *Lachnoclostridium* ↓ *Spirochaetaceae_unclassified* ↓

[a] Comparison of condition A vs. condition B: ↑, significant increase in condition A relative to condition B (Mann–Whitney *U* test, $P < 0.05$); ↓, significant decrease in condition A relative to condition B (Mann–Whitney *U* test, $P < 0.05$).

the balance of these phyla might amplify the gut absorption of these metals and further exacerbate toxicity. Microbial disturbances were also observed in the control group during the experimental period, which may be due to an age-dependent structural rearrangement of gut microbiota of mice [30,31].

At the genus level (Fig. 6), 8-week Cd exposure led to significantly increases in the relative abundances of *Alistipes* and *Odoribacter* ($P < 0.05$), two common populations in normal human and animal intestinal microbiota [32]. The former genus has been reported to associate with frail, less healthy subjects [33] and those with major depressive disorder [34]. However, recent studies have also shown that *Alistipes* may be candidate for the prevention and cure of intestinal disorders, including colorectal cancer and *Clostridium difficile* infection [35,36]. We therefore hypothesized that the increase in *Alistipes* might represent a self-adaption of the host to cope with adverse health effects (e.g., toxic metal exposure). Moreover, *Alistipes* strains have been reported to survive in heavy metal-containing wastewater [37,38], suggesting that these organisms exhibit superior metal resistance relative to other intestinal microorganisms. Accordingly, these strains may more readily survive in the Cd-exposed intestinal environment. Previous studies have correlated an abundance of *Odoribacter* strains inversely with inflammatory bowel disease (IBD) but positively with colorectal cancer [39,40]. Further studies are needed to determine the role of these microbes in Cd-induced intestinal disorders. In contrast, subchronic Cd exposure significantly reduced the abundances of *Mollicutes* and unclassified *Ruminococcaceae*. The *Mollicutes* class has been linked specifically to diet-induced obesity [41]. These strains, which lack a cell wall, may be very sensitive to metal stress [42], and a recent study confirmed a significant decrease in the abundance of *Mollicutes* in heavy metal-contaminated soil [43]. The *Ruminococcaceae* family is one of the

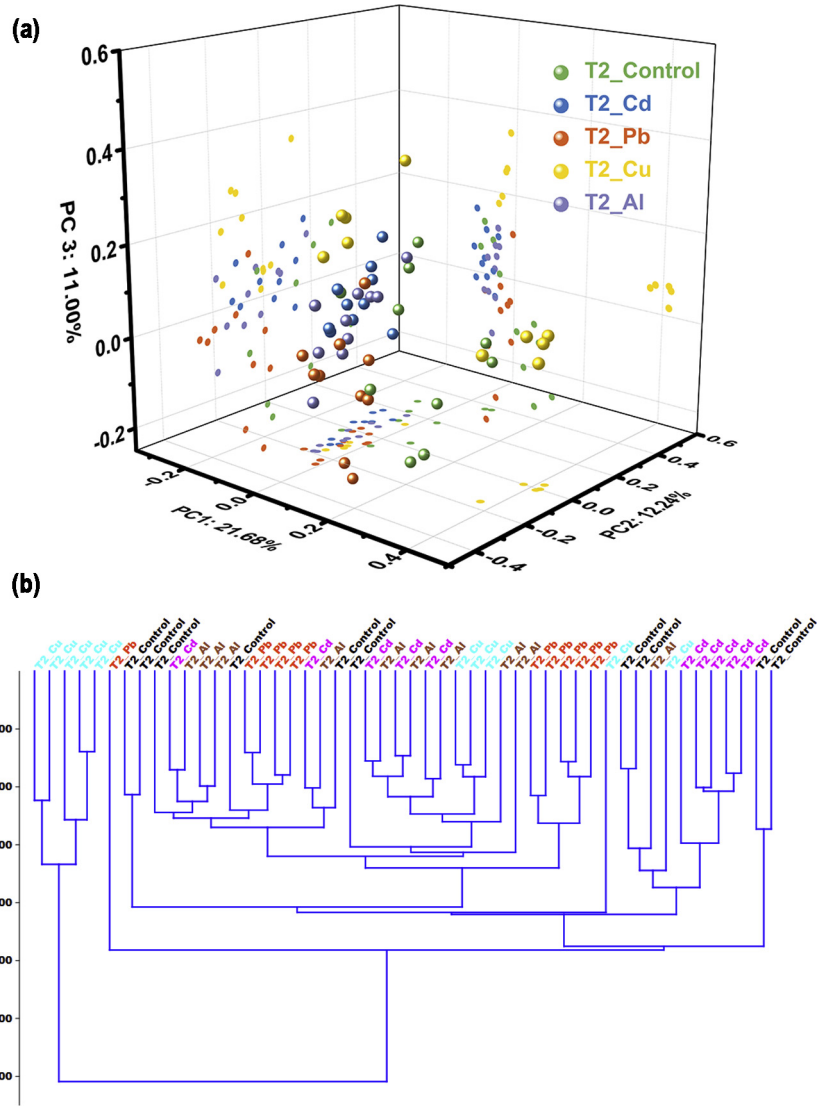

Fig. 4. Three-dimensional (3D)-principle coordinate analysis (PCoA) (a) and unweighted-pair group method with arithmetic mean (UPGMA) cluster analysis (b) of fecal bacterial communities in mice after an 8-week oral metal exposure period.

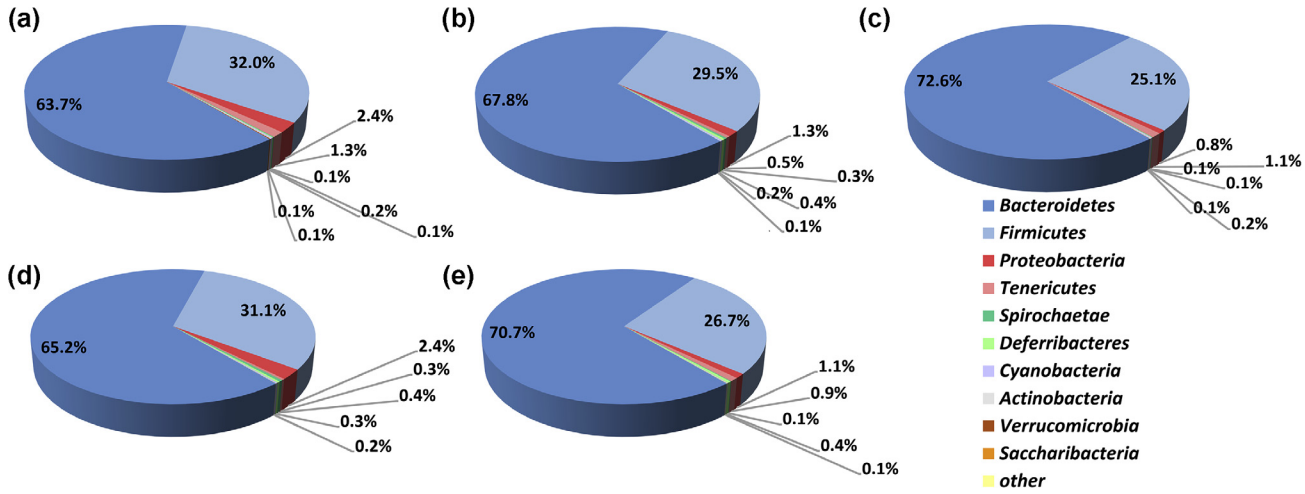

Fig. 5. Relative abundances of bacterial phyla in fecal samples of mice after an 8-week oral metal exposure period. (a) Control group. (b) Cd-treated group. (c) Pb-treated group. (d) Cu-treated group. (e) Al-treated group.

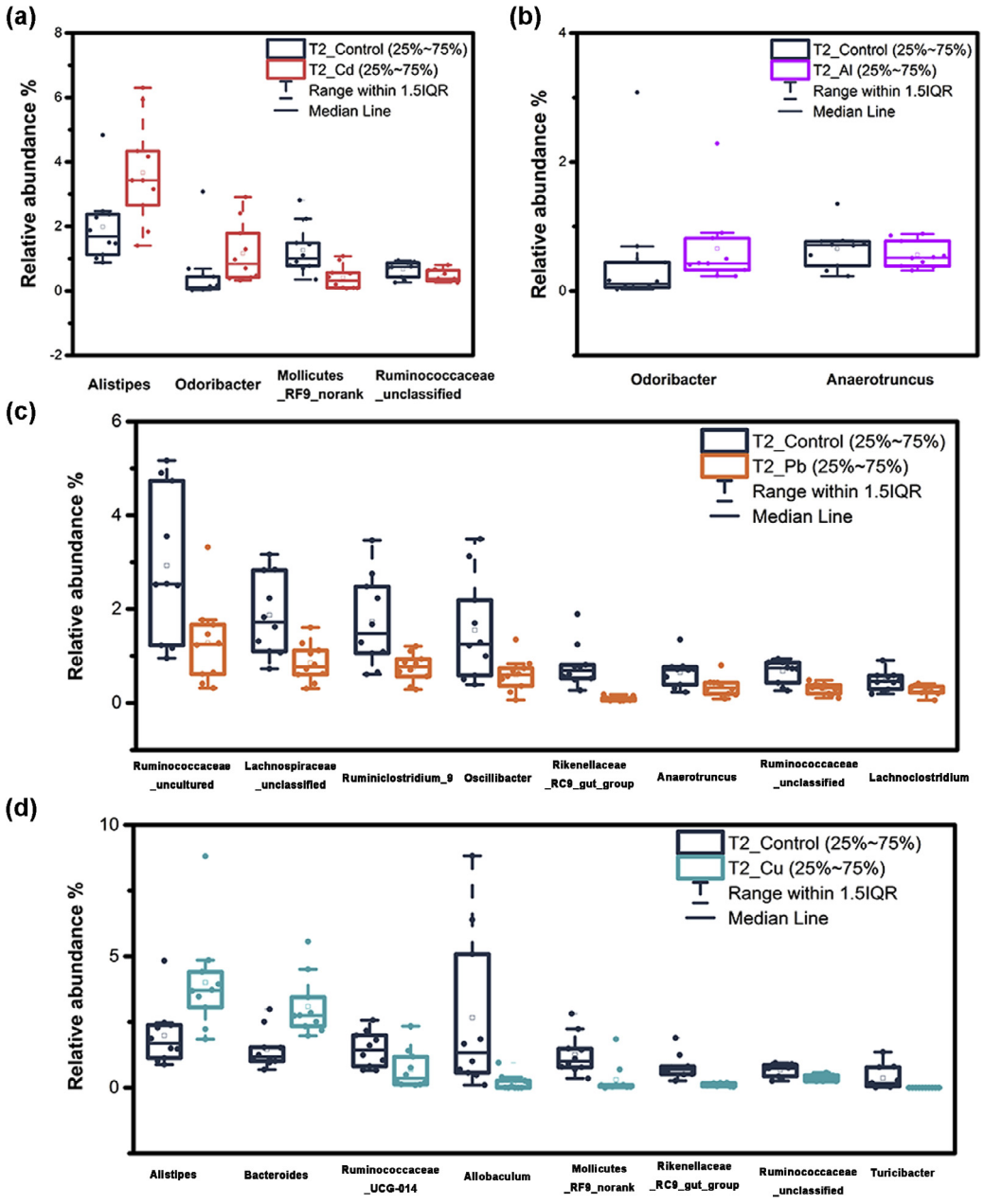

Fig. 6. (Color online) Relative abundances of significantly changed genera (core OTUs) after the 8-week oral metal exposure period. (a) Control vs. Cd-treated mice. (b) Control vs. Al-treated mice. (c) Control vs. Pb-treated mice. (d) Control vs. Cu-treated mice. Significant change was defined as a difference with a P value <0.05 (Mann-Whitney U test).

main mucosa-associated microbial populations in the human and mouse colon [44]. Consistent with our results, these strains (including unclassified *Ruminococcaceae*) are present at reduced levels in patients with non-alcoholic fatty liver disease, *Clostridium difficile* infection and IBD [45–47]. A recent study also confirmed significant decreases in the abundance of uncultured *Ruminococcaceae* in feces collected from *Peromyscus maniculatus* heavy metal-contaminated areas [48].

Compared with the control group, Pb exposure caused significant reductions in eight core OTUs at the end of the experimental period, whereas no marked upregulation was observed in any genera. Like Cd exposure, Pb treatment reduced the abundance of *Ruminococcaceae* family strains (uncultured and unclassified members), which may lead to further intestinal disorders. *Lachnospiraceae* is a dominant population in the human gut, and its abundance is associated with good health [49,50]. The *Rikenellaceae* genus could be considered an indicator of inflammation [28,50]. Consistent with our results, a previous study of mice indicated lower abundances of *Lachnospiraceae* and *Rikenellaceae* after heavy metal exposure [28]. The researchers hypothesized that these alterations correlated with intestinal inflammation and colitis [51]. Other studies have identified *Oscillibacter* strains as valeric acid-producing bacteria and noted a potential inverse correlation of their abundance with gut dysfunction (e.g., diarrhea) [52,53]. *Anaerotruncus* strains express enzymes required for butyrate production [54], and *Lachnoclostridium* strains exhibiting bile acid dehydroxylation activity play a role in the production of secondary bile acids [55]. Furthermore, significant decreases in the abundance of *Ruminoclostridium* strains have been reported in patients with IBD [56]. Reductions in these bacterial populations in Pb-exposed mice might indicate microbial dysbiosis and gut dysfunction.

As in Cd- or Pb-exposed mice, Cu-exposed mice also exhibited an increase in *Alistipes* strains and decreases in *Ruminococcaceae*, *Mollicutes* and *Rikenellaceae* strains after the 8-week exposure

period. Cu exposure also caused a significant increase in the *Bacteroides* population, a predominant genus in humans and other animals [57]. Excessive up-regulation of this genus has been associated with high risks of colon cancer and Crohn's disease [58,59]. Changes in these bacteria might explain some previously reported Cu-induced intestinal disorders [8]. Reductions in the *Allobaculum* and *Turicibacter* populations in Cu-exposed mice might indicate abnormal gut immune responses, given previous reports describing the reduced abundances of these bacteria in response to the induction of intestinal inflammation [60–62]. Furthermore, Al exposure led to an increase in *Odoribacter* and a decrease in *Anaerotruncus* strains. The potential effects of these alterations were discussed in the Cd and Pb exposure segments.

It is worth mentioning that exposure to all metals for 8 weeks led to significantly decreases in the abundance of *Akkermansia* ($P < 0.05$). This bacterial genus was recently shown to be universally distributed in the intestines of humans and other mammals [63,64]. These organisms are believed to play an immunoregulatory or anti-inflammatory role [65,66], and their abundance was found to correlate inversely with IBD severity [67]. These microbes might also enhance the repair of intestinal mucosal wounds and induce the expression of tight junction proteins [68,69], thus protecting the gut barrier and reducing gut permeability. Accordingly, a heavy metal-induced reduction in *Akkermansia* strains might contribute to subsequent intestinal inflammation and lesion formation.

In summary, this study demonstrated the effects of subchronic oral toxic metal exposure on the gut microbiota of mice. Cd, Pb, Cu and Al all induced metal-specific and time-dependent alterations in the gut microbiota. These results may provide a basis for further studies (e.g. fecal transplantation experiments or animal experiments focused on intestinal inflammatory diseases) of the toxic mechanisms of these metals in host species.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scib.2017.01.031.

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