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Ingestion of Remediated Lead-Contaminated Soils Affects the Fecal Microbiome of Mice

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Abstract

The relationship between ingestion of diets amended with a Pb-contaminated soil and the composition of the fecal microbiome was examined in a mouse model. Mice consumed diets amended with a Pb-contaminated soil in its native (untreated) state or after treatment for remediation with phosphoric acid or triple superphosphate alone or in combination with iron-waste material or biosolids compost. Subacute dietary exposure of mice receiving treated soil resulted in modulation of the fecal intestinal flora, which coincided with reduced relative Pb bioavailability in the bone, blood and kidney and differences in Pb speciation compared to untreated soil. Shifts in the relative abundance of several phyla including *Verrucomicrobia*, *Tenericutes*, *Firmicutes*, *Proteobacteria*, and TM7 (*Candidatus Saccharibacteria*) were observed. Because the phyla persist

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in the presence of Pb, it is probable that they are resistant to Pb. This may enable members of the phyla to bind and limit Pb uptake in the intestine. Families *Ruminococcaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Verrucomicrobiaceae*, *Prevotellaceae, Lactobacilaceae,* and *Bacteroidaceae,* which have been linked to health or disease, also were modulated. This study is the first to explore the relationship between the murine fecal microbiome and ingested Pb contaminated soils treated with different remediation options designed to reduce bioavailability. Identifying commonalities in the microbiome that are correlated with more positive health outcomes may serve as biomarkers to assist in the selection of remediation approaches that are more effective and pose less risk.

Keywords

Fecal microbiome; smelting soil; lead; triple superphosphate; phosphoric acid; biosolids; iron-rich waste; bioavailability

1. Introduction

For centuries, mining and smelting of lead (Pb) has contaminated soils, sediments and ground and surface waters worldwide (Moore & Luoma, 1990; Smith et al., 2021; Kozlov & Zvereva, 2007; Rowan et al., 1995; Eichler et al., 2014; Gamiño-Gutiérrez et al., 2013; Gillanet al., 2015; Jones et al., 1991). In children, soil and dust continue to be major sources of Pb exposure (Dong, Taylor, & Gulson, 2020; Fry, Wheeler, Gillings, Flegal, & Taylor, 2020). As an example, air emissions near the Port Pirie smelter site contained up to 16 $\mu g/m^3$ Pb which has been linked to elevated Pb in children (Taylor, 2019). In 2021, ~76% of two-year old Port Pirie children tested had blood lead levels > 5 μg/dL (Government of South Australia, 2022). The predominant role of these routes for Pb exposure is reflected in the integrated exposure uptake biokinetic model (IEUBK; USEPA, 1994; Hogan et al., 1998; Laidlaw et al., 2017) and in more recently developed models such as the Stochastic Human Exposure and Dose Simulation (SHEDS) Multimedia IEUBK model (Zartarian et al., 2017). Mouse (Kang et al., 2016; Juhasz et al., 2014; Bradham et al., 2018; Bradham et al., 2019; Breton et al., 2013), rat (Freeman et al., 1994; Brown et al., 2004), juvenile swine (Casteel, Weis, Henningsen, & Brattin, 2006), minipig (Marschner, Welge, Hack, Wittsiepe, & Wilhelm, 2006) and goat (Cretacci & Parsons, 2010) models have been used to assess Pb bioavailability, however the role of the gut microbiome in soil Pb bioavailability has not been explored. Both abiotic and biotic processes can alter the physical and chemical properties of Pb present in soil. Ultimately these transformations can affect the bioavailability of Pb that is ingested or inhaled. For example, phosphate solubilizing microbes in soil can promote the formation of poorly soluble Pb-phosphate compounds (Park et al., 2011; Teng et al., 2019; Yuan et al., 2017; Zhang et al., 2019). In addition, the presence of Pb in soil can eliminate sensitive species and select for resistant or tolerant organisms (Gillan et al., 2015; Hynninen et al., 2009; Jie et al., 2016; Van Houdt et al., 2009). These Pb-induced changes in the microbial landscape may affect the biogeochemical cycling of Pb in soil. Through resistance mechanisms, Pb is sequestered in less soluble forms (phosphates, sulfides, or carbonates) or can be adsorbed to exopolysaccharide or on the cell surface making it less available for biological uptake and transformation (George & Wan, 2020). Similarly, ingestion of Pb can alter the intestinal microbiome, producing both

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structural and functional anomalies that impact gut metabolism (Breton et al., 2013; Gao et al., 2017). For example, soil Pb exposure of individuals residing near a mining and smelting area correlated with changes in the intestinal microbiota (Shao & Zhu, 2020). Furthermore, increased Pb exposure has been shown to concurrently select for microbial Pb resistance genes and antibiotic resistance genes (El-Sayed, 2016; Kang & So, 2016; Koc et al., 2013; Matyar, 2012; Fatih Matyar et al., 2014). This Pb-induced change in intestinal microbiota may increase the risk of antibiotic failure in disease treatment (Bengtsson-Palme et al., 2018; Wright, 2010).

As part of a larger effort to understand the role of the gastrointestinal tract microbiota in the bioavailability of Pb in soil, various remediation strategies were examined for their role in modulating the composition of the fecal microbiomes of mice that ingested diets amended with remediated soils. Because soil Pb is an important source of Pb exposure in children, reducing the likelihood of soil ingestion has long been a central element in strategies to prevent childhood Pb poisoning (Gailey et al., 2020). This goal has often been attained by removal and replacement of Pb-contaminated soil (Henry et al., 2015). However, this is a complex and costly strategy that can be difficult to implement on a large scale. Alternatively, *in situ* solidification and stabilization of Pb can be used to remediate contaminated soils (National Research Council, 1997). Typically, chemical remediation of a Pb-contaminated soil involves *in situ* conversion of Pb into a poorly soluble form that, if ingested, will have decreased bioavailability (Bolan et al., 2014). Lead-contaminated soils are often remediated by treatment with phosphorus-containing compounds (e.g., rock phosphate, triple superphosphate [TSP], phosphoric acid [PA]) to promote formation of recalcitrant Pb phosphates, such as pyromorphite (Basta et al., 2001; Bradham et al., 2018; Bradham et al., 2016; Brown et al., 2004; Hettiarachchi et al., 2000; Scheckel et al., 2013).

Interest in remediation to reduce the bioavailability of Pb in treated soils has prompted the use of animal models to assess the efficacy of soil treatment. For example, the mouse model has been used to compare the relative bioavailability (RBA) of Pb in soil from a Superfund site in Joplin, Missouri, that was amended with phosphate alone on in combination with iron-rich waste material or biosolids compost (Bradham et al., 2018). This study found that treatment of soil with phosphate produced changes in the speciation of Pb in soil. When measured in the mouse model, the RBA estimates for Pb in treated soils were significantly lower than that obtained for Pb in the untreated soil. Treating soil with phosphoric acid reduced Pb RBA in bone by 32%. Triple superphosphate treated soil resulted in a 50% reduction in Pb RBA in bone; addition of iron waste or biosolids reduced RBA further to 64% and 74%, respectively. These findings suggested that remediation with phosphate could be an effective approach for the long-term reduction of Pb bioavailability. However, these studies do not provide any insights into other possible effects of soil treatment on potential interactions between Pb and organisms that ingest untreated or treated soil. Although there exists some information on the effects of ingested Pb on the intestinal microbiome (Breton et al., 2013; Gao et al., 2017; Xia et al., 2018), it is unclear how remediation-induced changes in Pb speciation might affect microorganisms resident in the gastrointestinal tract. This study is the first to explore the relationship between the fecal microbiome and ingested Pb contaminated soils with and without remediation treatments in a mouse model. While treating the soil with PA, or TSP with and without biosolids and/or iron-rich waste has

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been linked to a reduction in Pb bioavailability in the mouse, better understanding of the impact of remediated soil compared to the native soil on the mouse fecal microbiome has not been explored. Understanding this relationship between microbiome composition and the speciation of Pb in treated soils may provide new insights into the utility of various remediation methods. This information may help risk assessors and risk managers in selection of an optimal treatment for a specific contaminated site.

2. Materials and Methods

2.1 Soil and remediation treatment

Soils were obtained from a field trial designed to determine remediation alternatives for Pb smelter emission contaminated soil (Joplin, MO, USA). Site information, soil treatments, sample collection and processing are provided in Bradham et al. (2018) and summarized in Table 1. Briefly, three amendments were applied to field trial soil: (1) soil amended with 1% P as phosphoric acid (PA) , (2) 3.2% P as TSP (TSP), (3) 2.5% Fe as iron-rich waste material and 1% P as TSP (Fe/TSP), and (4) 10% biosolids compost and 1% P as TSP (C/TSP). Soil collected from the field trial site before treatment was designated as untreated soil (control). PA treated soil was collected 3 years after application and samples of soils treated with TSP, Fe/TSP, or C/TSP were collected 16 y after treatment. Metal concentrations in untreated and treated smelter impacted soil are provided in Table 1 (Bradham et al., 2018).

2.2 Assessment of Pb bioavailability in the mouse

A mouse-based assay for determination of soil Pb bioavailability has been described (Bradham et al., 2016). Briefly, twenty-eight day old female C57BL/6 mice (Charles River Laboratories, Raleigh, NC, USA) were acclimated for 12-13 days and then housed in groups of three in metabolic cages (3 cages per soil treatment [control, TSP, Fe/TSP, C/TSP, or PA] for a total of nine mice for each treatment; Lab Products, Seaford, DE). During the 9-day assay, mice had free access to drinking water and powdered AIN-93G rodent diet (Dyets, Bethlehem, PA) that was amended with 0.6% (w/w) of soil treated with PA, TSP with and with biosolids or iron rich waste or untreated soil (control). Test diets contained 21.6 (control), 22.2 (TSP), 14.3 (Fe/TSP), 24.2 (C/TSP), and 24.2 (PA) ppm Pb. For each metabolic cage, intakes of diet and water were monitored daily and each day's feces from each cage were combined to produce a cumulative cage sample (3 cumulative fecal samples for each soil treatment). Relative bioavailability of Pb was determined by calculating linear regression slopes (m) for cumulative Pb intake (mg) and tissue Pb level (mg kg^{-1} or mg l^{-1}) for treated and untreated soils and the following equation, $m_{treated}/m_{untreated}$ (Bradham et al., 2018). Homogenized fecal samples were stored at −20° C until processed for DNA extraction. Compared to mice that received control AIN-93G rodent diet, ingestion of soil-amended diets did not have significant adverse effects on absolute or relative changes in body weight or on diet or water consumption.

2.3 16S rRNA gene sequencing

Homogenized fecal samples were shipped frozen (−20° C) to the DNA Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Lemont, Illinois). There, DNA was extracted and the 16S rRNA gene V4 region of *Bacteria* and

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Archaea was amplified by polymerase chain reaction (PCR) with universal primers using extracted DNA as template. The PCR and amplicon sequencing were performed on a MiSeq DNA sequencer platform (Illumina, Inc., San Diego, CA) according to procedures used by the Earth Microbiome Project (Thompson et al., 2017).

2.4 Data analysis

DNA sequences were processed with mothur software using a standard set of commands (Schloss et al., 2009). The split.cluster command was used to construct the distance matrix and sequences having >97% nucleotide identities were clustered within an Operational Taxonomic Unit (OTU). Taxonomic identifications used the SILVA v135 non-redundant 16S rRNA sequence data base (Quast et al., 2012). Sequences were randomly subsampled to obtain a constant 5,000 sequences sample^{-1} for microbiome comparisons.

Statistical analyses by permutation of ranks, and calculations of diversity and evenness, were done with Primer-e 7 (Clarke & Warwick, 2001). 16S rRNA gene sequences OTU^{-1} sample^{-1} were transformed (fourth root) and a Bray-Curtis resemblance matrix and non-metric multidimensional scaling (nMDS) plots generated (Clarke etal., 2014). OTU, family, and phylum level dendrograms based on Bray-Curtis resemblance and similarity profile (SIMPROF) permutations were applied to highlight statistically significant clusters. PERMANOVA, a permutational multivariate analysis of variance, was used to test the significance of Bray-Curtis dissimilarities between treatments (Anderson, 2008). The community diversity (Shannon, H), evenness (Pielou, J), and richness (Margalef, S) indices were obtained from Primer for each replicate of the treatment. Treatment effects and difference among the means were determined with the one-way analysis of variance (ANOVA) followed by a Tukey test in R (R Core Team, 2014). A significance level of $p<0.05$ was selected for detection of treatment effects.

3. Results

3.1 Fecal microbiome similarities

Within replicate treatments, the fecal microbiomes were 82% to 93% similar to each other when aggregated at the genus, family and phylum levels for all treatments (Table S1). When considering all OTUs, similarity was reduced to 34-36%; when the 50 most prevalent OTUs were analyzed, similarity increased to 88-90%. Diversity (Shannon, H), richness (Margalef, S) and evenness (Pielou, J) indices, calculated at the genus and family levels, revealed only limited significant differences with C/TSP (Table 2). Diversity of the fecal microbiome differed in mice on an amended diet of C/TSP treated soil compared to control at the genus (p=0.0529) and family (p=0.0063) levels. At the family level, diversity in the microflora of mice that received diets amended with TSP (p=0.0895), Fe/TSP (p=0.0334), and PA (p=0.0633) were less similar to the C/TSP treated group; at the genus level, microflora from Fe/TSP (p=0.0198) and PA (p=0.0771) treated soil fed mice were different from those that received C/TSP treated soil. C/TSP treatment also contributed to changes in fecal community evenness compared to control (p=0.0471) and Fe/TSP (p=0.0739) treatments.

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Bray-Curtis dissimilarity analysis, using non-metric multidimensional scaling, examined the relatedness of fecal microbiomes from mice that consumed diets amended with untreated or treated soils at the OTU and the family levels (Fig. 1). OTUs from feces of mice that consumed Fe/TSP and PA amended diets were more closely related as were TSP and untreated soil groups. C/TSP separated from the other treatment groups. Similar results were observed when the OTUs were aggregated at the family level. However, fecal microbiomes from mice that consumed PA amended diets clustered more closely with those from mice that consumed diets amended with untreated and TSP treated soil. PERMANOVA analysis revealed an overall treatment effect at the OTU level and when aggregated at the genus, family, and phylum levels (Table 3). In pairwise comparisons at the OTU, genus, and family levels, fecal microbiomes of mice that consumed diets amended with TSP, Fe/TSP, C/TSP and PA treated soil differed significantly from those with untreated soil. All other pairwise comparisons among treatments revealed significant differences at all taxon levels except when aggregated at the phylum level, whereby only the ingestion of diet amended with PA-treated soil resulted in a significant change to the fecal microbiome compared to the fecal microbiome with untreated soil. Microflora from mice that ingested diet amended with PA treated soil also were significantly different from microflora in feces of mice that ingested diet amended with Fe/TSP or C/TSP treated soil.

3.2 Mice fecal microbiome composition

Phyla *Firmicutes* (44-52%), *Bacteroidetes* (39-49%), *Proteobacteria* (2-4%) and *Verrucomicrobia* (<5%) sequences were prevalent in the feces of mice from all treatments (Fig. 2A), with lower abundances of *Deferribacteres* (<2%), *Actinobacteria* (<1%), and TM7 (*Candidatus Saccharibacteria*; <1%). Relative to control soil, PA had a treatment effect across prevalent phyla; *Bacteroidetes and Proteobacteria* sequences were higher and *Firmicutes* and *Deferribacteres* sequences were lower. When analyzed individually, a treatment effect was observed for *Firmicutes* (p=0.0435) in feces from mice with all exposures; *Bacteroidetes* were not significantly different (p=0.3858). Relative abundance of *Firmicutes* was significantly lower in mice that consumed PA (p=0.0082) treated soil compared to its occurrence in the feces of mice that consumed diets amended with Fe/TSP (p=0.037), C/TSP (p=0.0429) treated soils or with untreated soil (p=0.0082).

Bacteroidaceae (*Bacteroidetes*), *Lachnospiraceae* (*Firmicutes*), *Lactobacillaceae* (*Firmicutes*), *Porphyromonadaceae* (*Bacteroidetes*), *Rikenellaceae* (*Bacteroidetes*), *Ruminococcaceae* (*Firmicutes*), *and Verrucomicrobiaceae* (*Verrucomicrobia*) were dominant families found in fecal microbiomes for all treatments (Fig. 2B; Table S2). Also observed were dominant members of the order *Clostridiales* (*Firmicutes*) that were not identifiable at the family level. Relative abundances of *Enterobacteriaceae* were elevated in feces from all treatment groups compared to control; *Lachnospiraceae* were elevated in mice that that ingested diets amended with Fe/TSP or C/TSP. Compared to effects of consumption of diet amended with untreated soil, consumption of diet amended with Fe/TSP or PA treated soil resulted in increased or reduced *Lactobacillaceae*, respectively. Consumption of diet amened with TSP, C/TSP and PA treated soil increased *Verrucomicrobiaceae* relative abundance almost 2-fold increase. Consumption of C/TSP treated soil increased *Prevotellaceae* (*Bacteroidetes*) relative abundance compared to untreated and treated soils.

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When aggregated at the genus level, *Lactobacillus* (*Firmicutes*), *Akkermansia* (*Verrucomicrobia)*, *Bacteroides* (*Bacteroidetes*), *Alistipes* (*Bacteroidetes*), *Mucispirillum* (*Deferribacteres*), *Parabacteroides* (*Bacteroidetes*), *Oscillibacter* (*Firmicutes*) dominated in all treatment groups (Fig. 2C, Table S3). Prevalent OTUs in all treatment identifiable at the genus level were *Lactobacillus* (OTU 1), *Akkermansia* (OTU 3), *Bacteroides* (OTU 4), *Alistipes* (OTU 8), *Mucispirillum* (OTU 23), *Oscillibacter* (OTU37), and *Parabacteroides* (OTU 25) (Table S3). *Barnesiella* (OTU 31 and/or OTU 35; *Bacteroidetes*) was identified in the top 20% of microflora from feces of animals that received dietary exposure to amended soils and at lower abundance in control soil. When aggregated at the genus level, these and additional genera contributing to similarity included *Dorea* (*Firmicutes*), *Allobaculum* (*Firmicutes*), *Enterococcus* (*Firmicutes*), *Parasutterella* (*Proteobacteria*), *Prevotella* (*Bacteroidetes*), *Pseudoflavonifractor* (*Firmicutes*), *Ruminococcus* (*Firmiutes*), *Staphylococcus* (*Firmicutes*), *Streptococcus* (*Firmicutes*), and *Clostridium* (*Firmicutes*) (Table S4).

3.3 Contributions of bacterial groups to fecal microbiome dissimilarity

An overall statistically significant difference among treatments was observed when the 16S rDNA data was aggregated at the phylum level. Pairwise analysis also revealed varying level of differences in microbiome composition after ingestion of diets amended with untreated, Fe/TSP, or C/TSP, and PA treated soils (Table 3). *Verrucomicrobia*, *Tenericutes*, *Acidobacteria*, TM7, *Cyanobacteria*, *Proteobacteria*, *Chloroflexi*, and an unidentified *Bacteria* accounted for 70% of the dissimilarity in the microbiomes from mice after consumption of diets amended with PA treated or untreated soils (Table S5). *Verrucomicrobia* (20.8%) and *Tenericutes* (20.81%) were the greatest contributors to the dissimilarity observed; similar results were observed when comparing Fe/TSP and C/TSP treated soil amended diets. *Verrucomicrobia* and *Tenericutes* were responsible for the dissimilarity in microbiome composition of mice that ingested diet amended PA treated soils and mice that ingested diets amended with Fe/TSP treated (28.7%) or C/TSP treated (33.0%) soil.

Family, genus and OTU level PERMANOVA and pairwise analysis also revealed significant treatment effects (Table 3). Seventy percent of the family contributions to dissimilarity are shown in Table S6. A small number of families contributed to dissimilarities in the fecal microbiome of mice that ingested diets amended with untreated or treated soil; *Verrucomicrobiaceae* contributions trended the highest and ranged from 2.4% to 4.35%, and *Staphylococcaceae* (*Firmicutes*), 1.7% to 2.8%. Relative increases and decreases in average abundance of families detected in all treatment groups are shown in Table S7. Families in the class *Rhizobiales* (*Proteobacteria*) accounted for 0.8% to 1.6% in feces from mice treated with Fe/TSP, C/TSP, and PA; the family *Deferribacteraceae* (*Deferribacteres*) decreased following dietary exposure to soils treated with TSP, Fe/TSP, and C/TSP; *Nocardioidaceae* (*Actinobacteria*) was elevated in Fe/TSP and PA; and *Geodermatophilaceae* (*Actinobacteria*) contributed 0.8% and 1.0%, respectively, to dissimilarity in C/TSP and PA treatments compared to control. Average abundance of *Gemmatimonadacea*e (*Gemmatimonadetes*) was elevated and contributed 0.90% to dissimilarity in feces of mice treated with TSP treated soil; conversely, when TSP was supplemented with biosolids, consumption of an

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amended diet reduced the average abundance of *Gemmatimonadaceae*, contributing 0.8% to dissimilarity.

When aggregated at the genus level for all treatments, primary contributors to dissimilarity included *Allobaculum* and *Ruminococcus*, ranging from 2.2% to 3.7% and 2.2% to 2.7% contribution (Table S8). *Clostridium* contributed 3.1% and 3.0%, respectively, to the fecal microbiome in mice that consumed diet in which TSP was combined with Fe or C treatment. *Akkermansia* contributed 2.0% to dissimilarity to the fecal microbiome in PA amended soil treated mice compared to control; TSP, Fe/TSP, and C/TSP, 1.7%, 1.7%, and 1.5%, respectively. When comparing to controls, C/TSP treated soil dietary exposure of mice resulted in a two-fold increase in *Akkermansia* contribution to dissimilarity (2.2%) compared to the other treatments (1.0%).

Data from all OTUs (37,993) indicated that dissimilarity of fecal microbiomes between mice consuming diet amended with treated soils and those with untreated soil ranged from 64.2% to 66.5%. When analysis was restricted to the 50 most abundant OTUs the dissimilarity was reduced to 10.4% to 12. 5%, accounting for only about 3.2% of the total. Within the top ten OTUs, only OTU 34 (*Lachnospiracea*e) was evident in all treatment types. However, when the range was expanded to the top 50 contributors to dissimilarly comparing the treatment groups to control, 12 OTUs were common across the 4 diets amended with treated soils (Table S9). Of those, 75% were identified as *Lachnospiraceae*; 17% as *Ruminococcaceae*; and 8% as *Allobaculum.* The average abundances of *Lachnospiraceae* were generally greater in feces of mice that received dietary treated soils compared to control (Fig. 2C).

3.4 Indices of metabolic change: *Firmicutes*/*Bacteroidetes* and *Bacilli*/*Clostridia*

Intestinal *Firmicutes*/*Bacteroidetes* and *Bacilli*/*Clostridia* ratios have been linked to physiological and metabolic changes in the host. Therefore, statistical analyses of these phyla, classes, and their ratios were performed. When analyzed together, no overall treatment effect on the relative abundance of fecal *Firmicutes* and *Bacteroidetes* was observed in the feces of mice that received the treated soils in their diet (p=0.1616) (Table 4). However, pairwise analysis found significant differences (p=0.0071) in relative abundance of *Firmicutes* and *Bacteroidetes* in feces of mice that consumed diets amended with untreated and PA-treated soil. When phyla were examined individually, there was an overall significant difference (p=0.0435) for *Firmicutes* for all treated soils. Pairwise analysis indicated that PA treated soil reduced abundance of *Firmicutes* compared to untreated soil (p=0.0082) and decreased the *Firmicutes*/*Bacteroidetes* ratio from 1.17 to 0.91. Similarly, TSP amended soil marginally reduced the relative abundance of *Firmicutes* (p=0.0679), decreasing the *Firmicutes*/*Bacteroidetes* ratio to 1.08.

An overall treatment effect was observed for fecal classes *Bacilli* and *Clostridia* (p=0.0002; Table S10). Furthermore, consumption of diets amended with Fe/TSP, C/TSP, or PA treated soil had a significant effect on fecal microflora compared to untreated soil group (Table S10). Relative abundance of *Clostridia* decreased in the three treatment groups; relative abundance of *Bacilli* was increased when mice received diet amended with Fe/TSP and C/TSP treated soil and decreased with PA treated soil, thus changing the *Bacilli*/*Clostridia* ratio from 0.12 to 0.5, 0.21, and 0.09, respectively (Table 4).

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4. Discussion

While mammalian factors contribute to Pb transformation, such as dissociation of Pb species in the acidic stomach (Juhasz et al., 2014), the intestinal microbiome is well poised to contribute. Lead is toxic to many microorganisms causing the Pb resistant community to dominate and marginalizing sensitive members, thus altering gut morphology and barrier permeability. These changes can affect the physiological state by modulating carbohydrate, energy, nitrogen and vitamin E and bile acid metabolism and create inflammatory and prooxidant states (Gao et al., 2017; Xia et al., 2018). Modulations in the fecal microflora indicate that the toxic components in the soil matrix may be bioavailable. The shift to a more resistant microbial community may impact Pb bioavailability due to increased expression of resistance mechanisms which may result in Pb adsorption to the gut microbiome and subsequent excretion. Here, we examined effects of ingestion of diets amended with Pb-contaminated soil subjected to different remediation treatments on the microbiome at descending levels of taxonomic organization. The potential health impacts associated with the microflora community shift and their role in transformation of Pb species and bioavailability is discussed.

4.1 Potential health implications of fecal microbiome modulation.

One approach to assessing the effects of toxins on the fecal microbiome is examination of changes in relative abundance at the phylum level. For example, modulation of the *Firmicutes*/*Bacteroidetes* ratio has been used as an indicator of obesity, exercise effects, aging, diet, carbohydrate metabolism and inflammation (Breton et al., 2013; Cheng et al., 2018; Denou et al., 2016; Kim et al., 2016; Murphy et al., 2010; Zhang et al., 2021). Similarly, changes in the *Bacilli*/*Clostridia* ratio have been linked to an inflammatory response (Breton et al., 2013; Cheng et al., 2018; Denou et al., 2016; Kim et al., 2016; Lee et al., 2020; Murphy et al., 2010; Pearson-Leary et al., 2020). In the present study, the change in the *Firmicutes*/*Bacteroidetes* ratio was greatest in mice that consumed diet amended with untreated soil. Smaller changes in the *Firmicutes*/*Bacteroidetes* ratio were found in the fecal microbiomes of mice that consumed diets amended with PA or TSP treated soil. This reduction in effect on the ratio may correlate with the lowering of Pb RBA produced by treatment of soils to reduce Pb solubility (Bradham et al., 2018). In a fifteen week drinking water study, treatment with Pb acetate (10, 30 or 100 mg L^{-1}) resulted in an increase in the *Bacteroidetes*/*Firmicutes* ratio (i.e. decrease in *Firmicutes*/*Bacteroidetes* ratio) which correlated with liver triglyceride, pyruvate and serum triglyceride, total cholesterol and glucose fluctuations (Xia et al., 2018). Interestingly, microflora effects were mediated by the addition of ferric chloride (5 mg L^{-1}) and resulted in changes to the abundance of several antimicrobial resistance genes. Eight weeks of Pb treatment (100 ppm or 500ppm; 1.83 g/L Pb acetate) administered in drinking water revealed no change in fecal *Bacteroidetes* or *Firmicutes* relative abundance compared to control, indicating a possible temporal association with their modulation (Breton et al., 2013; Zhai et al., 2017). Differences in the physical and chemical state of Pb in drinking water and soil make it difficult to compare the observed effects on the fecal microbiome. For soil-borne Pb, it may be more appropriate to compare fecal microbiomes found in mice that ingested untreated

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or treated soils. This approach makes it possible to correlate changes in the RBA of Pb produced by treatment of soils with changes in the composition of the fecal microbiome.

The effect of soil treatment on the fecal microbiome also was examined by comparing the relative abundance differences of classes *Bacilli* and *Clostridia*. When compared with the composition of the fecal microbiome from mice that consumed diet amended with untreated soil, there were significant differences (p=0.002) which reflected an increase in *Bacilli* in microbiomes of mice that consumed diets amended with Fe/TSP or C/TSP treated soil and a decrease in *Bacilli* in microbiomes of mice that consumed diet amended with PA treated soil (Table 4, Table S10). Similarly, compared with the composition of the fecal microbiome from mice that consumed diet amended with untreated soil, relative abundance of *Clostridia* was also reduced in fecal microbiomes from mice that consumed diets amended with the three treated soils. Reduced *Bacilli* to *Clostridia* ratio is indicative of an elevated inflammatory response (Lee et al., 2020; Pearson-Leary et al., 2020), suggesting that ingestion of diet amended with Fe/TSP or C/TSP treated soil may reduce inflammation below the level found after consumption of diet amended with untreated soil. In contrast, consumption of diet amended with PA treated soil may increase the inflammatory response. If an elevated inflammatory response is considered an adverse effect, then it may be prudent to use fluctuations in *Bacilli*/*Clostridia* and *Firmicutes*/*Bacteroidetes* in the selection of a soil treatment strategy used in remediation of Pb contaminated soil.

Other differences at the phylum level were seen in fecal microbiomes of mice that consumed diet amended with treated soils. Consumption led to an upward trend in the relative abundance of *Verrucomicrobia* in fecal microbiomes (Fig. 2A). Notably, this phylum made the largest contribution to dissimilarity between microbiomes from mice that consumed diets amended with untreated or treated soils. Similarly, relative abundance of *Tenericutes* and candidate phylum TM7 (*Candidatus Saccharibacteria*) contributed between 6.0% to 13.1% and 6.9% to 9.5%, respectively, to dissimilarity observed in mice that consumed diets amended with untreated or treated soils (Table S5). Compared to consumption of untreated soil, TSP or PA treated soil exhibited reduced relative abundance of *Tenericutes*; Fe/TSP or C/TSP treated soil increased the relative abundance of *Tenericutes*. For candidate phylum TM7 the relative abundance was elevated in all mice that consumed diets amended with treated soil (Fig. 2A). Relative abundances of *Verrucomicrobia*, *Tenericutes*, and candidate phylum TM7 (*Candidatus Saccharibacteria*) have been linked to inflammatory response (Kuehbacher et al., 2008). In humans, improved kidney function, evidenced by reduced serum creatinine levels and increased glomerular filtration, is correlated with elevated abundance of candidate phylum TM7 in the feces (Xu et al., 2020). In the horse, levels of *Verrucomicrobia* and *Clostridiales* in ileum are associated with induction of regulatory immunity, suggesting a role in modulating inflammatory response (Lindenberg et al., 2019). An eight-week exposure to Pb acetate in drinking water reduced the abundance of *Verrucomicrobia* which may be indicative of a heightened inflammatory state following Pb exposure (Zhai et al., 2017). Biomarkers of aging and inflammation that are associated with increased levels of IL-6 and IL-17 cytokines and tumor necrosis factor alpha have been reported to accompany decreased abundance of *Tenericutes* (Huang et al., 2013; Cao et al., 2020; Li et al., 2019). Consumption of diets amended with treated soil marginally increased *Proteobacteria* abundance in fecal microbiomes (Fig. 2A). The phyla *Proteobacteria* have

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been correlated with gut dysbiosis, aging, and inflammation (Huang et al., 2013; Cao et al., 2020; Litvak et al., 2017; Shin et al., 2015). Elevated abundance of *Proteobacteria* is associated with gut dysbiosis and in aged mice, lower abundance of *Proteobacteria* is reported (Huang et al., 2013; Cao et al., 2020). An eight-week exposure to Pb acetate in drinking water also reduced *Proteobacteria* abundance (Zhai et al., 2017). Modulation of the *Proteobacteria* community associated with consumption of diet amended with a treated soil may be an adverse effect associated with soil remediation. Careful consideration of the full range of effects of consumption of complex treated soils on the composition of the microbiome of the gastrointestinal tract may be part of assessment of treatment and development of a risk assessment framework to evaluate different soil treatments.

Because the contaminated soils are complex matrices containing a wide array of inorganic and organic components, to include metals such as cadmium, chromium, arsenic, and zinc, it is difficult to attribute changes to microbiome composition to a single contaminant, such as Pb. However, evidence from one ecological study suggests a relationship between exposure to Pb in soil and changes in intestinal microflora. A study in deer mice (*Peromyscus maniculatus*) trapped at a previously remediated site in the former Tri-State Mining District (Missouri, USA) reported to have 270-732 mg kg^{-1} soil Pb, found significant differences in intestinal microflora community structure compared to *P. maniculatus* trapped from a reference site (Coolon et al., 2010). In mice that resided on Pb-contaminated soil, *Ruminococcaeceae* abundance was significantly reduced. Conversely, in a cross sectional study in humans residing near a mining and smelting site who were exposed long term to Pb and other heavy metal contaminated soil, the relative abundances of families *Ruminococcaceae*, *Lachnospiraceae*, and *Erysipelotrichaceae* were elevated in stool (Shao & Zhu, 2020). Intestinal *Ruminococcaeceae* has been related to health promotion in humans (Hattori & Taylor, 2009); its reduction in mice from Pb contaminated sites may contribute to smaller body size and mass and reduced body fat.

In the present study, ingestion of diets amended with soil for nine days decreased fecal *Ruminococcaceae,* comparable to the *P. maniculatus* study and *Lachnospiraceae*; *Erysipelotrichaceae* levels declined after consumption of diets amended with Fe/TSP, C/TSP or PA treated soils (Table S2). *Verrucomicrobiaceae* contributed 2.4% to 4.4% to microbiome dissimilarity between mice consuming diets amended with untreated or treated soil; relative abundance was increased in groups consuming diets amended with treated soils. *Verrucomicrobiaceae* (as well as genera *Akkermansia* and *Dorea*) have been reported to have a causal relationship with inflammatory bowel disease, although the role of these microorganisms in the disease process has not been elucidated (Zhang et al., 2021). Ingestion of diets amended with treated soils were associated with other changes in microbiome composition. Elevated *Prevotellaceae* levels were observed in mice that consumed diet amended with C/TSP treated soil. Increased levels of *Lactobacilaceae*, which have been linked with an anti-inflammatory response in the gut (Ojo et al., 2019; Vandana et al., 2020), were found in mice that consumed a diet amended with Fe/TSP treated soil; in these mice *Bacteroidaceae* levels were marginally decreased. Other studies have reported changes in microflora composition with Pb exposure. Members of the *Prevotellaceae* family were detected in the feces of mice exposed to 500 ppm of Pb in drinking water for 8 weeks; in contrast, members of this family were absent from the feces of mice not exposed

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to Pb (Breton et al., 2013). Studies have linked elevated *Prevotellaceae* levels in humans to a higher genetic risk score associated with body mass index and obesity, suggesting an association with alterations in gut metabolism (Cuevas-Sierra et al., 2020). These results demonstrate the integral role of the microbiome in maintaining gut homeostasis and the complexities in associating function to one or more families.

Aggregating the OTU data at the genus level revealed additional changes in the fecal microbiome of mice that consumed soil-amended diets. For all mice consuming diets amended with treated soils, the relative abundance of *Pseudoflavonifractor* (*Firmicutes*) was reduced as compared to its abundance in fecal microbiome of mice that consumed diet amended with untreated soil. *Pseudoflavonifractor* abundance has been positively correlated with obesity induced Type 2 diabetes and hypertension (Wang et al., 2020; Zuo et al., 2019); reduced *Pseudoflavonifractor* abundance observed in the current study may be a potential positive health indicator. Consumption of diet amended with C/TSP treated soil increased the relative abundance of *Ruminococcus* (*Firmicutes*). Furthermore, *Allobaculum* (*Firmicutes*) and *Ruminococcus* were key contributors to dissimilarity between the microbiomes of mice consuming diets amended with untreated soil and those consuming diets amended with treated soils. *Allobaculum* abundance has been correlated with a high fat diet and elevated levels of short chain fatty acids, simple carbohydrates, and acetate in the gut (Zhang et al., 2021; Balakrishnan et al., 2021). The mucin degrader *Ruminococcus gnavus*, which metabolizes complex polysaccharides and is a key symbiont in the mammalian intestinal tract (Crost et al., 2013; Henke et al., 2019), has been associated with Crohn's disease and inflammation (La Reau & Suen, 2018). *Akkermansia* (*Verrucomicrobia*) which contributed approximately 1.7% to dissimilarity in microbiomes in mice consuming TSP +/− Fe and C amended diets was elevated in mice consuming all diets amended with treated soil compared to untreated soil. Therapeutic use of *Akkermansia muciniphila*, a mucin degrader, has been reported to have beneficial metabolic and immunologic effects including reduced incidence of diabetes mellitus and prevention of age-related colonic mucus layer decline and inflammation (de Vos, 2017; Hänninen et al., 2018; Jayachandran et al., 2020; Van Der Lugt et al., 2019). While not definitive, modulation of the microbiome may be implicated in potential positive or negative health outcomes.

Among the top 50 OTUs contributing to the fecal microbiome dissimilarity between mice consuming diet amended with untreated soil and treated soils, 12 OTUs occurred in all groups consuming diets amended with treated soils (Table S9). Of these, most were either *Lachnospiraceae* or *Ruminococcaceae*, which were dominant members of the fecal microflora found in mice receiving diets with treated soils. Notably, OTU 84, identified as *Allobaculum*, was a prevalent contributor to dissimilarity in fecal microflora found in mice receiving diets with treated soils. Future research on the use of biomarkers of effect to evaluate the influence of ingestion of remediated soils on the composition of the fecal microbiome might focus on use of *Allobaculum* as candidate biomarker as it is a prevalent contributor at both the OTU and genus level. Additional studies also are needed to determine whether *Lachnospiraceae* or *Ruminococcaceae* OTUs can be used as biomarker for changes in the microbiome.

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4.2 Relationship of the fecal microbiome to Pb transformation and bioavailability

Microorganisms have evolved survival mechanisms to evade the toxic effects of heavy metals. The related genes are plasmid or chromosomally linked and can co-occur with antibiotic resistance genes in humans and animals (Li et al., 2017). Many phyla, including *Proteobacteria*, *Firmicutes*, and *Actinobacteria* harbor Pb and metal resistance genes (Bharagava et al., 2014; El-Sayed, 2016; Koc et al., 2013). Expression of these genes can result in Pb binding extracellularly to microbial siderophores, exopolysaccharides, and cell phosphoryl-, carboxyl-, or sulfhydryl-groups or sequestration intracellularly by binding to metallothionein (George & Wan, 2020). Hui et al. (2018) demonstrated that Pb resistance genes can sequester Pb *in vivo*. When a Pb binding protein, PbrR, was engineered to express on the cell surface of *Escherichia coli* and subsequently introduced into mice, Pb was immobilized at both acidic and neutral pH and correlated with a decrease in bioavailability. Because exposure to Pb and other heavy metals selects for resistance strains, more opportunities for adsorption or precipitation may be available and contribute to reduced Pb bioavailability. *Verrucomicrobiaceae* and *Proteobacteria*, which qualitatively increased in relative abundance in mice that received treated soil, have been reported to co-occur with the Pb resistance gene *pbrT* and other genes associated with heavy metal resistance expression in the environment (Roberto et al., 2019). Members of these phyla could adsorb or bind Pb and make less available for mammalian adsorption. In addition, the higher concentration of available Pb in the untreated soil may have exceeded a microbial Pb resistance threshold, resulting in a reduction of *Verrucomicrobiaceae* and *Proteobacteria* populations, which, in turn, resulted in increased mammalian adsorption.

Microbial Pb resistance mechanisms also generate less soluble Pb species such as sulfides, phosphates, and carbonates which have been leveraged in bioremediation strategies to reduce bioavailability (George & Wan, 2020). The untreated soil diet used in this study contained anglesite, galena, organic-Pb, and adsorbed-Pb; no anglesite or galena were detected in the feces and relative amounts of adsorbed and organic Pb were increased (Fig. 3; Bradham et al., 2018). Pb phosphate also was present in the feces of mice provided the untreated soil diet. The low pH in the stomach, or microbial phosphatases or organic acids may have solubilized the Pb species as they transited through the stomach and small intestine. As pH increased in the colon, Pb phosphate may have been formed either abiotically or through phosphate generating microbial processes. In addition, adsorbed and organic Pb also were relatively greater in feces compared to that in the diet which may be a result of Pb adsorbing or binding to exopolysaccharide or the cell surface. Diets prepared with soil treated with PA, TSP, and Fe/TSP contained relatively similar amounts of anglesite, Pb phosphate, pyrophosphate, and organic-Pb; anglesite was not recovered from the feces and relatively more Pb phosphate and organic-Pb and less pyrophosphate was detected, supporting a possible microbiome role. Adsorbed Pb was elevated in feces from mice that received dietary Fe/TSP treated soil compared to other phosphate treatments (TSP, C/TSP, PA) suggesting that Pb may have adsorbed to iron minerals. Feces from mice receiving a diet containing C/TSP had relatively more organic-Pb compared to the other treatments (TSP, Fe/TSP, PA); pyromorphite was relatively less abundant, presumably due to the prevalence of organic associated Pb. Animals that received similar amounts of Pb in their diet from C/TSP and PA treated soils (24.2 ppm), excreted more (184.7 ppm) and less (162.6 ppm)

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Pb in the feces, respectively (Table S11). Lead excretion was inversely correlated to RBA. Lead was less bioavailable in animals that received C/TSP treated soil (28%) whereas it was more bioavailable (69%) in those that received PA treated soil. Therefore, both the biosolids treatment and the intestinal flora may have adsorbed Pb leading to increased excretion in the feces.

C/TSP soil treatment affected fecal microbiome diversity compared to PA and Fe/TSP treated soils and control when aggregated at the genus and family levels; C/TSP soil treatment fecal microbiome richness was significantly different than that observed for Fe/TSP treated soil and untreated control (Table 2). Compared to control, phyla *Actinobacteria* (*Pseudonocardiaceae*), *Bacteroidetes* (*Chitinophagaceae*, *Cytophagaceae*, *Prevotellaceae*), *Firmicutes* (*Alicyclobacillaceae*, *Bacillaceae*, *Clostridiaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Paenibacillaceae*, *Planococcaceae*), *Proteobacteria* (*Enterobacteriaceae*, *Sutterellaceae*), *Tenericutes* (*Anaeroplasmataceae*), and *Verrucomicrobia* (*Verrucomicrobiaceae*) were elevated in feces from mice that received dietary C/TSP relative to control (Table S7). Members of the associated phyla have been reported to be resistant to high levels of Pb, and therefore have the potential to bind Pb and be excreted in the feces (Adler, Devarajan, Wildi, & Poté, 2016). Feces from mice that received dietary C/TSP treated soil compared to PA treated soil had elevated average abundance of *Verrucomicrobia* which contributed 19% to the dissimilarity associated with the two treatments and may have more efficient Pb binding. The microbial community differences are suggestive of a microflora role in Pb transformation which results in less Pb bioavailability and higher Pb fecal excretion rate.

5. Conclusions

In summary, this study provides unique insight into the effect of soil remediation alternatives on the murine fecal microbiome and implications for potential health associated outcomes and Pb bioavailability due to change in microbiome equilibrium. Changes in the fecal microbiome may be a consequence of exposure to Pb present in soil. Assessing the significance of those changes in the gastrointestinal tract associated with ingestion of Pb-contaminated soils is an aspect of estimating adverse health effects caused by ingestion of Pb. As shown in the present study, remediation of Pb-contaminated soils by treatment with agents that reduce the solubility and bioavailability of Pb can produce changes in Pb speciation that affects the composition of the fecal microbiome. The consequences of these changes in microbiomes after ingestion of treated soils have not been well characterized, however a link to altered physiology and metabolism and expression of Pb resistant microbial mechanisms can be inferred. Future work is needed to improve our understanding of the relationship between Pb speciation in treated soils and to assess the adverse health effects of these changes. To better delineate treatment effects, future studies will take discrete fecal samples during dietary exposure and while an uncontaminated reference soil may be desirable, its inclusion may introduce another variable to the study because it would be sourced separately. Development of biomarkers of effect to monitor critical changes in microbiome composition will be critical to this work.

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Animals

The protocol for use of mice for the determination the bioavailability of Pb in soil was reviewed by the Institutional Animal Care and Use Committee of the US Environmental Protection Agency, Research Triangle Park, North Carolina. These studies reported here conformed to the approved protocol that complied with National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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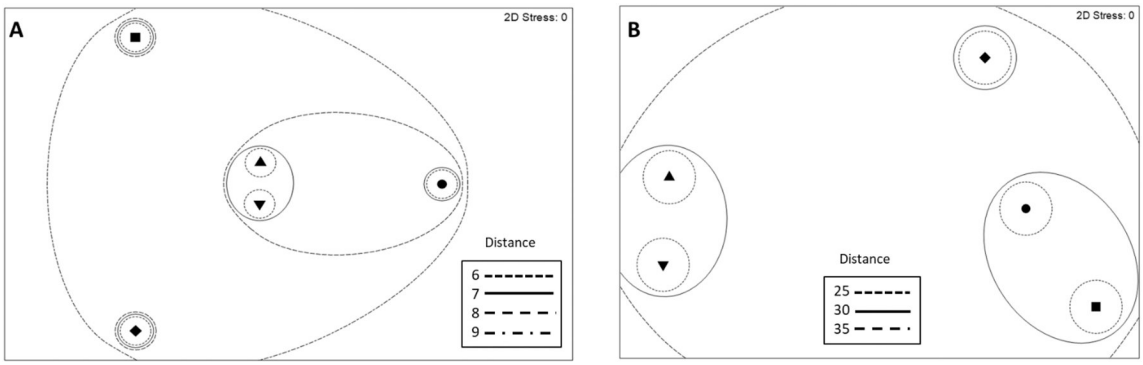

Fig. 1.

Bray-Curtis resemblance matrix and non-metric multidimensional scaling (nMDS) of fecal microbiome among remediated and control treatments for all OTUs (A) and when aggregated at the family level (B). Dietary amendment of soil treatments are as follows: untreated, triangle; TSP, inverted triangle; Fe/TSP, square; C/TSP, diamond; PA, circle.

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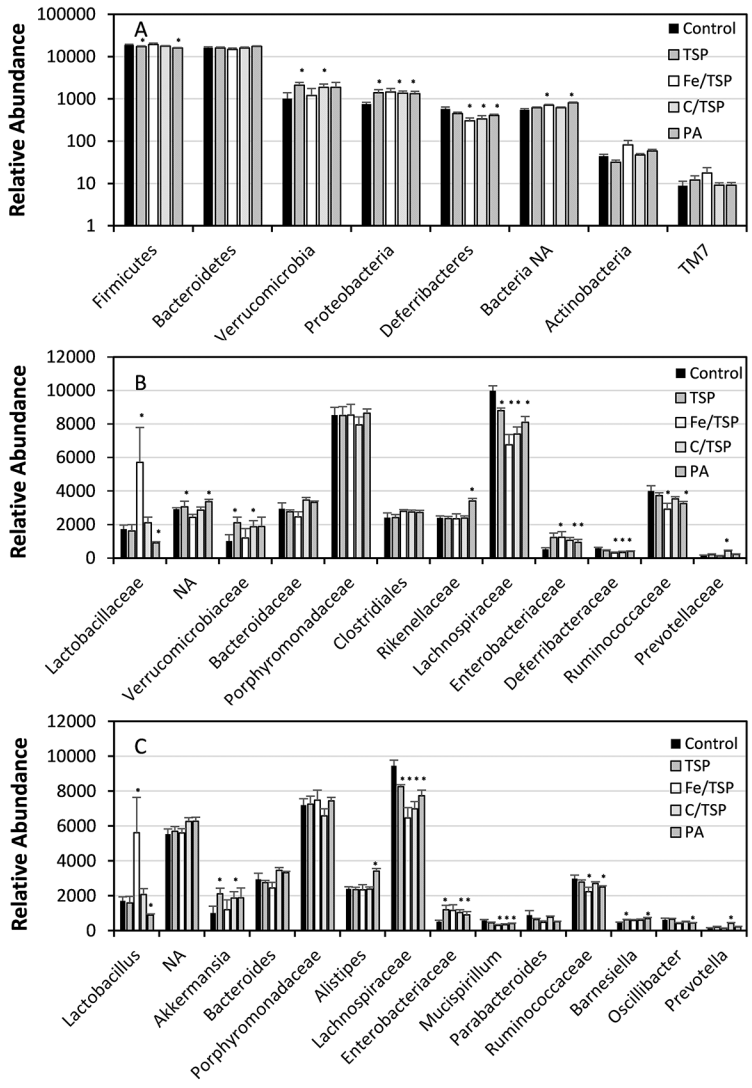

Fig. 2.

Relative abundance of dominant phyla (A); families(B); genera (C) (fourth root transformed) in feces of mice receiving dietary amendments of treated and untreated (control) soil. Error bars are standard errors of means. * denotes significant difference of the treatment against the control (p<0.05).

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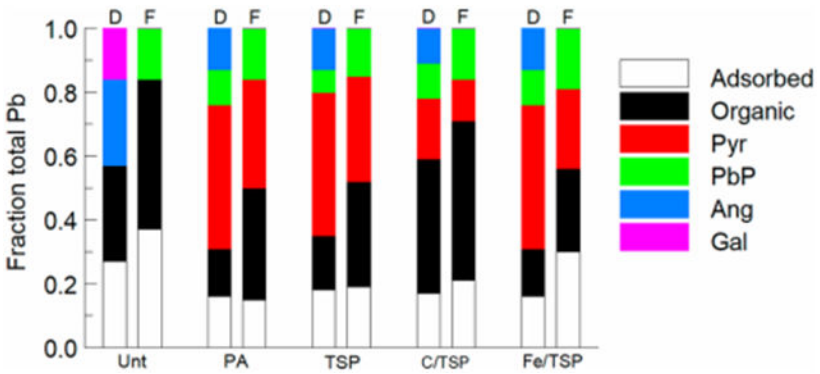

Fig. 3.

Pb speciation in diet consumed and feces excreted by mice ingesting soil-amended diets. Relative amounts of each Pb species (fraction of total Pb) present in diet (D) and in feces (F) during the standard assay period were calculated using Pb speciation data derived from spectral analysis of diet and feces. Treatments as identified in Table 1. PbP is trilead diphosphate ($Pb_3(PO_4)_2$), Ang is anglesite ($PbSO_4$), Gal is galena (PbS), Pyr is pyromorphite. Reprinted with permission from Bradham, K. D., Diamond, G. L., Nelson, C. M., Noerpel, M., Scheckel, K. G., Elek, B., Chaney, R. L., Ma, Q, and Thomas, D. J. Long-term in situ reduction in soil Pb bioavailability measured in a mouse model. *Environmental Science & Technology*, 2018, 52(23), 13908-13913. Copyright 2018 American Chemical Society.

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Table 1.

Summary of soil treatments, elemental analysis (mean±95% confidence limits) and relative lead bioavailability (means ± standard error). Reduction in lead bioavailability related to each soil treatment is calculated relative to untreated soil [($slope_{treated}$/$slope_{untreated}$) x 100] and shown as a percent (Bradham et al., 2018).

Soil Treatments	**Control**	**3.2% P as TSP Triple Superphosphate**	**2.5% Fe as Fe rich waste + 1% P as Triple Superphosphate**	**10% C as Biosolids + 1% P as Triple Superphosphate**	**1% P as Phosphoric Acid**
Abbreviation	**Control**	**TSP**	**Fe/TSP**	**C/TSP**	**PA**
Duration (years)	0	16	16	16	3
Elemental Analysis of Soils (ppm)					
Pb	3055.2 ± 1321.1	2136.8 ± 1291.3	1992.5 ± 1743.8	2684.7 ± 1763.9	3088.9 ± 1079.5
Zn	3813.3 ± 1316.8	2950.6 ± 1363.2	2587.9 ± 2060.2	3354.5 ± 1787.6	3579.7 ± 1094.1
Cd	17.1 ± 6.0	12.9 ± 5.7	10.5 ± 6.8	14.5 ± 7.2	15.7 ± 4.1
Cu	34. 8 ± 16.5	21.1 ± 6.9	19.6 ± 6.4	38.0 ± 9.8	28.2 ± 10.0
Ni	13.6 ± 1.8	13.0 ± 2.3	19.2 ± 4.9	11.5 ± 2.3	11.5 ± 0.6
As	7.8 ± 2.4	6.2 ± 0.8	5.2 ± 1.0	5.9 ± 0.6	4.1 ± 0.4
P	683.2 ± 190.8	15024.0 ± 9177.1	3785.6 ± 2503.4	8085.4 ± 2796.3	6687.2 ± 1979.4
Soil Treatment Effect on Relative Lead Bioavailability					
Bone	100	54 ± 0.09	45 ± 0.11	28 ± 0.09	69 ± 0.09
Blood	100	57 ± 0.07	45 ± 0.08	47 ± 0.07	52 ± 0.08
Kidney	100	68 ± 0.09	53 ± 0.10	55 ± 0.08	67 ± 0.07

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Table 2.

Fecal microbiome diversity indices. Richness (Margalef, S), evenness (Pielou, J), and diversity (Shannon, H) and were determined for the fecal microbiome of mice that received treated or untreated (control) soils in their diet. ANOVA, $p<0.05$, significant**; $0.05<p<0.08$ marginally significant*; Tukey multiple comparison of means, a significantly different than b within index p<.

Taxon	Diversity Index	Control		TSP		Fe/TSP		C/TSP		PA	
		Value	sem[1]	Value	sem	Value	sem	Value	sem	Value	sem
Genus	S	81.5	0.7	80.7	4.7	89.5	5.4	83.7	1.0	75.2	3.4
	J*	0.5	0.0	0.5	0.0	0.5	0.0	0.6	0.0	0.5	0.0
	H**	2.3[b]*	0.0	2.4	0.0	2.3b**	0.1	2.5[a]	0.0	2.3[b]*	0.0
Family	S	56.50	0.77	56.33	3.41	62.00	4.84	56.17	1.34	52.17	1.85
	J**	0.54[b]**	0.01	0.56	0.01	0.54[b]*	0.02	0.59[a]	0.01	0.56	0.01
	H**	2.16[b]**	0.02	2.23b*	0.03	2.21[b]*	0.04	2.37[a]	0.04	2.22[b]*	0.03

[1] Standard error of mean, sem

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Table 3.

Treatment effects on the murine fecal microbiome. PERMANOVA and t-tests were performed to determine differences in the fecal microbiome at each taxon level among treatments with dietary treated (TSP, Fe/TSP, C/TSP, PA) or untreated (control) soil; $p<0.05$, significant; $0.05<p<0.07$ marginally significant (italics).

Group Comparison	df	t	P(perm)	Unique permutations
OTU				
All Amendments	29		0.0001	9219
control x TSP	10	1.1267	0.0054	460
control x Fe/TSP	10	1.2146	0.0027	462
control x C/TSP	10	1.1921	0.0018	461
control x PA	10	1.2206	0.0034	461
TSP x Fe/TSP	10	1.2157	0.0025	461
TSP x C/TSP	10	1.2274	0.0026	458
TSP x PA	10	1.2316	0.0017	462
Fe/TSP x C/TSP	10	1.1743	0.0029	459
Fe/TSP x PA	10	1.1689	0.002	460
C/TSP x PA	10	1.2172	0.0015	461
Genus				
All Amendments	29		0.001	999
control x TSP	10	1.1778	*0.053*	407
control x Fe/TSP	10	1.3951	0.004	408
control x C/TSP	10	1.4807	0.004	421
control x PA	10	1.5277	0.003	418
TSP x Fe/TSP	10	1.4744	0.001	401
TSP x C/TSP	10	1.4431	0.008	403
TSP x PA	10	1.4206	0.003	414
Fe/TSP x C/TSP	10	1.4467	0.003	412
Fe/TSP x PA	10	1.541	0.002	410
C/TSP x PA	10	1.8144	0.001	414
Family				
All Amendments	29		0.0001	9814
control x TSP	10	1.202	0.028	407
control x Fe/TSP	10	1.4994	0.006	414
control x C/TSP	10	1.7341	0.003	420
control x PA	10	1.6124	0.002	412
TSP x Fe/TSP	10	1.4627	0.017	401
TSP x C/TSP	10	1.438	0.005	401
TSP x PA	10	1.3524	0.01	406
Fe/TSP x C/TSP	10	1.5588	0.001	403
Fe/TSP x PA	10	1.6851	0.004	416
C/TSP x PA	10	1.8469	0.002	404

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Group Comparison	df	t	P(perm)	Unique permutations
Phylum				
All Amendments	29		0.0031	9909
control x TSP	10	1.3545	0.126	410
control x Fe/TSP	10	1.2784	0.164	413
control x C/TSP	10	1.5367	*0.066*	411
control x PA	10	1.842	0.013	408
TSP x Fe/TSP	10	1.3952	*0.067*	421
TSP x C/TSP	10	0.94343	0.524	402
TSP x PA	10	1.2544	0.175	414
Fe/TSP x C/TSP	10	1.5042	*0.054*	409
Fe/TSP x PA	10	1.5151	0.04	408
C/TSP x PA	10	1.5619	0.05	413

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Table 4.

Relative abundance of phyla *Firmicutes* and *Bacteroidetes* and classes *Bacilli* and *Clostridia* and associated indices. Statistical analyses comparing fecal flora from mice that received treated (TSP, Fe/TSP, C/TSP, PA) or untreated (control) soil in their diet were performed to determine differences in the specific phyla and classes; $p<0.05$, significant (**); $0.05<p<0.08$ marginally significant (*). See Supplemental Table 9 for PERMANOVA and pairwise t-test results.

Phylum	Relative Abundance				
	Control	TSP	Fe/TSP	C/TSP	PA
Firmicutes	18900	17331*	19403	17673	15947**
Bacteroidetes	16128	16032	14818	16058	17551
Firmicutes/ Bacteroidetes	1.17	1.08	1.31	1.1	0.99**
Class					
Bacilli	2053	1900	6321	3002	1313
Clostridia	16504	15042	12727	14200	14209
Bacilli/Clostrida	0.12	0.13	0.5**	0.21**	0.09**