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Research Article

Perinatal Lead Exposure Alters Gut Microbiota Composition and Results in Sex-specific Bodyweight Increases in Adult Mice

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ABSTRACT

Heavy metal pollution is a principle source of environmental contamination. Epidemiological and animal data suggest that early life lead (Pb) exposure results in critical effects on epigenetic gene regulation and child and adult weight trajectories. Using a mouse model of human-relevant exposure, we investigated the effects of perinatal Pb exposure on gut microbiota in adult mice, and the link between gut microbiota and bodyweight changes. Following Pb exposure during gestation and lactation via maternal drinking water, bodyweight in A^{vy} strain wild-type non-agouti (*a*/*a*) offspring was tracked through adulthood. Gut microbiota of adult mice were characterized by deep DNA sequencing of bacterial 16S ribosomal RNA genes. Data analyses were stratified by sex and adjusted for litter effects. A Bayesian variable selection algorithm was used to analyze associations between bacterial operational taxonomic units and offspring adult bodyweight. Perinatal Pb exposure was associated with increased adult bodyweight in male ($P < .05$) but not in female offspring ($P = .24$). Cultivable aerobes decreased and anaerobes increased in Pb-exposed offspring ($P < .005$ and $P < .05$, respectively). Proportions of the 2 predominant phyla (Bacteroidetes and Firmicutes) shifted inversely with Pb exposure, and whole bacterial compositions were significantly different (analysis of molecular variance, $P < .05$) by Pb exposure without sex bias. In males, changes in gut microbiota were highly associated with adult bodyweight ($P = .028$; effect size = 2.59). Thus, perinatal Pb exposure results in altered adult gut microbiota regardless of sex, and these changes are highly correlated with increased bodyweight in males. Adult gut microbiota can be shaped by early exposures and may contribute to disease risks in a sex-specific manner.

Key words: gut microbiota; microbiom; perinatal lead (Pb) exposure; bodyweight; Bacteroidetes and Firmicutes.

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, nutrition and other environmental factors during prenatal and early postnatal development influence developmental plasticity, thereby altering susceptibility to adult chronic diseases, including obesity (Barker, 2004; Bateson *et al.*, 2004). Although data from animal models and human epidemiology support the DOHaD hypothesis, the underlying biological mechanisms linking early exposures to later in life disease risk are poorly understood (Lucas, 1991; McMillen and Robinson, 2005; Waterland and Garza, 1999). Growing evidence shows that transient exposure to environmental toxicants during the perinatal period influences the developmental establishment of epigenetic gene regulation and links epigenetic dysregulation with later in life disease risk, such as obesity (Faulk *et al.*, 2013, 2014b; Feil, 2006). More studies are needed using a multidisciplinary approach, including studies on changes in the gut microbiome, to fully understand mechanisms linking developmental exposures and later in life disease risk.

Pb is a highly toxic metal with no safe blood level identified by the Centers for Disease Control and Prevention (CDC) (Betts,

2012). However, environmental Pb is ubiquitous, and its effects are worldwide due to its use in various goods, such as tetraethyl lead in gasoline, and its persistence in the environment over time. Therefore, air, soil, water, old paint, and food are all potential exposure routes for Pb uptake via ingestion, inhalation, and dermal absorption. The U.S. Centers for Disease Control and Prevention recently lowered the blood lead level (BLL) of concern from 10 to 5 μg/dl (Betts, 2012), and increasing evidence suggests that adverse health effects can occur at even at lower levels (Gilbert and Weiss, 2006). When compared with oral ingestion in adults, transfer of Pb from mother to offspring occurs transplacentally and lactationally (Snyder *et al.*, 2000). Pb has also been implicated in contributing to obesity by interrupting energy production and other metabolic processes (Beier *et al.*, 2015; Katzen-Luchenta, 2007). Recent animal evidence shows that early life Pb exposure is highly associated with increased bodyweight and other health effects in later life (Faulk *et al.*, 2013, 2014a; Leasure *et al.*, 2008). Little is known, however, about the mechanism(s) of these health outcomes following early-life exposure.

Recently, it has been recognized that gut microbiota play a critical role in maintaining human health, and alteration of normal gut microbiota may increase susceptibility to infectious diseases and different types of chronic metabolic diseases (e.g., obesity, diabetes, and cancer; Clemente *et al.*, 2012; Flint *et al.*, 2012; Villanueva-Millan *et al.*, 2015). When humans ingest Pb gastro-orally, the microorganisms in the digestive tract are exposed to Pb as well. In the field of environmental microbiology, microbial communities change composition in Pb-contaminated soil (Sobolev and Begonia, 2008), with certain bacterial species more resistant or tolerant to Pb being more active and found in higher abundance than those in intolerant of Pb (Chen *et al.*, 2011). However, few studies have examined interactions between metals and microbiota in the animal or human gut (Lu *et al.*, 2014), especially the effects of early-life exposure on gut microbiota and its relationship with health risks later in life. Thus, our hypothesis was that perinatal exposure to Pb in mice impacts gut microbiota composition and function later in life.

Here, by using wild-type non-agouti (*a/a*) mice of the A^{vy} strain isogenic mouse model of perinatal environmental exposures (Dolinoy, 2008), we evaluated the effects of physiologically and environmentally relevant levels of Pb, resulting in a mean maternal BLL of 32 μg/dL (Faulk *et al.*, 2013). We examined the interaction of Pb exposure with bodyweight and gut microbiota, measured via observations of adult bodyweight and pyrosequencing of the gut microbiota. We evaluated associations among Pb exposure, gut microbiota, and body weight, stratified by sex and adjusting for litter. Our results suggest that perinatal exposure to Pb induces differences in gut microbiota composition, and these changes contribute to increased bodyweight in adult male mice.

MATERIALS AND METHODS

Animals and diet. Animals and diet were the same as previously reported (Faulk *et al.*, 2013, 2014a). The control group was provided Pb-free distilled water, and the Pb exposure group was supplied with Pb-acetate water. Pb (II) acetate trihydrate (Sigma-Aldrich, MO) was dissolved in a single batch of distilled water to a final concentration of 32 ppm, and the concentration was verified by inductively coupled plasma mass spectrometry (ICPMS; NSF International, MI). The water bottles were changed at least weekly. Animals were maintained on a phytoestrogen-free AIN-93G diet (TD.95092, 7% Corn Oil Diet; Harlan Teklad, Madison, WI) throughout the duration of the experiment.

Following 2 weeks on Pb-acetate water, non-agouti A^{vy}-strain wild-type *a/a* dams of 7–10 weeks of age were mated with age-matched viable yellow agouti A^{vy}/a males. The animals were provided with access to chow and water *ad libitum*. Pb-acetate water was provided to dams throughout pregnancy and lactation. Following weaning at postnatal day 21, a subset of control and Pb-exposed *a/a* wild-type non-agouti offspring (approximately 1 male and 1 female per litter) were maintained on Pb-free water and followed until 40 weeks of age. Mice were conventionally housed in polycarbonate-free cages, sex-separated, with littermates combined in cages.

The microbiome study herein included 13 (4 females and 9 males) control mice and 15 (7 females and 8 males) Pb-exposed mice. Details of mice information are listed in Supplementary Table S1. Mice were weighed in weekly intervals except during weeks when they were transported to the Michigan Metabolic and Obesity Center (MMOC) facility for metabolic measurements as well as measurement of lean percentage, fluid percentage, and fat percentage by using an nuclear magnetic resonance analyzer (Minispec LF90II, Bruker Optics), as a part of a separate study (Faulk *et al.*, 2014a). We considered the weeks prior to, during, and after transport to the MMOC to be "stressed" time points. Thus, for purposes of this study, the final nonstressed bodyweight time point was measured at 33 weeks of age, rather than at age at sacrifice (40 weeks).

Mice were monitored by the University of Michigan Unit for Laboratory Animal Medicine (MI), and our study protocols followed the guidelines for the care and use of laboratory animals during maintenance and sacrifice. The protocol for the study was reviewed and approved by the University of Michigan Committee on Use and Care of Animals.

Colon collection, bacterial cultivation and DNA extraction. Mice were sacrificed at 40 weeks of age, and colons were collected aseptically and weighed for further processing. For counting total bacterial numbers and extraction of total DNA, 2 ml of 1 × PBS buffer was mixed with tissue sample and homogenized using an OMNI probe homogenizer (Omni TH; Omni International) to release bacterial cells. The homogenized mixture was used for culturing total aerobic and anaerobic bacteria as well as total DNA extraction. A total of 100 μl of homogenized mixture was serially diluted and plated on LB agar and Schaedler agar plates (Schaedler *et al.*, 1965), respectively. LB agar plates were incubated at 37°C aerobically, and Schaedler agar plates were incubated at 37°C in an anaerobic jar with Gas-pak sachets (B-260680; Fisher Scientific, Waltham, MA). Colonies formed on both agar plates were recorded after incubating plates for 3–5 days. Total DNA was extracted from 1 ml of the homogenized mixture using the QIAamp DNA Stool Mini Kit (51540; Qiagen, Valencia, CA) according to the manufacturer's instructions.

High-throughput 16S ribosomal RNA gene pyrosequencing. Gut microbiota were examined using 454 DNA-pyrosequencing methodology. The variable region (3 to 5') of the bacterial 16S ribosomal RNA (rRNA) gene was amplified using 3 primer sets: forward primers combined in equal ratios in solution (Bac-338F1: CCTACGGGRGGCAGCAG; Bac-338F2: ACWYCTACGGRW GGCTGC; and Bac-338F3: CACCTACGGGTGGCAGC) and reverse primer Bac-909R (5′-CCGTCAATTYHTTTRAGT-3′), which was tagged with a unique 10-nt barcode (Pinto and Raskin, 2012). The PCR reactions were conducted in triplicate and were limited to 20 cycles to minimize formation of PCR artifacts. Each PCR reaction contained 10 μl of PfuULtra II hotstart mastermix (Stratagene, Santa Clara, CA), 0.2 μM of equimolar mix of the

forward primers, 0.2 μM reverse primer, 0.3 μg/ml of bovine serum albumin (Invitrogen, Carlsbad, CA), and a final DNA template concentration of 4 ng/μl of DNA. The amplification conditions were as follows: 95°C for 2 min, 20 cycles of 95°C for 20 sec, 50°C for 20 sec, and 72°C for 40 sec, followed by a final extension at 72°C for 5 min. After PCR amplification, triplicate PCR products for each sample were pooled and purified using a QiaQuick PCR purification kit (Qiagen Inc, Valencia, CA). Purified amplicons with different barcodes were pooled in equimolar ratios and cleaned. Amplicon pyrosequencing was done using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Branford, CT) at Macrogen Inc, South Korea.

DNA sequence data analysis. DNA sequencing data processing was conducted using Mothur (Schloss *et al*., 2009) and focused primarily on α and β diversity-based analyses. A total of 291,663 sequences were obtained for the 28 samples within this study. The sequences were trimmed to remove primers and barcodes, quality filtered, and chimera checked as described previously (Pinto and Raskin, 2012), resulting in a total of 264,269 sequences in the final data library. A total of 2,468 sequences in each sample were used for further statistical analysis.

α and β diversity and sequence statistical analyses. The sequences were clustered using the average neighbor approach (Quince *et al*., 2009) to form operational taxonomic units (OTUs) at 97% sequence similarity cutoff (3% sequence divergence) by using SILVA (from Latin silva, forest)-compatible alignment database. Prior to further analyses, all samples were normalized to ensure an equal number of sequences in each sample. Phylogenetic trees were constructed using the Clearcut program (Sheneman *et al*., 2006). An OTU-based approach was used for the beta diversity measurements. A heatmap of the relative abundance of each OTU across the 28 samples was generated using log2 scaling the relative abundance values of top 50 OTUs. Molecular analysis of molecular variance (AMOVA) statistical analysis (Schloss and Handelsman, 2006) was performed to determine significance of structural similarity among communities across sampling groups. The UniFrac analysis (Hamady et al., 2010) was used to estimate weighted and unweighted UniFrac metrics (Lozupone and Knight, 2005). Constrained ordination redundancy analysis (RDA) was calculated (Borcard *et al*., 2011), and the significance of environmental variables was checked by forward selection (Blanchet *et al*., 2008). The characterization of microorganismal features differentiating the gut microbiota specific to Pb exposure group was performed using the linear discriminant analysis effect size method (http://huttenhower.sph.harvard.edu/lefse/, accessed January, 2016) for biomarker discovery, which emphasizes both statistical significance and biological relevance (Segata *et al*., 2011).

Statistical analysis. Litter effects were estimated by using linear-mixed models (Lazic and Essioux, 2013). When the litter effects were significant ($P < .05$), power was analyzed (Wickham, 2011) and outcomes were adjusted for litter. Associations between the bodyweight and microbiota composition were assessed. Specifically, a Bayesian variable selection algorithm (Wen, 2014) was used to identify OTUs associated with bodyweights controlling for the sex effect. Although the OTUs are generally correlated in various degrees, this algorithm allows identifying independent association signals that jointly predict the bodyweight. After the variable selection, the effects of the selected OTUs on bodyweights were estimated by fitting a multiple linear regression model.

Sequence accession numbers. The raw pyrosequencing data were deposited at NCBI Sequence Read Archive (http://trace. Ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession No. SRP058304.

RESULTS

Altered Adult Bodyweight in Male Offspring Exposed to Pb

Overall, male mice weighed more than female mice (46.2 ± 3.5 and 36 ± 4.3 g, respectively; $P < .05$). Stratifying by sex, male offspring (but not female offspring) displayed sustained differences in bodyweight between control and exposure groups (Figure 1). Male mice exposed to Pb were heavier than controls ($P < .05$; effect size = 2.59). When compared with the average weight of adult control male mice, Pb exposure resulted in about 11% increase in bodyweight. No significant differences were observed in female mice ($P = .24$).

Changes in Numbers of Cultivable Gut Bacteria in Offspring Exposed to Pb

Significant exposure group differences in both aerobic and anaerobic bacterial numbers were noted, as measured by total cultivable gut bacteria (Figure 2). Control mice had more cultivable aerobes than Pb-exposed mice ($P < .005$), whereas there were dramatically more anaerobes in Pb-exposed mice than control mice ($P < .05$). No significant sex effects were noted for cultivable bacteria ($P = .45$).

No Significant Differences in the Richness and Diversity of Gut Microbiota

Overall, a total of 291,663 sequences with an average length of 440 bp were obtained. After removing unmatched primers and barcodes, quality filtering, and chimera checking, a total of 264,269 bacterial sequences and 5,997 unique sequences were obtained for all 28 samples. The lowest sequence count was 2,468, and this number was used as the cutoff in all 28 samples for further analysis. The rarefaction curves (Supplementary Figure S1a) depict unprecedented levels of bacterial complexity in all pyrosequenced colon samples. There were no significant differences in rarefaction of gut microbiota between control and Pb exposure groups, suggesting that bacterial richness in those 2 groups was similar. The average inverse Simpson index of the Pb-exposed group was slightly lower than control, but the differences were not statistically significant ($P = .23$) (Supplementary Figure S1b), indicating the perinatal Pb exposure does not affect the richness and diversity of gut microbiota in adult mice.

Changes in Adult Gut Microbiota in Offspring Exposed to Pb

In a heatmap depicting the top 50 OTUs (Figure 3), blue and red dots after each row represent control or Pb-exposed mice, respectively. Gut microbiota clustered into 2 groups based on their similarity. One cluster contains 8 mice separated into 2 subgroups, which perfectly aligns with exposure grouping. The second cluster shows 20 samples separated into 2 subgroups according to Pb exposure with few exceptions. When the branch length from the dendrogram was incorporated (weighted Unifrac method), the difference in gut microbiota was significant between control and Pb-exposed mice ($P < .001$). When the branch length from dendrogram was not incorporated (unweighted Unifrac method), no significant difference was

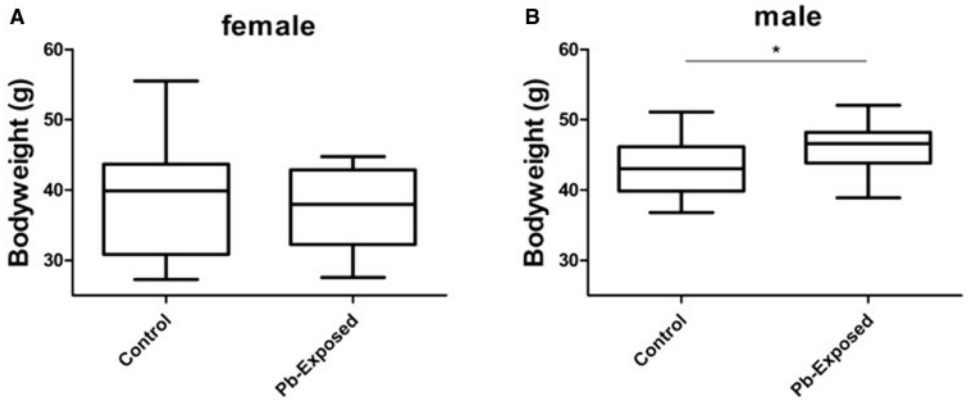

FIG. 1. Adult bodyweight of week 33 female and male mice. Student's *t* test was used for significance testing. *$P < .05$.

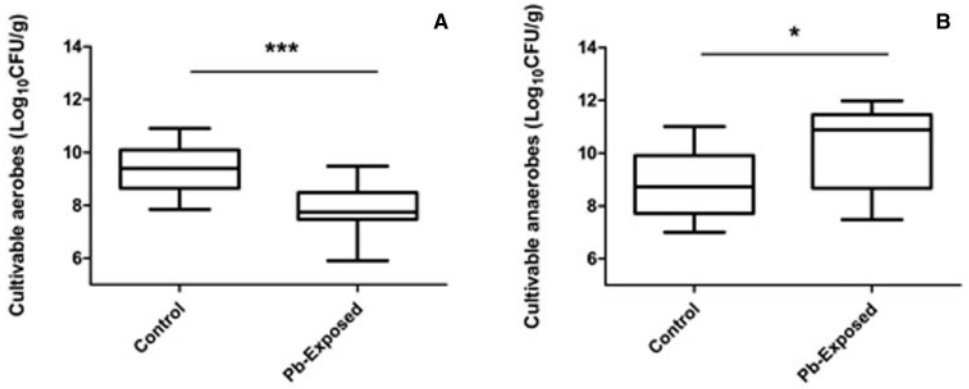

FIG. 2. Cultivable aerobic (A) and anaerobic (B) bacteria recovered from colon tissue. Data are presented in Log10 unit per g of wet tissue. Student's *t* test was used for significance testing. *$P < .05$, ***$P < .005$.

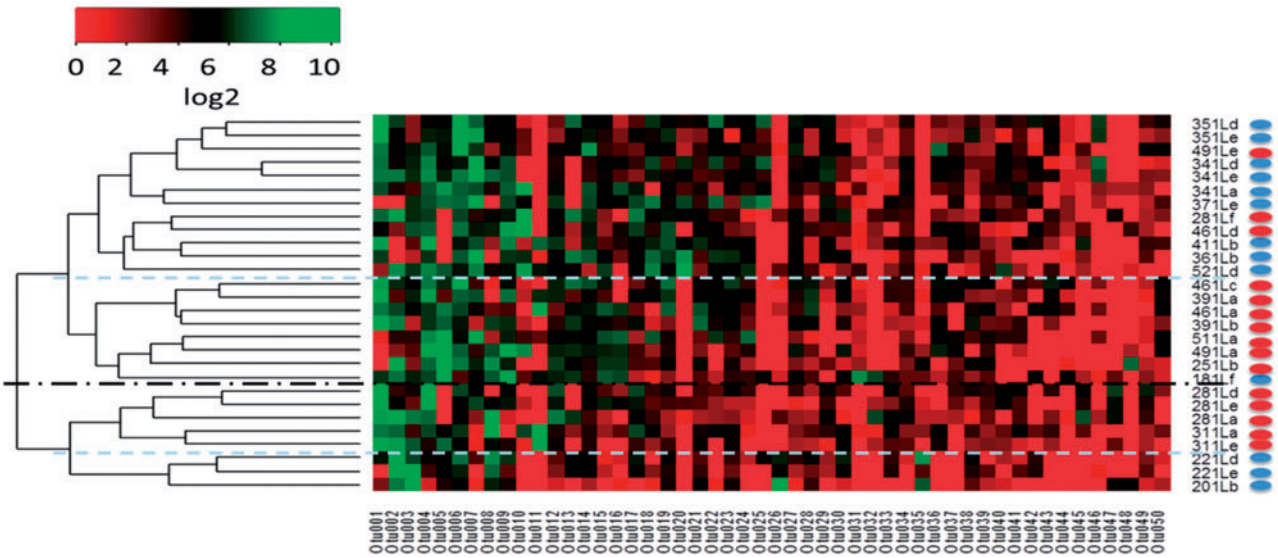

FIG. 3. A heatmap dendrogram generated with the top 50 OTUs. The values of OTUs were log2 transformed. The red colors in the heatmap indicate communities that were more similar than those with black colors. Red and blue dots after each row presents control and Pb-exposed mice, respectively.

observed ($P = .19$). The AMOVA result confirmed that the difference between control and Pb exposure groups contained a significantly different centroid for this study ($P < .05$). No significant sex effects were observed in this analysis ($P = .32$). Bodyweight, lean percentage, fluid percentage, fat percentage, sex, litter, and Pb exposure were used as environmental variables for the RDA analysis. Among them, exposure, litter, and fluid percentage had more power as driving factors, whereas

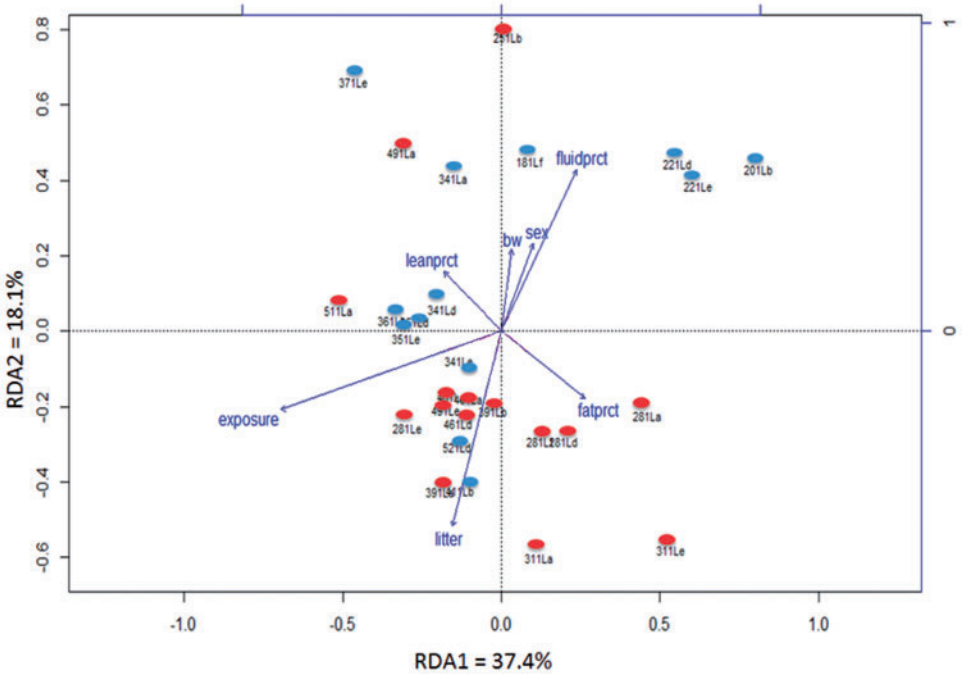

FIG. 4. The gut microbiota RDA plot with environmental variables. Pb exposure was negatively correlated with RDA1 axis, which could explain 37.4% proportion of the separation. The red dots in the RDA plot represent Pb-exposed offspring, and blue dots represent control offspring. Fluidprct, fluid percentage; fatprct: fat percentage; leanprct, lean percentage; bw, bodyweight.

TABLE 1. Forward Selection of Variables Associated With Bacterial Community

Variables	AIC	*F*
Exposure	−45.2	2.72**
Fat percentage	−43.7	1.20
Litter	−43.7	1.24
Fluid percentage	−43.7	1.19
Lean percentage	−43.6	1.12
Bodyweight	−43.2	0.79
Sex	−43.1	0.69

**$P \leq .05$.

sex, bodyweight, and lean percentage had less impact (Figure 4). Among them, Pb exposure alone was significant ($P \leq .05$) by forward selection analysis (Table 1). Moreover, the direction of exposure was negatively correlated with RDA1 axis, which could explain 37.4% proportion of the separation (Figure 4). This suggests that Pb exposure is the major factor in separating gut microbiota in control and Pb-exposed mice.

Differences in Taxonomic Composition in Gut Microbiota in Offspring Exposed to Pb

Overall, 395 OTUs were detected from all 28 gut microbiota samples. Bacteroidetes and Firmicutes were the 2 predominant phyla detected. They accounted for 37.9 ± 15.8% and 56.5 ± 15.7% of total bacteria, respectively. Actinobacteria and Proteobacteria were the less represented in mice gut microbiota, accounting for 0.56 ± 0.84% and 2.0 ± 1.8% of total bacteria, respectively. However, Bacteroidetes and Firmicutes had significant inverse exposure-related changes. When compared with control (46.0 ± 15.5%), Bacteroidetes were dramatically reduced to 29.2 ± 11.3% in Pb-exposed mice ($P < .005$) (Figure 5A). Conversely Firmicutes were significantly increased in Pb-exposed mice, 64.8 ± 13.4%, when compared with control mice, 48.2 ± 13.7% ($P < .005$) (Figure 5B). Exposure-related differences were only observed at the phylum level, and these differences were not sex related. No significant differences were observed at lower taxonomic levels with the exception of the order of Desulfovibrionales (Figure 5C), which belongs to the phylum of Proteobacteria. Pb-exposed mice displayed significantly more Desulfovibrionales (2.3 ± 1.6%) than control mice (0.6 ± 1.2%; $P < .005$). A cladogram representative of the structure of the gut microbiota and their predominant bacteria is shown in Figures 6A and B. The greatest differences in taxa between the control and Pb exposure groups are displayed, e.g., a greater abundance of Desulfovibrionaceae, Barnesiella, and Clostridium XIVb was found in Pb-exposed mice, and a greater abundance of Lactococcus, Enterorhabdus, and Caulobacterales was found in control mice. Intriguingly, *Akkermansia nuciniphila*, the only species in genus *Akkermansia*, an obesity-associated bacterium (Cani and Everard, 2014; Everard *et al.*, 2013; Lukovac *et al.*, 2014), was nearly undetected in Pb-exposed mice. No significant differences were observed in Actinobacteria and Proteobacteria between control and Pb-exposed mice. Detailed differences in OTUs are listed in Supplementary Table S2.

Associations Among the Gut Microbiota Composition, Pb Exposure, and Bodyweight in Males

Spearman correlation analysis showed that Pb exposure and specific OTUs were highly associated. OTU20 (Bacteroides from phylum Bacteroidetes) and OTU63 (Lactococcus from phylum Firmicutes) displayed significant correlation with Pb exposure (*F* statistic: 7.669; $P = .003$), and no significant sex effects were found ($P = .77$). The Bayesian variable selection procedure identified 2 associated OTUs, OTU72 (unclassified in phylum Bacteroidetes) and OTU78 (Enterorhabdus from phylum

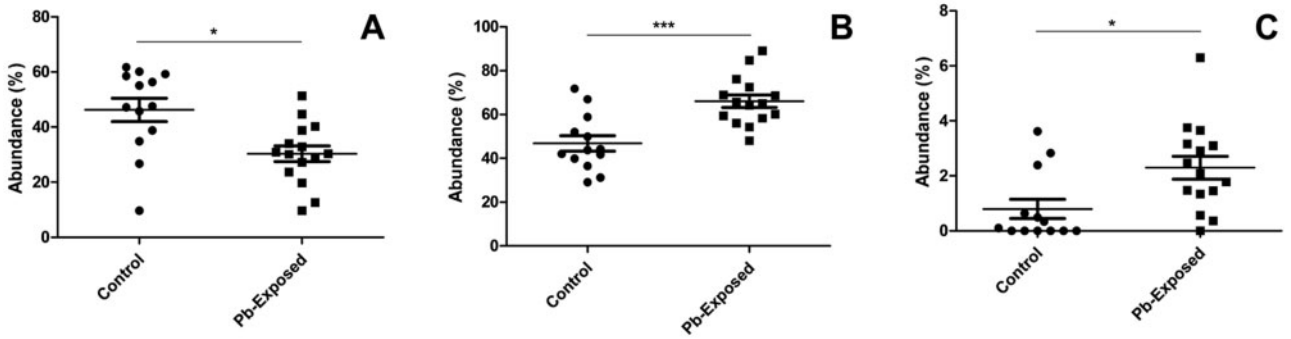

FIG. 5. The abundance of phylum Bacteroidetes (A), phylum Firmicutes (B), and order Desulfovibrionales (C) in mice gut microbiota. Student's *t* test was used for significance testing. $^{*}P < .05$, $^{***}P < .005$.

Actinobacteria). The correlation between these 2 OTUs and bodyweight is modest ($r^2 = .02$ or $\rho = -.15$). By fitting a multiple linear regression model including both of the selected OTUs (also controlling for sex), the association *P* values for OTU72 and OTU78 are 1.6×10^{-4} and 1.2×1^{-4}, respectively.

DISCUSSION

Epidemiological studies show mixed evidence in the association between blood Pb level in childhood and obesity later in life (Cassidy-Bushrow *et al.*, 2015; Scinicariello *et al.*, 2013; Wang *et al.*, 2015). However, these studies are cross-sectional, and therefore have reduced power to potential confounding cohort effects in determining Pb's long-term effects. A longitudinal epidemiological study shows a strongly positive relationship of bone Pb level and obesity in children that may persist in adulthood (Kim *et al.*, 1995). In the current study, we report changes in gut microbiota and sex-dependent response of bodyweight in adult mice following perinatal exposure to an environmentally relevant concentration of Pb acetate (32 ppm, mean dam BLL 32 μg/dL) in drinking water. These findings of altered microbiota and bodyweight in adulthood following perinatal Pb exposure represent a potential mechanism of the developmental origins of health and disease paradigm and may underlie the sex-specific effects on adult food intake, fat, weight, and insulin response across the murine life-course following perinatal Pb exposure, reported by our group in 2014 (Faulk *et al.*, 2014a).

First, we observed perinatal Pb exposure was significantly associated with increased adult bodyweight in males ($P < .05$) but not in females ($P = .24$). Second, we observed that perinatal exposure to Pb shifted gut microbiota composition in adult (age 40 weeks) offspring, despite cessation of Pb exposure at 3 weeks of age. The total cultivable aerobic and anaerobic bacterial numbers were significantly different by exposure group. Bacteria in the genus Pseudomonas, Enterobacter, and Desulfovibrio had greater abundance in adult mice exposed perinatally to Pb than controls ($P < .05$). These same 3 genera of bacteria have been demonstrated to be more resistant to Pb in contaminated soil (Roane and Kellogg, 1996; Sani *et al.*, 2003; Sobolev and Begonia, 2008), suggesting that they may be under positive selection in the gut microbiota after Pb exposure. Although the inverse Simpson index did not reach a significant level, the inverse Simpson of Pb exposure group was slightly less than the control, implying that the diversity of gut microbiota in Pb exposure group was disrupted and was not as resilient as control gut microbiota. Taken together, this study indicates that Pb may have long-lasting impact on the bacterial composition in the adult digestive system following early life exposure.

Second, we found the proportions of 2 predominant major phyla, Bacteroidetes and Firmicutes, had significant inverse changes associated with Pb exposure. The ratio of Bacteroidetes/Firmicutes has been demonstrated to be important in maintaining human health, including determining bodyweight (Krznaric *et al.*, 2012). Obese people are more likely to have a smaller proportion of Bacteroides than Firmicutes, whereas the ratio of Bacteroides/Firmicutes increases as fat mass decreases (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). This "obese microbiota" has increased capacity to harvest energy from the diet. Most importantly, this trait is transmissible between subjects (Musso *et al.*, 2010, 2011; Ray, 2012; Turnbaugh *et al.*, 2006). Interestingly, we found that 2 health-associated genus, *Akkermansia* and *Desulfovibrio*, were significantly correlated with Pb exposure ($P < .05$). *Akkermansia* was also completely abolished in the Pb exposure group, whereas *Desulfovibrio* was enriched in the Pb exposure group. *Akkermansia* has been recently demonstrated as being partial protective against adiposity and inflammation (Cani and Everard, 2014; Caputo *et al.*, 2015; Everard *et al.*, 2013; Lukovac *et al.*, 2014) and improving metabolic obesity (Dao *et al.*, 2015). *Desulfovibrio* was first studied *in vitro* and found to have the ability to convert choline to trimethylamine (TMA) (Baker *et al.*, 1962) which is further oxidized in the liver to TMA *N*-oxide (TMAO). Colonization with TMA producers results in both reduction in bioavailability of choline and the accumulation of TMAO, which has high correlation with cardiovascular disease and colon cancer (Bae *et al.*, 2014; Tang *et al.*, 2013; Wang *et al.*, 2011, 2014). Thus, our data suggest that perinatal exposure to Pb results in altered gut microbiota, which may be an additional contributing factor to increased adult bodyweight, and other diseases as well. We acknowledge that our sample size is small and the power to detect associations with small effects is generally limited. Instead of focusing on individual OTUs, our analysis pooled phylogenetically similar OTUs into higher level of taxonomy and examined and measured its proportion in each gut bacteria population. At this measurement level, we observed greatly reduced variation across mice, the similar phenomenon is also confirmed by Benjamini (2010).

Third, we explored the correlation of Pb exposure and the gut microbiota as well as gut microbiota and bodyweight. The gut microbiota compositions in the control and Pb exposure groups displayed significant differences by weighted Unfrac and AMOVA analysis in regardless of sex. Forward selection and RDA analysis showed Pb exposure, but not litter or other factors, was the main factor driving the separation in gut microbiota. Association of Pb exposure and OTU analysis revealed that OTU20 and OTU63 were highly correlated with Pb exposure regardless of sex, suggesting that OTU20 (Bacteroides from

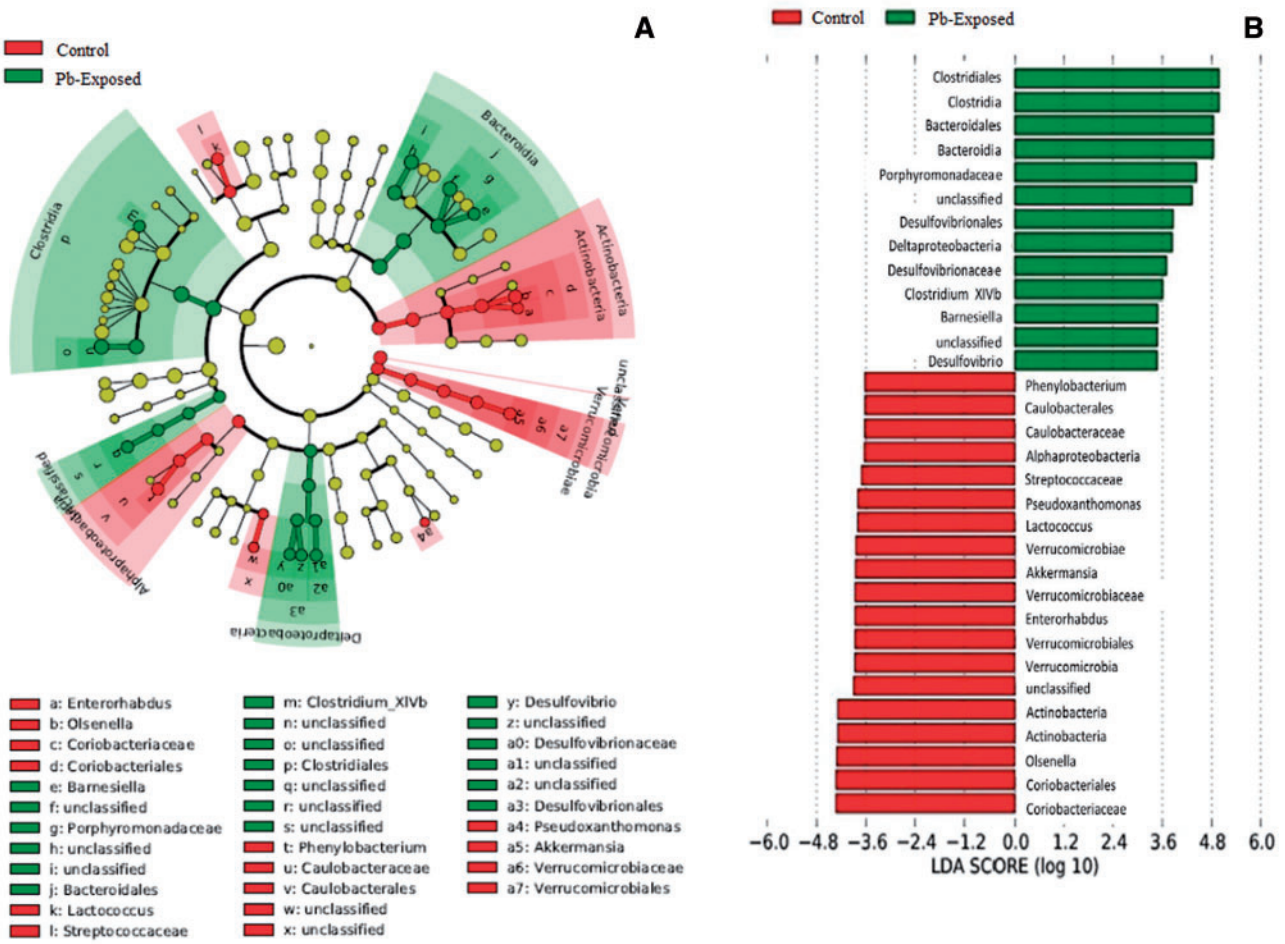

FIG. 6. Linear discriminant analysis effect size (LEfSe) cladogram showed the most differentially abundant taxons between control and Pb-exposed groups. Taxonomic cladogram obtained from LEfSe analysis of pyrosequencing sequences. Red, Taxa enriched in controls. Green, Taxa enriched in exposures. The brightness of each dot is proportional to its effect size (A). Only taxa meeting an linear discriminant analysis (LDA) significant threshold > 2 are shown (B).

phylum Bacteroidetes) together with OTU63 (Lactococcus from phylum Firmicutes) can be potentially used as a joint OTU marker for prediction of Pb exposure. Joint variable selection for association of weight and OTUs suggested that OTU72 and OTU78 had predicting power for bodyweight in males (*F* statistic: 8.23; $P = .0001$) but not in females.

Previous research show that sex plays an important role in the pathophysiology of obesity and hormones and other sex-relevant factors were postulated as part of reasons (Faulk *et al.*, 2013, 2014a; Leasure *et al.*, 2008; Uceyler *et al.*, 2010). Similar to these findings, here we show exposure-dependent sex effects with bodyweight in adult mice. In contrast, gut microbiota analysis in adult mice reveals similar effects by exposure on both male and female mice. Because the altered gut microbiota was reported to highly associate with weight gain, this may imply that Pb exposure is a direct factor in changing gut microbiota, and gut microbiota, with other sex-relevant factors, mediate the effects of Pb on adult bodyweight. Insight into the role of protective factors in female mice may be relevant to the prevention and treatment of obesity in humans.

We used 16S rDNA sequences because numerous studies have demonstrated that gut microbiota play a key role in the development of obesity, and the bacterial composition reflects its functional characteristics (Ley *et al.*, 2005, 2006; Ridaura *et al.*, 2013; Turnbaugh *et al.*, 2006, 2009). However, direct functional characterization would enhance our understanding of the consequences of these changes of gut microbiota. Recent studies in mice and humans reveal the correlation between obesity and the Firmicutes/Bacteroidetes ratio. A high-fat diet promotes an increase of Firmicutes and a relative reduction of Bacteroidetes. The shift in those 2 phyla is associated with more effective caloric intake that leads to weight gain and higher likelihood of obesity (Ley *et al.*, 2006; Tilg *et al.*, 2011). In our study, we find similar trends following perinatal Pb exposure. Specifically, perinatal Pb exposure was associated with a higher Firmicutes/Bacteroidetes ratio in adult gut microbiota of exposed offspring compared with unexposed offspring. In addition, the association of alteration of specific groups of bacterial species, such as *Akkermansia*, to weight gain, is also in agreement with previous reports (Cani and Everard, 2014; Caputo *et al.*, 2015; Lukovac *et al.*, 2014). We further demonstrate that the alteration of gut microbiota has a strong association with the weight gain in a sex-specific manner. Future functional metagenomic and/or metabolomic analyses of Pb-altered gut microbiota and their relationship with bodyweight are necessary to fully understand the long-term toxicity of perinatal Pb exposure. Additional study of the responses of the identified genera or OTUs like *Akkermansia* and their impact in metabolism and weight gain of adult mice would confirm our observations based on the composition of gut microbiota.

The interaction between pathogens and toxic agents and the impact on disease development in humans have recently been recognized by the National Institute of Environmental Health Sciences as a new research paradigm (Birnbaum and Jung, 2010; Feingold et al., 2010). Our study and others focusing on the association of perinatal exposures and adult microbiota composition are needed to increase the understanding of environmental impacts on the gut microbiota (Betts, 2011). Enhanced recognition of gut microbiota composition, especially at early-stage development and its response to toxic exposures may increase our understanding of how the environment affects health, including obesity (Cho and Blaser, 2012; Metsala *et al.*, 2015; Murk *et al.*, 2011; Patrick *et al.*, 2011). Although much work remains to be done, this study provides insight into how the adult gut microbiota is shaped by Pb exposure in early life, and how that may contribute to disease risk in later life.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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