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Arsenic Exposure Perturbs the Gut Microbiome and Its Metabolic Profile in Mice: An Integrated Metagenomics and Metabolomics Analysis

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Running Title: Arsenic perturbs the gut microbiome and its function

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Abstract

Background: The human intestine is host to an enormously complex, diverse, and vast microbial community, the gut microbiota. The gut microbiome plays a profound role in metabolic processing, energy production, immune and cognitive development, epithelial homeostasis, and so forth. However, the composition and diversity of the gut microbiome can be readily affected by external factors, which raises the possibility that exposure to toxic environmental chemicals leads to gut microbiome alteration or dysbiosis. Arsenic exposure affects large human populations worldwide and has been linked to a number of diseases, including cancer, diabetes and cardiovascular disorders.

Objectives: We investigated the impact of arsenic exposure on the gut microbiome composition and its metabolic profiles.

Methods: We used an integrated approach combining 16S rRNA gene sequencing and mass spectrometry-based metabolomics profiling to examine the functional impact of arsenic exposure on the gut microbiome.

Results: 16S rRNA gene sequencing discovered that arsenic significantly perturbed the gut microbiome composition in C57BL/6 mice following exposure to 10 ppm arsenic for 4 weeks in drinking water. Moreover, metabolomics profiling revealed a concurrent effect, with a number of gut-flora-related metabolites being perturbed in multiple biological matrices.

Conclusions: These data clearly illustrated that arsenic exposure not only alters the gut microbiome community at the abundance level, but also substantially disturbs its metabolic profiles at the function level. These findings may provide novel insights regarding perturbations of the gut microbiome and its functions as a potential new mechanism by which arsenic exposure leads to or exaggerates human diseases.

Introduction

The human body is host to 100 trillion gut microbes, ~ 10-times more than all human cells (Ley et al. 2006). It is estimated that the ~ 500-1000 species residing in the human gut encode 100-fold more unique genes than the human genome. The gut microbiota has important functions in metabolic processing, energy production, immune cell development, food digestion, epithelial homeostasis, and so forth (Young et al. 2008). Mounting evidence indicates that dysregulated gut microflora contributes in a significant way to a variety of diseases, including diabetes, obesity, cardiovascular diseases, allergies, inflammatory bowel disease and others (Ley et al. 2005; Wang et al. 2011; Qin et al. 2012). For example, obese individuals exhibit a remarkable reduction in the abundance of *Bacteroidetes* and a relative increase in *Firmicutes* compared to lean individuals (Turnbaugh et al. 2006). Likewise, a metagenome-wide association study revealed that beneficial butyrate-producing bacteria are less abundant and that opportunistic pathogens are more abundant in individuals with diabetes than in healthy controls (Qin et al. 2012). The microbiome evolves through several transitions during the first years of life and thereafter remains relatively constant if no significant perturbations occur. However, the composition of the gut microbiome is highly diverse, and this diversity can be readily affected by external factors, such as environment, diet, bacterial/viral infection and antibiotics. This raises the possibility that exposure to toxic environmental chemicals leads to gut microbiome alteration or dysbiosis as a mechanism by which environmental agents exert their detrimental effects on human health.

Exposure to arsenic affects large human populations worldwide, with contamination of drinking water by geological sources of inorganic arsenic being the primary route of exposure. Hundreds of millions of people around the world, especially in South and East Asia, drink water with arsenic levels that far exceed the 10 µg/L guideline, established or accepted by WHO and US

EPA (Hughes et al. 2011). In the United States, as many as 25 million people are estimated to drink water with an arsenic level above 10 $\mu\text{g/L}$, as private wells are not regulated by EPA and other agencies (Kozul et al. 2009). Arsenic exposure has been associated with a number of diseases, such as skin, bladder, lung, and liver cancers, diabetes, as well as cardiovascular disorders (Hughes et al. 2011; Van de et al. 2010). More recently, arsenic exposure has been linked to an increased incidence of diabetes in animal models and human population studies (Paul et al. 2011). Numerous mechanisms have been proposed to be responsible for arsenic-induced diseases, including the interaction with sulfur, oxidative stress, genotoxicity, altered DNA repair and signal transduction, cell proliferation and epigenetics (Hughes et al. 2011; Smeester et al. 2011; Ren et al. 2011; Hou et al. 2012). Accumulating evidence indicates that perturbations of the gut microbiome and its influence on metabolic and physiological functions may play an important role in the development of human diseases. Given the essential role of the gut microbiome in a variety of aspects of human health coupled with the high toxicity of arsenic, there is a need to elucidate the effects of arsenic exposure on the gut microbiome and its functions. In particular, several previous seminal studies have reported interactions between the gut microbiome and environmental chemicals, such as arsenic, mercury, polycyclic aromatic hydrocarbons and polychlorinated biphenyls (Pinyayev et al. 2011; Liebert et al. 1997; Van de et al. 2005; Van de et al. 2010; Choi et al. 2013).

The gut microbiome has profound roles in modulating host metabolism. For example, non-digestible carbohydrates are degraded via fermentation by the gut bacteria to yield energy for microbial growth and microbial end products that act as energy substrates, inflammation modulators and signaling molecules (Holmes et al. 2011). Therefore, the reach of the gut microbiome on host metabolism extends well beyond local effects in the gut to diverse remote

organ systems, such as liver, brain, adipose and muscle (Claus et al. 2011; Diaz et al. 2011). Accumulating evidence indicates that metabolic perturbations associated with gut microbiome composition changes are important risk factors for developing diseases (Wang et al. 2011; Jones et al. 2008). For example, gut flora-generated trimethylamine N-oxide (TMAO) from dietary choline and carnitine has been strongly associated with atherosclerosis in animal models and clinic cohorts (Wang et al. 2011). Therefore, it is of particular interest to probe the gut microbiome-related metabolic changes associated with arsenic-perturbed gut microbiota community. In this aspect, mass spectrometry-based metabolomics profiling is highly attractive because of its high sensitivity, ability to detect molecules with diverse structures, wide dynamic range, quantitative capability and the ease of interfacing with other separation techniques such as liquid chromatography (Lu et al. 2012).

In this study, we applied an integrated approach combining 16S rRNA gene sequencing and liquid chromatography-mass spectrometry (LC-MS) metabolomics to analyze the effects of arsenic exposure on the gut microbiome and its metabolite profiles. Metagenomics sequencing revealed that arsenic exposure significantly perturbed the gut microbiome composition in C57BL/6 mice. Our non-targeted metabolomics profiling revealed a notable effect of arsenic exposure in these mice, with diverse perturbed metabolites being correlated with gut microbiome changes.

Materials and Methods

Animals and exposure

Specific pathogen free C57BL/6 female mice (~ 6 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were provided pelleted rodent diet (ProLab 3000; Purina Mills, MO) and filtered water ad libitum and were maintained in AAALAC accredited facilities. Mice (total number = 20, 10 mice/group, body weight = 20 ± 3 g) were housed in static microisolator cages (5 mice/cage) on heat-treated hardwood bedding, under environmental conditions of 22 °C, 40-70% humidity and a 12:12h light:dark cycle. All experiments were approved by the MIT Committee on Animal Care. The animals were treated humanely and with regard for the alleviation of suffering. Inorganic arsenic (arsenic, 10 ppm) was administered to mice (~ 8 weeks old) as sodium arsenite (Fisher Scientific, Waltham, MA) in drinking water for 4 weeks. Freshly prepared arsenic-containing water (10 ppm) was provided to mice every Monday and Thursday.

Animal monitoring and histological analysis

Throughout the experiments, mice were assessed daily for evidence of diarrhea, dehydration, and deteriorating body condition. Mice were euthanized with CO₂ and necropsied after 4 weeks of arsenic consumption. Formalin-fixed tissues were routinely processed, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and evaluated by a board-certified veterinary pathologist blinded to the sample identity. Inflammation, edema, epithelial defects, hyperplasia, and dysplasia of multiple regions of liver (left lateral lobe, media lobe, right lateral lobe and caudate lobe) and colon (distal, transverse and proximal colon) were scored on an ascending scale (0 to 4, with 0 being normal) of severity and invasiveness of the lesion if any. Pathological scores did not show any significant difference between the control and arsenic-

treated mice and were accordingly not presented herein. We also did not observe any significant changes in body weights, mortality and food intake.

16S rRNA gene sequencing

DNA was isolated from fecal pellets collected during necropsy using a PowerSoil® DNA Isolation Kit as instructed by the manufacturer (MO BIO Laboratories, CA). The resultant DNA was quantified by a UV spectroscopy and stored at -80°C for further analysis. DNA was amplified using universal primers of U515 (GTGCCAGCMGCCGCGGTAA) and E786 (GGACTACHVGGGTWTCTAAT) to target the V4 regions of 16S rRNA of bacteria. Individual samples were barcoded, pooled to construct the sequencing library, followed by sequencing with an Illumina Miseq (Illumina, San Diego, CA) to generate pair-ended 150 x 150 reads.

Analysis of 16S rRNA sequencing data

The raw mate-paired fastq files were quality-filtered, demultiplexed and analyzed using Quantitative Insights into Microbial Ecology (QIIME) (<http://qiime.org>). For quality filtering, the default parameters of QIIME were maintained in which reads with a minimum Phred quality score less than 20, containing ambiguous base calls and containing less than 113bp of consecutive high quality base calls, were discarded. Additionally, reads with three consecutive low quality bases were truncated. The samples sequenced were demultiplexed using 8bp barcodes, allowing 1.5 errors in the barcode. UCLUST (<http://www.drive5.com/uclust>) was used to choose the Operational Taxonomic Units (OTUs) with a threshold of 97% sequence similarity. A representative set of sequences from each OTU was selected for taxonomic identification of each OTU using the Ribosomal Database Project (RDP) classifier (<http://rdp.cme.msu.edu>). The Greengenes OTUs (4feb2011 build) reference sequences (97% sequence similarity) were used as the training sequences for RDP. A 0.80 confidence threshold was used for taxonomic

assignment. The taxonomic assignment of 16S rRNA sequencing data could be achieved at different levels, including *phylum*, *class*, *order*, *family* and *genus*. Our analyses were typically conducted at the *family* level due to higher confidence in the assignment of taxa based on the sequencing reads, therefore, a significant change at the *family* level may reflect changes of multiple gut bacteria at *genus* and *species* levels. The sequencing data have been deposited in the MG-RAST server (<http://metagenomics.anl.gov/>).

Sample processing for metabolomics

Urine samples were collected one day before the euthanasia of mice using a metabolic cage with dry ice placed around the urine collection vessel to prevent oxidation or degradation of metabolites during the collection period (~ 16 h). Fecal pellets were also collected from individual animals. Plasma samples were collected during necropsy. Metabolites were isolated from urine using methanol as described previously (Lu et al. 2012). Cold methanol (80 μ L) was added to 20 μ L of urine or plasma. After vortexing for 1 minute, the samples were incubated at 4°C for 20 minutes and then centrifuged for 10 minutes at 12,000 rpm. The supernatant was collected, dried in a SpeedVac (Thermo Electron, Savant SC110A, Waltham, MA), and then resuspended in 30 μ L of 98:2 water:acetonitrile for MS analysis. Metabolite extraction from fecal pellets was conducted in a similar manner. Fecal pellets (25 mg) were dissolved in 400 μ L of cold methanol solution (methanol: water = 50:50), followed by vortexing at maximum speed for 10 minutes using a flat-bed vortex (MO BIO Laboratories, Carlsbad, CA). The supernatant was centrifuged for 10 minutes at 12,000 rpm, dried in a SpeedVac, and then resuspended in 30 μ L of 98:2 water:acetonitrile for metabolomics profiling.

Metabolomics profiling

LC-MS analyses were performed on a Quadrupole-time-of-flight (Q-TOF) 6510 mass spectrometer from Agilent (Santa Clara, CA) with an electrospray ionization (ESI) source. The mass spectrometer was interfaced with an Agilent 1200 High Performance Liquid Chromatography system. The Q-TOF was calibrated daily using the standard tuning solution from the Agilent Technologies. The typical mass accuracy of the Q-TOF was less than 10 ppm. Metabolites were analyzed in the positive mode only over a range of 80-1000 m/z using a C18 T3 reverse phase column from Waters (Milford, MA) due to higher numbers of detected molecular features as demonstrated previously (Lu et al. 2012). Metabolomics profiling data were processed as previously (Lu et al. 2012). MS/MS was generated on the Q-TOF to confirm the identity of perturbed metabolites. The metabolomics data were submitted to the XCMS Online server (<https://xcmsonline.scripps.edu/>).

Data processing of metabolomics data

Data acquired in Agilent .d format were converted to mzXML using MassHunter Workstation software from Agilent (Santa Clara, CA). Data were filtered by intensity; only signals with intensities larger than 1000 were considered. The converted data were processed by XCMS (Scripps, La Jolla, CA) (<https://xcmsonline.scripps.edu/>) for peak picking, alignment, integration and extraction of the peak intensities. To profile individual metabolite differences between control and arsenic treatment groups, a 2-tailed Welch's t-test was used ($p < 0.05$). The exact masses of molecular features with significant changes were searched against the Human Metabolome Database (HMDB, <http://www.hmdb.ca/>), METLIN (<http://metlin.scripps.edu>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases with a 10 ppm mass accuracy

threshold. The matched exact masses were stored and used for the generation of MS/MS data to identify the metabolites.

Statistical analysis of data

Principal component analysis (PCA) was performed to examine intrinsic clusters of metabolomics data. To identify potential outliers in samples, 95% confidence interval of all samples was used as the threshold. In addition, heat maps were generated using a hierarchical clustering algorithm to visualize the metabolite difference within the dataset. Principal Coordinate Analysis (PCoA) was used to compare the gut microbiome profiles between the control and treatment. The difference in the gut microbiome composition was further assessed using a non-parametric test via Metastats as described previously (White et al. 2009) (<http://metastats.cbcb.umd.edu/>). The correlation matrix between the gut flora-related metabolites and gut bacterial species was generated using Pearson's correlation coefficient.

Results

Workflow to probe functional changes of the gut microbiome

The experimental workflow combines 16S rRNA gene sequencing and metabolite profiling to examine the impact of arsenic exposure on the gut microbiome and its metabolic profiles (see Supplemental Material, Figure S1). Briefly, DNA was isolated from fecal pellets, amplified by PCR using 16S rRNA specific primers, followed by 150 x 150 bp paired-end sequencing using the Illumina Miseq platform. The resultant sequencing reads were processed by the QIIME and Metastats software packages to reveal exposure-induced gut microbiome changes. For metabolomics analysis, metabolites from fecal pellets, urine and plasma were extracted and analyzed by Q-TOF. Molecular features, i.e., all signals associated with a given analyte were

further processed and statistically analyzed with XCMS software to profile metabolites with significant changes (1.5 fold change, $p < 0.05$) between the control and arsenic-treated animals. The resultant peak list with exact masses was searched against metabolite databases including HMDB and METLIN. Next, the matched exact masses and associated retention times were used to generate MS/MS spectra to confirm the metabolite identities, followed by metabolic pathway or function analysis with the KEGG and HMDB database. Finally, correlations between the gut microbiome changes and shifted metabolome were examined to establish the functional impacts of arsenic exposure on the gut microbiome.

Arsenic-induced gut microbiome changes

Figure 1A shows the identified gut bacteria assigned at the *family* level from 16S rRNA sequencing reads, with each color representing an individual bacterial family (see Supplemental Material, Figure S2A and Table S1). In terms of the assignment at the *phylum* level, *Firmicutes* (52.79%) and *Bacteroidetes* (41.57%) are predominant in the gut bacteria of mice, followed by *Tenericutes* (3%), *Actinobacteria* (0.18%), *Cyanobacteria* (0.023%) and *Proteobacteria* (0.0042%), with 2.41% sequences unmatched with the database (see Supplemental Material, Figure S2B). The observations and assignments of gut bacteria at *phylum* level are consistent with previous reports that the gut microbiome consists of only several *phyla* (Turnbaugh et al. 2006). The taxonomic assignments and fold changes of gut bacterial components that were significantly changed ($p < 0.05$) are listed in Figure 1B. The difference in the gut microbiome patterns arising from arsenic is readily differentiated using multivariate statistical analysis, as shown by the PCoA plot in Figure 1C. The control and treated animals are well separated, with 19.95% and 10.66% variation explained by PC1 and PC2, respectively. Consistent with the PCoA plot, the Jackknifed beta diversity and hierarchical clustering analysis via Unweighted

Pair Group Method with Arithmetic Mean demonstrate that all control and treated animals cluster in their own groups, as shown in Figure 1D. In addition, control mice cluster into two sub-groups in the PCoA plot (Figure 1C) and UPGMA hierarchical clustering analysis (Figure 1D), which was attributed to individual variations of gut microbiome profiles, as illustrated in Figure 1A.

Arsenic-induced changes in metabolic profiles of the gut microbiome

The combination in feces of a large quantity of gut bacteria and their metabolic products creates an ideal biological sample to assess functional changes of the gut microbiome. Figure 2A illustrates that arsenic exposure perturbed the metabolic profiles of the gut microbiome, with 146 increased and 224 decreased molecular features, respectively. As shown in Figure 2B, the control and arsenic groups could be differentiated readily using metabolite fingerprints, with an excellent separation of the control and treated animals using the first two components of PCA. The hierarchical clustering heat map in Figure 2C also shows similar clustering patterns of detected molecular features within each group. Clear separations of metabolite profiles between control and arsenic-treated mice were also observed for the urine and plasma samples with a large number of perturbed molecular features (see Supplemental Material, Figure S3). A number of metabolites with greater than 1.5 fold changes between the control and treated mice were identified (see Supplemental Material, Tables S2-4) using the MS/MS approach (see Supplemental Material, Figure S4). The structures of these metabolites are diverse, including amino acid derivatives, bile acids, lipids, fatty acids, isoflavones, indole-derivatives, glucuronide and carnitine conjugates, with many being either directly generated or modulated by the gut bacteria.

Correlation between the gut microbiome and metabolites

To explore the functional correlation between the gut microbiome changes and metabolite perturbations, a correlation matrix was generated by calculating the Pearson's correlation coefficient, as shown in Figure 3A. Clear correlations could be identified between the perturbed gut microbiome and altered metabolite profiles ($\rho > 0.5$ or < -0.5 , $p < 0.05$). Figure 3B lists several typical gut flora-related metabolites that are highly correlated with specific gut bacteria to demonstrate the functional correlation between the gut microbiome and metabolites. For example, indolelactic acid that decreased 11.6-fold in arsenic-treated mice positively correlates with B9 family (*p_Tenericutes; f_Erysipelotrichaceae*), but negatively correlates with B8 family (*p_Firmicutes; f_Clostridiales Family XIII Incertae Sedis*). Likewise, daidzein positively correlates with the B4 (*p_Firmicutes; Other*) and B7 (*p_Firmicutes; f_Clostridiaceae*), respectively. Phenylpyruvic acid, indole-3-carbinol and glycocholic acid positively correlate with B2 (*p_Cyanobacteria; f_unassigned*), B5 (*p_Firmicutes; f_unassigned*), and B7 family (*p_Firmicutes; f_Clostridiaceae*), respectively, while dihydrodaidzein negatively correlates with B8 family (*p_Firmicutes; f_Clostridiales Family XIII Incertae Sedis*). In summary, arsenic exposure induces a significant taxonomic perturbation in the gut microbiome, which in turn substantially alters the metabolomic profile of the gut microbiome, as evidenced by changes of diverse gut-flora-related metabolites.

Discussion

In this study, we used high-throughput 16S rRNA gene sequencing and metabolomics profiling to study the impact of arsenic exposure on the gut microbiome and its metabolic profiles. The data clearly demonstrated that arsenic exposure induces a significant change in the gut microbiome composition of mice. In addition, these perturbed gut bacteria were strongly

associated with changes of a number of gut flora-related metabolites, indicating that arsenic exposure not only disturbs gut bacteria at the abundance level, also substantially alters the metabolomic profile of the gut microbiome resulting in disturbance of host metabolite homeostasis following exposure to arsenic. These findings may provide mechanistic insights regarding perturbations of the gut microbiome as a new mechanism of environmental chemical-induced human disease.

Accumulating evidence suggests that metabolic changes associated with gut microbiome perturbations are important risk factors for inducing abnormal tissue functions resulting in diseases, such as obesity, insulin resistance and cardiovascular disease (Wang et al. 2011; Jones et al. 2008; Turnbaugh et al. 2006). The gut microbiome could directly change its metabolic capacity and affect intestinal function locally through microbial products. For example, the gut microbiota has a strong effect on energy homeostasis in the colon, attributed to the utilization of butyrate produced by gut bacteria as the primary energy source for colonocytes (Donohoe et al. 2011). Likewise, altered gut microbiota can also trigger systemic effects on host metabolism in remote tissues, such as liver, brain, adipose and muscle, since the products of microbial metabolism could serve as signaling molecules or act in conjunction with the host on the metabolism of diverse chemicals to affect individual susceptibility to different diseases. For instance, TMAO, a gut flora-generated metabolite, has been identified to be strongly associated with atherosclerosis in a large clinical cohort (Wang et al. 2011). Atherosclerosis enhanced by dietary choline could be inhibited by suppressing intestinal microflora in atherosclerosis-prone mice, further highlighting the role of perturbations of gut-flora-generated metabolites in disease development.

It has been known that the gut microbiome plays a key role in the energy metabolism of the host. The non-digestible carbohydrates are degraded via fermentation in the colon by the gut microbiota to yield energy for the host and metabolic end products, such as short-chain fatty acids. Previous studies have documented that the imbalanced gut microbiome may be associated with human disease, such as obesity and diabetes (Qin et al. 2012; Turnbaugh et al. 2006). For example, Turnbaugh, *et al.*, found that obesity was associated with a large shift in the relative abundance of the specific taxa present, with a statistically significant reduction in *Bacteroidetes* and a significantly greater proportion of *Firmicutes* in obese mice (Turnbaugh et al. 2006). Likewise, transplantation of the caecal microbiota from obese mice fed on high fat diets into germ-free recipients increases adiposity significantly more than transplantation of a lean microbiota (Turnbaugh et al. 2006), further highlighting the role of the gut microbiome in affecting the efficiency of harvesting energy and defining obese/lean phenotypes. Another study also demonstrated the influence of gut microbiome perturbations on tissue adiposity of the host by delineating the effect of antibiotics use in early life (Cho et al. 2012). They discovered substantial taxonomic changes in the gut microbiome, changes in primary genes responsible for the metabolism of carbohydrates to short-chain fatty acids, and marked alterations in hepatic metabolism of lipids and cholesterol (Cho et al. 2012). In our study, bacteria in the family of *Bacteroidetes* were not increased by arsenic exposure, whereas four *Firmicutes* families were significantly decreased. Arsenic is not an obesogen and, but rather has anti-obesogenic properties, although the mechanism remains unknown (Paul et al. 2011). Excess accumulation of white adipose tissue in obesity is a risk factor for insulin resistance and the development of diabetes, since they regulate energy balance, lipid and glucose homeostasis, and releases a variety of signaling factors to affect insulin sensitivity and inflammation (Rosen and Spiegelman

2006). However, defects in adipogenesis in adipose tissue can also lead to insulin resistance and diabetes (Vigouroux et al. 2011; Hou et al. 2012). We identified several decreased *Firmicutes* families following arsenic exposure, and a number of *Firmicutes* species, such as *Eubacterium*, *Faecalibacterium* and *Roseburia*, are known to be butyrate producers (Tremaroli and Backhed 2012). Thus, arsenic-altered gut microbiome may affect energy harvesting, short-chain fatty acid production and adipogenesis. Another example to support that arsenic exposure impairs energy metabolism stems from the observation that fatty acid-carnitines were significantly reduced in urine of mice exposed to arsenic (see Supplemental Material, Table S3). Fatty acid-carnitines are used to transport fatty acids into mitochondria for fatty acid oxidation to generate metabolic energy; this process may be used to compensate for insufficient energy harvest due to disturbed gut microbiota following arsenic exposure. In agreement with this potential mechanism, germ free mice and lean individuals also have increased fatty acid oxidation and decreased lipogenesis (Backhed et al. 2007).

Indole-containing metabolites were significantly altered in fecal and urine samples following exposure to arsenic (see Supplemental Material, Figure S5A). Many of them are highly correlated with perturbed gut bacterial families. For example, 3-indolepropionic acid may serve as a specific indicator of imbalanced gut bacteria since a gut bacterial metabolic process is needed to synthesize this compound. A recent study identified *Clostridium sporogenes* as the only species, among 24 intestinal flora tested, to produce 3-indolepropionic acid (Wikoff et al. 2009). Thus, increased excretion of 3-indolepropionic acid in urine (+1.8 fold) may indicate that *Clostridium sporogenes* or species with similar functions have increased. In fact, among six significantly perturbed *Firmicutes* families, one *Firmicutes* family significantly increased following arsenic exposure (*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiales Family*

XIII Incertae Sedis, B8 in Figure 1B, fold change=+2.9). In particular, *Clostridium sporogenes* and this increased gut bacteria family are classified into the same *order* of *Clostridiales*. The formation of indole-containing metabolites is likely through the generation of indole by the intestinal bacteria that produce the enzymes to catalyze the conversion of tryptophan to indole, followed by further enzymatic processing. For example, absorbed indole could be converted to indoxyl in the liver, which is then sulfated to allow for urinary excretion. An increased amount of indoxyl in urine (+2.9 fold in mice exposed to arsenic) suggests an increased indole production by gut bacteria, which also supports increased amounts of other urine indole-related metabolites, such as IPA, indole-3-carboxylic acid and indoleacrylic acid. However, increased excretion of indoxyl in urine may also suggest that phase I/II biotransformations, including hydroxylation, sulfation and glucuronidation, were changed during exposure. An elegant previous study demonstrated a broad phase II metabolic response of the host to metabolites generated by the microbiome (Wikoff et al. 2009), as evidenced by the exclusive presence of numerous sulfated, glycine-conjugated, and glucuronide adducts in the serum of mice with normal gut microbiota compared to germ-free mice. In our study, we also found several glucuronide metabolites significantly reduced in urine (see Supplemental Material, Table S3), which may reflect an impaired glucuronidation capacity affected by a perturbed gut microbiome resulting from arsenic.

Isoflavone metabolites are also sensitive to gut microbiome changes (see Supplemental Material, Figure S5B). Previously, we discovered that daidzein was significantly increased in the serum of *Rag2^{-/-}* mice infected with *Helicobacter hepaticus* (Lu et al. 2012). Increased amount of daidzein was attributed to an altered gut microflora induced by *Helicobacter hepaticus* infection consistent with previous studies which have demonstrated that *Helicobacter* spp. infections

perturb the gut microflora in mice (Whary et al. 2006). Daidzein is converted by anaerobic bacteria in the large intestine to several metabolites, including dihydrodaidzein, O-desmethylangolensin, and equol, depending on the gut bacterial composition of the host. In this study, we detected significantly decreased daidzein and dihydrodaidzein, but increased O-desmethylangolensin in feces, suggesting that the formation of O-desmethylangolensin is favored in arsenic-treated mice. Of particular interest, O-desmethylangolensin is positively correlated with a *Firmicutes* family (*p_Firmicutes;c_Bacilli;o_Bacillales;Other*, +9.2 fold in arsenic-treated mice). It has been suggested that several candidate bacteria are participating in daidzein metabolism (Hur et al. 2000), although the intestinal bacteria responsible for daidzein metabolism in humans have not been unambiguously identified. Nevertheless, altered isoflavone metabolites may be used as bioindicators to probe changes in the gut microbiome composition arising from diverse environmental factors, such as bacterial infections (Lu et al. 2012) and toxic chemicals.

Bile acids and intermediates were significantly perturbed in arsenic-treated mice (see Supplemental Material, Table S2). Bile acids are cholesterol derivatives synthesized in the liver, and they undergo extensive enterohepatic recycling and gut microbial modification, including deconjugation and dehydroxylation. Bile acids not only have important functions in solubilizing cholesterol and facilitating the absorption of cholesterol, fat-soluble vitamins, and lipids from the gut, but also act as signaling molecules to regulate metabolic homeostasis via activating diverse nuclear receptors (Nguyen and Bouscarel 2008). In this study, 7- α -hydroxy-3-oxo-4-cholestenoate and its degradation product were excreted at a higher amount in exposed mice compared to controls. 7- α -hydroxy-3-oxo-4-cholestenoate is involved in the biosynthesis of primary bile acids (Kanehisa et al. 2012), and an increased excretion of 7- α -hydroxy-3-oxo-

4-cholestenoate and its degradation product may impair the synthesis and enterohepatic recycling of bile acids. Likewise, glycocholic acid decreased -2.3 fold in fecal samples of mice exposed to arsenic. These altered bile acid species indicate that arsenic exposure affects the homeostasis of bile acids. The underlying mechanisms remain elusive, but arsenic-induced gut microbiome perturbations may play a role in this process. Previous studies demonstrated a large effect of the gut flora on primary and secondary bile acid profiles in tissues of antibiotic treated rats (Swann et al. 2011). Moreover, specific microbial bile acid co-metabolites present in peripheral tissues, and pathway changes regulated by farnesoid X receptor indicate a broad signaling role for bile acids and highlight the symbiotic microbial influences in bile acid homeostasis in the host (Swann et al. 2011). Moreover, the role of gut bacteria in the regulation of bile acids has raised the question whether bile acids modulated by the gut microbiome are associated with regulation of the host immune system (Brestoff and Artis 2013). Previous studies have demonstrated that bile acid signaling via their receptors is linked to a common anti-inflammatory response in macrophages and monocytes (Brestoff and Artis 2013). In particular, it has been reported that chronic arsenic exposure significantly compromises the immune response to influenza A infection (Kozul et al. 2009). Clearly, further research is needed to shed light on the role of the gut microbiome and its associated metabolites, including bile acids, in arsenic-induced impaired immune response and other diseases.

In this study, we have demonstrated that arsenic exposure altered the gut microbiome and associated metabolomic profiles. However, future studies are warranted to delineate the mechanistic basis of these perturbations. As shown by the correlation analysis between the gut bacteria and metabolites, arsenic exposure can induce changes in the gut microbiome in terms of abundance, resulting in a shifted metabolome in the gut microbiome. Of equal importance,

arsenic exposure may also cause an altered metabolome by affecting the physiology of the gut bacteria without changing the species and abundance. Therefore, the changes in the metabolic profiles of the gut microbiome may not entirely depend on shifts in the spectrum of microbes revealed by 16S rRNA gene sequencing since metabolic changes could be achieved via other mechanisms in addition to altering the types and numbers of bacteria present in the gut, such as the regulation of gene and protein expression in a bacterium. A recent study has demonstrated that xenobiotics significantly altered the physiology and gene expression of the human gut microbiome (Maurice et al. 2013). Therefore, metatranscriptomics and metaproteomics profiling are warranted in the future to elucidate the role of arsenic exposure in altering the functions of the gut microbiome that encodes 100-fold more unique genes than the human genome and profoundly modulates host metabolism (Ley et al. 2006).

Our data show that arsenic exposure perturbs the gut microbiome composition and associated metabolic profiles, which represents an initial and critical step towards understanding how arsenic exposure affects the gut microbiome and its functions. Future studies are needed to address many intriguing questions in this exciting field. For example, it remains to be determined whether any changes to the gut microbiome and its associated metabolites occur in exposed humans since there are differences in the gut microbiome between mice and humans. The use of epidemiological studies combined with experiments in humanized gnotobiotic mice will allow researchers to better address species differences and elucidate the interaction between arsenic exposure and the gut microbiome in humans. In addition, while we treated mice by 10 ppm arsenic for 4 weeks, humans are usually exposed to much lower doses of arsenic for longer periods. Thus, the dose- and time-dependent effects of arsenic exposure on the gut microbiome need to be defined. Of particular importance is how arsenic impacts the gut microbiome during

the windows of susceptibility, as the establishment of the gut microflora is a temporal process after birth (Palmer et al. 2007). Thus, arsenic-induced perturbations in the gut microbiome may have age-related effects. Likewise, answers to other issues, such as gender-specific influence, persistent effects of arsenic on the microbiome after cessation of exposure, and correlation between gut microbiome changes and toxicity phenotypes, all await future studies.

Conclusions

In summary, we have combined 16S rRNA sequencing and metabolomics to analyze the impact of arsenic on the gut microbiome and its metabolic profiles. The sequencing revealed that arsenic exposure significantly altered the gut microflora composition, while the metabolomics experiments discovered that a number of metabolites involved in diverse metabolic pathways were substantially perturbed following exposure to arsenic. In addition, correlation analysis identified that some gut bacteria families were highly correlated with altered gut-flora-related metabolites. Taken together, these data indicate that arsenic exposure not only perturbs the gut microbiome at the abundance level, but that it also alters metabolic profiles of the gut microbiome, supporting the hypothesis that perturbations of the gut microbiome may serve as a new mechanism by which arsenic exposure leads to or exaggerates human diseases. Furthermore, these modulated gut-flora-related metabolites may be potential biomarkers to probe the functional impacts of arsenic and other diverse environmental chemicals on the gut microbiome.

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Figure Legends

Figure 1. The gut microbiome composition profiles at *family* level in the control and arsenic-treated mice revealed by 16s rRNA sequencing (each color represents one bacterial family) (A); The fold changes and taxa assignments of significantly perturbed gut bacteria ($p < 0.05$) in arsenic-treated mice compared to controls (*p*: *phylum*; *c*: *class*; *o*: *order*; *f*: *family*) (B); The gut microbiome patterns of control samples (Red) and arsenic-treated (Blue) mice are readily differentiated by Principal Coordinate Analysis (C); Hierarchical Clustering analysis by the Unweighted Pair Group Method with Arithmetic Mean indicates that controls and arsenic-exposed mice cluster in their own groups, with the UPGMA distance tree constructed at distance of 0.03 (D).

Figure 2. Arsenic exposure perturbed the metabolic profile of fecal samples of mice, with 370 molecular features being significantly changed compared to controls (fold change > 1.5 and $p < 0.05$) (Red: decreased metabolites; Green: increased metabolites) (A); Control animals are separated from arsenic-treated mice using metabolite profiles by Principal Component Analysis (B); The hierarchical clustering heat map constructed using molecular features with 1.5 fold changes ($p < 0.05$) shows a consistent clustering pattern within individual groups (C). (Control: sample 1 to 9; Arsenic-treated group: sample 10 to 19)

Figure 3. The correlation plot, calculated by Pearson's correlation coefficient, demonstrates the functional correlation between perturbed gut bacteria families and altered fecal metabolites (A); The scatter plots illustrate statistical association ($\rho > 0.5$ or < -0.5 and $p < 0.05$) between the relative abundance of altered gut bacteria families and the mass spectrum intensities of some

typical gut-flora-related metabolites, including indole-containing compounds, isoflavone metabolites and bile acids (B). (Pink: control samples; Blue: arsenic-treated mice.)

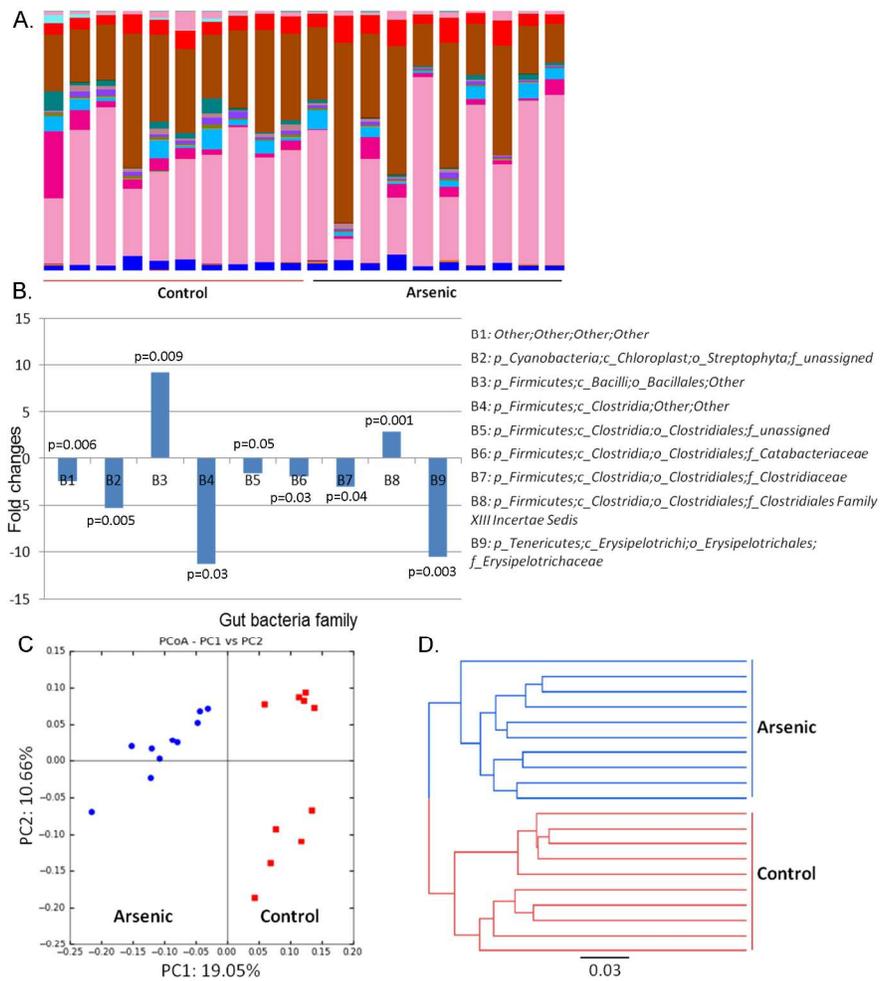


Figure 1
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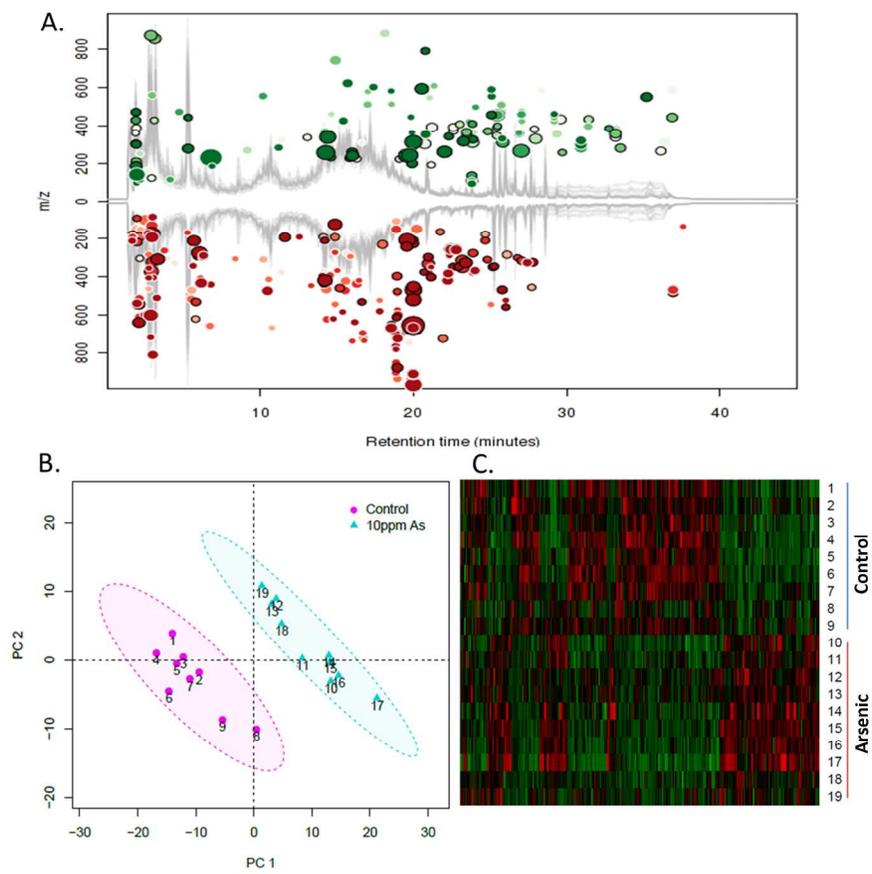


Figure 2
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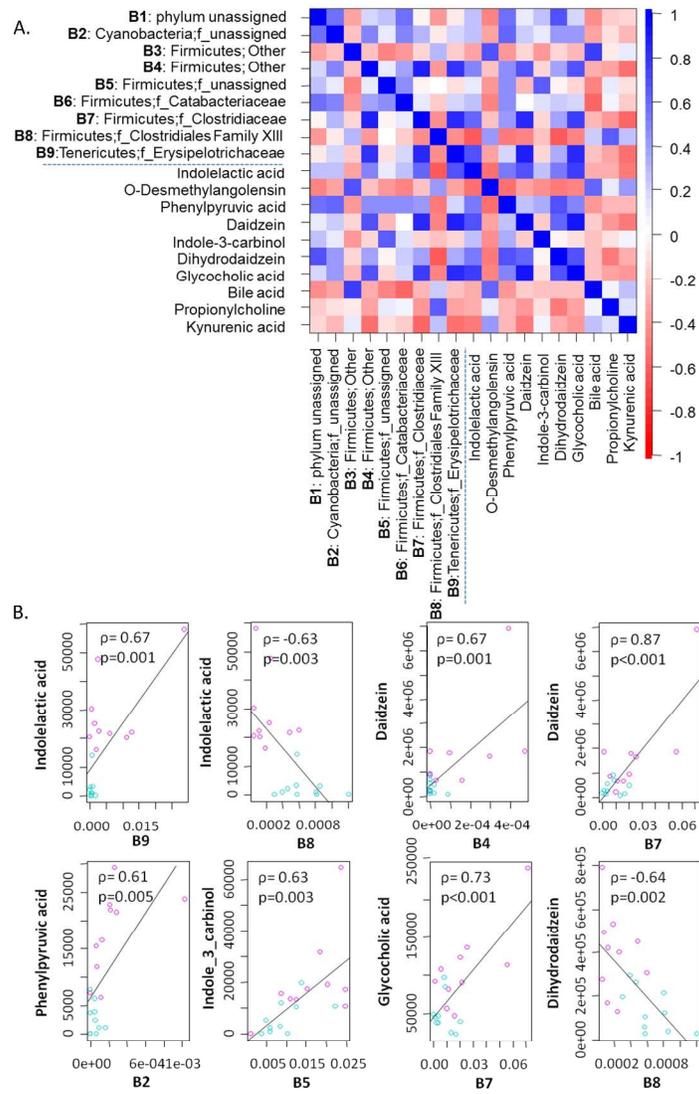


Figure 3
215x279mm (300 x 300 DPI)