



Exposure to Arsenite in CD-1 Mice during Juvenile and Adult Stages: Effects on Intestinal Microbiota and Gut-Associated Immune Status

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ABSTRACT Intestinal microbiota composition and gut-associated immune response can contribute to the toxicity of arsenic. We investigated the potential toxicity of short-term arsenic exposure on gut microbiome composition, intestinal immune status, microbial arsenic resistance gene, and arsenic metabolic profiles in adult and developmental stages of CD-1 mice. The potential toxicity of arsenite [As(III)] was determined for two life stages: (i) adult animals at 24 or 48 h after single gavage (0.05 mg/kg body weight [b.w.] [low dose], 0.1 mg/kg b.w. [medium dose], and 0.2 mg/kg b.w. [high dose]) and repeated exposure at 1 mg/liter for 8 days and (ii) postnatal day 10 (PND10) and PND21 after single gavage (0.05 mg/kg b.w.). Dose- and time-dependent responses in bacterial recovery/microbial composition were observed in adults after a single gavage. Repeated exposure caused a transient decrease in the recovery of intestinal bacteria, a shift in the bacterial population with abundance of arsenic resistance genes, and evidence for host metabolism of arsenite into less-reactive trivalent methylated species. Arsenic exposure in adult animals induced high levels of CC chemokines and of proinflammatory and anti-inflammatory cytokine secretion in intestine. Arsenic exposure at PND21 resulted in the development of distinct bacterial populations. Results of this study highlight significant changes in the intestinal microbiome and gut-associated immune status during a single or repeated exposure to arsenic in juvenile and adult animals. The data warrant investigation of the long-term effects of oral arsenic exposure on the microbiome and of immune system development and responses.

IMPORTANCE Transformation of organic arsenic to toxic inorganic arsenic (iAs) is likely carried out by intestinal bacteria, and iAs may alter the viability of certain microbial populations. This study addressed the impact of arsenic exposure on intestinal microbiota diversity and host gut-associated immune mediators during early development or adulthood using scenarios of acute or repeated doses. During acute arsenic exposure, animals developed defense functions characterized by higher abundances of bacteria that are involved in arsenic resistance or detoxification mechanisms. Arsenite had a negative effect on the abundance of bacterial species that are involved in the conversion of protein to butyrate, which is an alternative energy source in the intestine. The intestinal mucosal immune cytokine profile reflected a mechanism of protection from arsenic toxicity.

KEYWORDS arsenic, arsenic resistance genes, arsenic speciation, developmental toxicity, immune response, intestinal microbiome

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The health risks associated with environmental arsenic exposure have been an area of interest for a long time. Due to the presence of this element in soil and water, the interaction of arsenic with the environmental microbial population had gained much attention (1–3). The U.S. Environmental Protection Agency (EPA) has set regulatory standards for the arsenic level in drinking water at 0.01 mg/liter or 10 ppb (4). In 2016, FDA proposed an action level of 100 ppb for iAs (inorganic arsenic) in infant rice products (5). Larger amounts of arsenic are known to be associated with gastrointestinal disturbance and other adverse effects (6–8). These adverse health hazards could include cancer or other health disorders (5, 7, 9–12). Specifically, exposure to arsenic by ingestion is known to be associated with liver, lung, kidney, and bladder cancer (see reference 13). The lifetime risk of dying from cancer of the liver, lung, kidney, or bladder from drinking 1 liter/day of water was predicted to be as high as 13 per 1,000 persons for a level of arsenic in drinking water of 50 ppb (13). These data need to be reevaluated with the current regulatory standards (50 ppb versus 10 ppb).

Transformation of organic arsenic to more-toxic iAs is likely carried out by the intestinal gut bacteria and some of the host enzymes involved in methylation (14, 15). Arsenic toxicity is related to its metabolism from iAs into organic forms, particularly the trivalent species (monomethylarsonic acid [MMA] and dimethylarsinic acid [DMA]) (16, 17). The metabolism of arsenic is very complicated; however, in general, iAs is more toxic than organic As (MMA and DMA), and the toxicity of arsenite [As^{III}] is higher than that of As^V, but there is interconversion of As^{III} and As^V. Intestinal microbiota play a key role in the speciation of the arsenic that enters the gastrointestinal tract (GIT) via consumption of contaminated water and food (16, 18). Bacterial genera, including *Bacteroides*, *Clostridium*, *Alistipes*, and *Bilophila*, carry As resistance genes, with an ability to methylate As (19, 20). *Bacteroides vulgatus* possesses an arsenic resistance operon consisting of eight continuous genes, with functions as an As(III)-responsive transcriptional repressor (*arsR*) and in detoxification of inorganic arsenic (*arsDABC*), whereas the functions of the other three genes (*orf1*, *orf2*, and *orf3*) in arsenic resistance are unknown (21).

Introduction of a prebiotic (oligofructose) and a probiotic (*Lactobacillus rhamnosus* GR-1) was shown to be protective for arsenic-induced alterations in the gastrointestinal microbiome (22). *Lactobacillus rhamnosus* GR-1 was shown to decrease the bioaccumulation of arsenic in pregnant women and children (23). Arsenic can also cause adverse effects in progeny (24, 25). Newborns (both laboratory animals and human) are more prone to develop metabolic diseases, cardiovascular irregularities, and behavioral, cognitive, and motor disabilities if the mother is exposed (chronically) to arsenic during gestation (26–35).

A change in the microbiome composition of C57BL/6 mice was observed after exposure to 10 ppm arsenic in drinking water for 4 weeks (36). Mice exposed for 2, 5, or 10 weeks to 0, 10, or 250 ppb arsenite (As^{III}) showed changes in the colonic microbial population, metabolic phenotype, and levels of metabolites in the tissue and serum (37). Another study showed that 100 ppb arsenic exposure for 13 weeks changed the gut microbial composition and altered important microbial functional pathways (carbohydrate metabolism [especially pyruvate fermentation], short-chain fatty acid synthesis, and starch utilization) that could influence host metabolism (38). Moreover, child gut microbiota exhibit high levels of As^{III}, the more toxic form of arsenic, which could result in increased health risk (39).

The immune response to arsenic exposure shows a spectrum ranging from immune-suppressive to immune-stimulatory effects depending on the type of experimental model, dose, concentration, the duration of exposure of arsenic (40), and on the levels of trivalent arsenic species (41). In a population-based longitudinal study, arsenic exposure was shown to be associated with oxidative stress, inflammation, and immune disruption in human placenta and cord blood (42). The results of those studies clearly depict multifaceted interactions of arsenic with the microbiome and immune system that could result in a compromised health status. However, further studies are needed

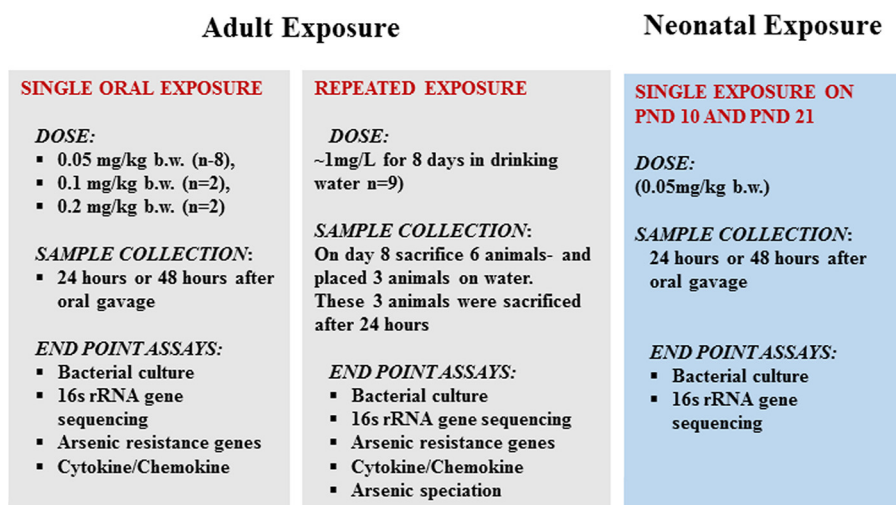


FIG 1 Arsenic exposure strategy during different life stages of CD1 mice. The figure depicts the experimental strategies regarding exposure to arsenic during two life stages (adult and postnatal) of animals. The rectangles represent phases of the life cycle as indicated, and each rectangle presents a description of the detailed experimental plan.

for assessment of the risk represented by arsenic during single or repeated exposures in adults as well as during developmental phases.

The experimental approach in the current investigation addressed the following questions. (i) Does a single or repeated exposure to arsenic have any effect on the commensal microbiota, arsenic resistance, and on the gut mucosa-associated immune status? (ii) Are the responses to repeated arsenic exposure transient and correlated with arsenic speciation in the GIT? (iii) Does exposure of neonates to arsenic lead to any changes in the gut bacterial population?

RESULTS

Rationale for study design. This study aimed to assess changes in the murine intestinal microbial population, relative expression levels of arsenic resistance genes in gut bacteria, and the intestinal mucosal secretion of chemokines and cytokines during exposure to arsenite under different conditions in two different life stages as described in Fig. 1. Typical arsenic levels in highly contaminated drinking water could be in the milligram-per-liter range, thus reaching a total daily intake of ~50 $\mu\text{g}/\text{kg}$ body weight (b.w.) (43). For this reason, we selected three different doses for the single-gavage exposure effect (50 $\mu\text{g}/\text{kg}$ b.w., 100 $\mu\text{g}/\text{kg}$ b.w., and 200 $\mu\text{g}/\text{kg}$ b.w.). Note that the exposure in the present study was performed only once (single dose [SD]) or for 8 days (repeated dose [RD]). During the repeated dosing, the sodium arsenite content (1 mg/liter or 1 ppm as arsenic) in drinking water predicted the total daily intake in the range of 100 to 200 $\mu\text{g}/\text{kg}$ b.w. According to our recently published study (44), this arsenite concentration reflected the finding that individual drinking events throughout the day would produce acute intakes similar to a 50 $\mu\text{g}/\text{kg}$ b.w. dose. This concentration is also similar to the upper limits of arsenic contamination in drinking water in heavily contaminated communities across the world (43).

The first phase of the study investigated the differential effects of arsenite in the adult female with single-gavage exposure (0.05 mg/kg b.w. [low dose; LD], 0.1 mg/kg b.w. [medium dose; MD], and 0.2 mg/kg b.w. [high dose; HD]) or a repeated-dose (RD) 8-day exposure (1 mg/liter, ~200 $\mu\text{g}/\text{kg}$ b.w./day) via drinking water. The second phase of the study investigated the effects of a single exposure of arsenite on microbial populations in neonates. Moreover, the samples were collected 24 and 48 h post-exposure, a strategy that allowed the bacterial population in the intestine to stabilize after a single gavage of arsenic.

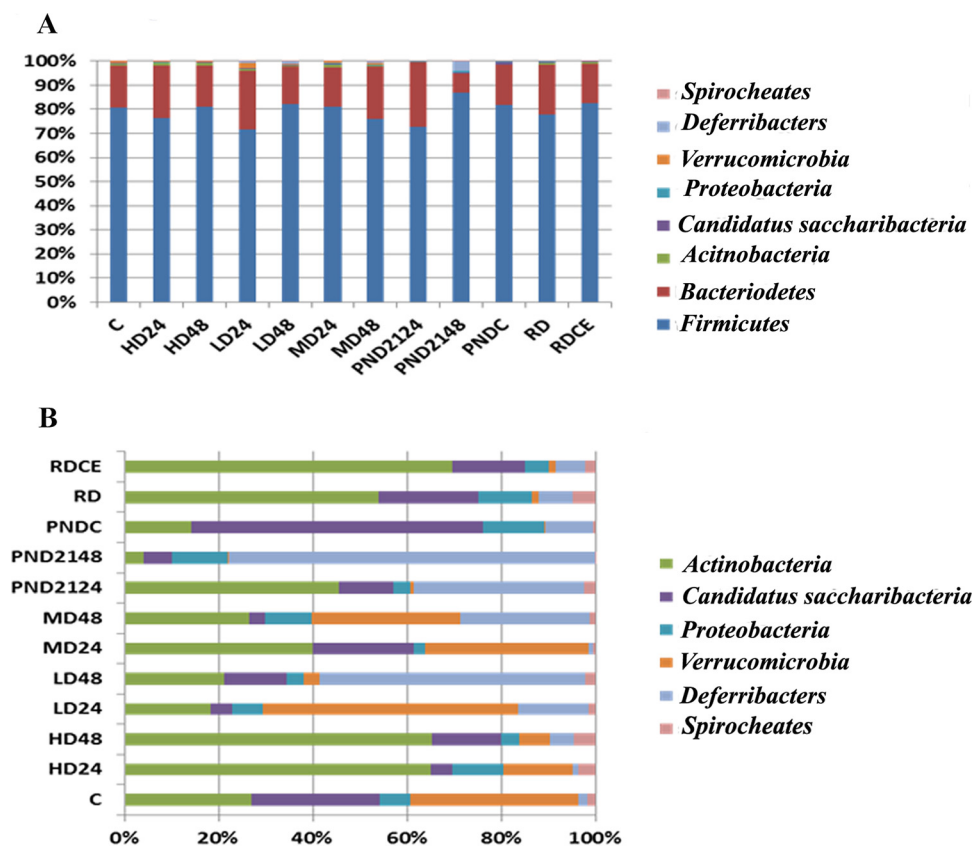


FIG 2 Effect of single or repeated arsenic exposure on the abundance of bacteria representative of specific phylum. Data represent the relative abundances of bacterial populations representing the phyla *Firmicutes* and *Bacteroidetes* (A) or other phyla (A and B) in the fecal samples of mice included in the study. The y axis in panel A shows a 100% stack column to emphasize the ratio of *Firmicutes* and *Bacteroidetes* phyla in the total bacterial population measured. The stack column values represent results from an average of two to six experimental animals.

16S rRNA sequencing analysis at the bacterial phylum level. The predominant bacterial phyla in all the samples were *Firmicutes* and *Bacteroidetes* as assessed by 16S RNA sequencing (Fig. 2A). The percent abundance range for *Firmicutes* and *Bacteroidetes* for various experimental groups is provided as Table S1 in the supplemental material. There was a decrease of about 50% in the abundance of bacteria that belong to *Bacteroidetes* in PND21 animals after 48 h. Bacteria that belong to the phyla *Actinobacteria*, *Proteobacteria*, "*Candidatus Saccharibacteria*," *Verrucomicrobia*, *Deferribacters*, and *Spirochaetes* were also present in the control and treatment groups and were classified as "other phyla" (Fig. 2B). There was a predominance of bacteria that belong to the phylum "*Candidatus Saccharibacteria*" in control neonatal mice. Bacteria that belong to the phylum *Deferribacters* were significantly higher in abundance and contributed to more than 70% of the "other phyla" category in the PND21 mice 48 h after exposure to arsenite (Fig. 2B).

16S rRNA sequencing analysis at the bacterial genus level. To assess the separation between different experimental groups and to observe the similarities within an experimental group at the genus level, we used partial least-squares discriminant analysis (PLS-DA) (Fig. 3A). Some distinct features that could be observed from these data were as follows: (i) bacterial populations in adult controls were quite distinct from those in the PND21 control at the genus level; (ii) a single arsenite dose had an effect distinct from that seen with a repeated dose; (iii) during the single-dose exposure, the bacterial populations within a treatment group were more similar to each other at 48 h of exposure than at 24 h postexposure; (iv) repeated dosing with arsenite

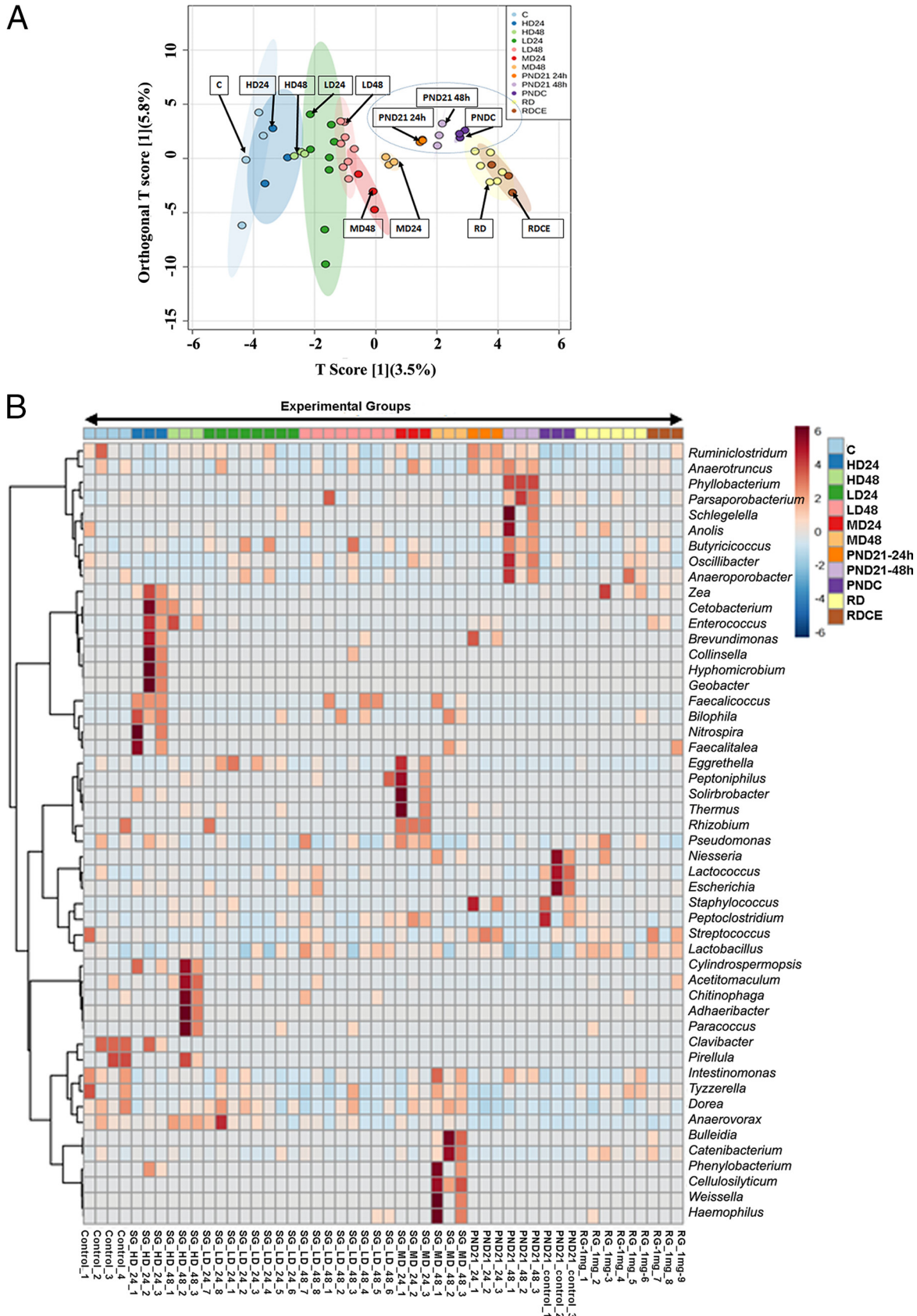


FIG 3 Effect of arsenic exposure on the abundance of bacteria representative of specific genera. PLS-DA was conducted to classify the metagenomics data at the genus level (A). Here the x abscissa presents the bacterial genera, and the y ordinate variables represent results (Continued on next page)

resulted in increased abundance of bacteria that belong to distinct genera compared with exposure to arsenite using single gavage; and (v) the levels of bacterial diversity did not differ much between the individuals on PND21 within an experimental group.

To provide a clear view of the predominance of the bacteria representing specific genera, we focused on the top 50 genera after applying analysis of variance (ANOVA) (Fig. 3B) and representing the data as a heat map. Despite sample-to-sample variation, bacterial genera in control adults showed a predominance of *Clavibacter*, *Pirellula*, and *Intestinimonas*, whereas the PND21 control showed a predominance of *Neisseria*, *Lactococcus*, *Escherichia*, *Staphylococcus*, and *Peptoclostridium*. There was a lower abundance of bacteria of the genus *Intestinimonas* in animals exposed to arsenite; this decrease was most prominent in the RD group.

Experimental groups exposed to a single dose or repeated doses of arsenite showed the presence of bacteria belonging to the *Bilophila* genus; however, these were almost absent in the control adults as well as in PND21 animals. The genera that were exclusively predominant in PND21 after 48 h of arsenic exposure were *Butyricoccus*, *Phyllobacterium*, and *Parasporobacterium* (Fig. 3B).

16S rRNA sequence analysis at the bacterial species level. The adult experimental animals gavaged with single doses of arsenite showed a clear separation with respect to the presence of bacterial species compared to control animals (Fig. 4A). There was a clear difference in the abundances of species in PND21 animals. The alpha and beta diversity data are provided here as the measure of intensity in the heat map for clear visualization. The heat map data indicate the number of species (i.e., the species richness) in the treatment groups (individual columns in one class, top row). Comparisons of all the experimental groups (with classes as depicted in heat map) reveal the change in species diversity between the experimental groups and thus the beta diversity. Beta diversity was higher at the species level (Fig. 4B). The bacterial species *Dorea formicigenerans* was predominant in the adult animals. Responses to the arsenite exposure (single gavage) differed significantly among the mice within the same treatment groups. *Clostridium sulfatireducens* bacteria were consistently detected in most of the single-gavage LD animals after 24 h. However, the abundance of these bacterial species was only transient, as they were absent when the sampling was conducted at 48 h. *Alistipes massiliensis* and *Lactobacillus johnsonii* emerged following repeated exposure to arsenite.

Arsenite exposure during development (PND21) resulted in the appearance of a very distinct population of bacterial species. This difference was more noticeable in the samples collected 48 h after exposure by a single gavage than in those collected from 24 h after exposure. Some predominant species in this group were *Eubacterium plexicaudatum*, *Lachnoclostridium*, and *Mucispirillum schaedleri*, along with several species of the genera *Roseburia* and *Parasporobacterium*.

Arsenic speciation in blood and ileal tissue. The three gavage dosing levels used in acute treatments produced fluxes of “free” pentavalent and “bound” trivalent arsenic metabolites that peaked after ~1 h and were largely absent by 24 h (44). For example, the maximal plasma concentrations of “free” pentavalent DMA (DMA^v) produced by the low, medium, and high arsenite doses were ~200, 400, and 900 nM at 1 h. Peak erythrocyte levels of bound DMA^{III} were 300, 400, and 800 nM at 1 to 2 h. In ileal tissue,

FIG 3 Legend (Continued)

of comparisons of observations of different treatment groups. The group designations were as follows: low dose (LD), 0.05 mg/kg b.w.; medium dose (MD), 0.1 mg/kg b.w.; high dose (HD), 0.2 mg/kg b.w. Animals were sacrificed at 24 or 48 h postgavage and are designated LD24, LD48, MD24, MD48, HD24, or HD48. Another group of adult females were given continuous exposure (dosed water; 1 mg/liter) for 8 days (repeated dose [RD]), and some animals from this group were provided only water during the last 24 h (RDCE). Postnatal day 21 (PND21) female mice were exposed to single doses of arsenic (oral gavage, 0.05 mg/kg b.w.), and the microbiome was analyzed at 24 h and 48 h postexposure (PND21 24h and PND21 48h, respectively). The controls were an adult control (C) and a PND21 control (PNDC). (B) A heat map was generated to show the alpha, beta, and gamma diversity in the animals across all the experimental groups. The data for the top 50 genera were determined after applying ANOVA. Here the distance was measured by applying Pearson correlation and the Ward hierarchical clustering algorithm. The heat map provides an inbuilt visualization of the OTU abundance. Each colored cell in each column corresponds to an individual OTU abundance within specific samples (alpha diversity), and the rows correspond to individual samples within each of the experimental groups/samples (beta diversity).

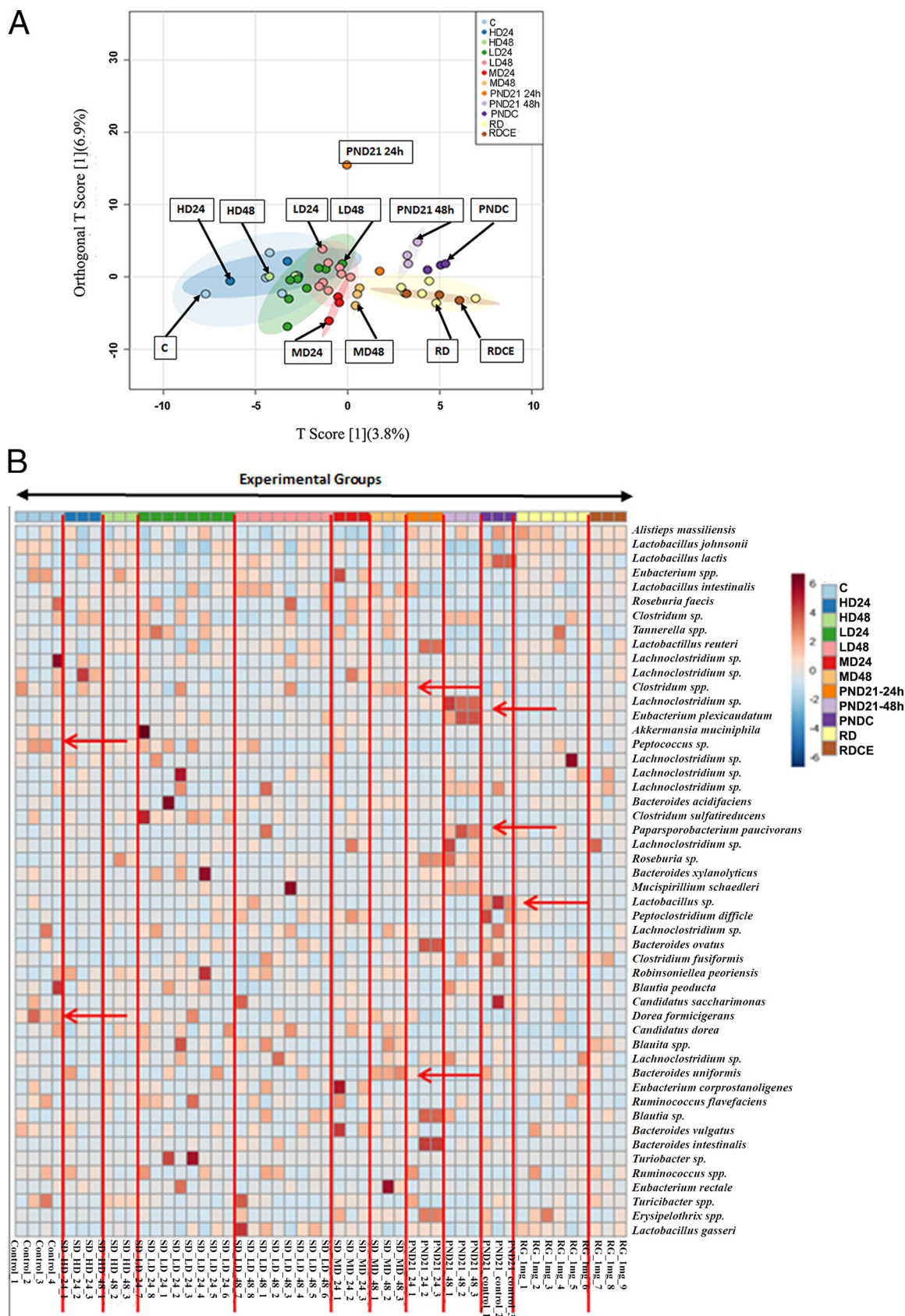


FIG 4 Effect of arsenic exposure on the abundance of bacterial species. (A) PLS-DA was conducted to classify the metagenomics data at the species level. The group designation are as follows: low dose (LD), 0.05 mg/kg b.w.; medium dose (MD), 0.1 mg/kg b.w.; high dose (Continued on next page)

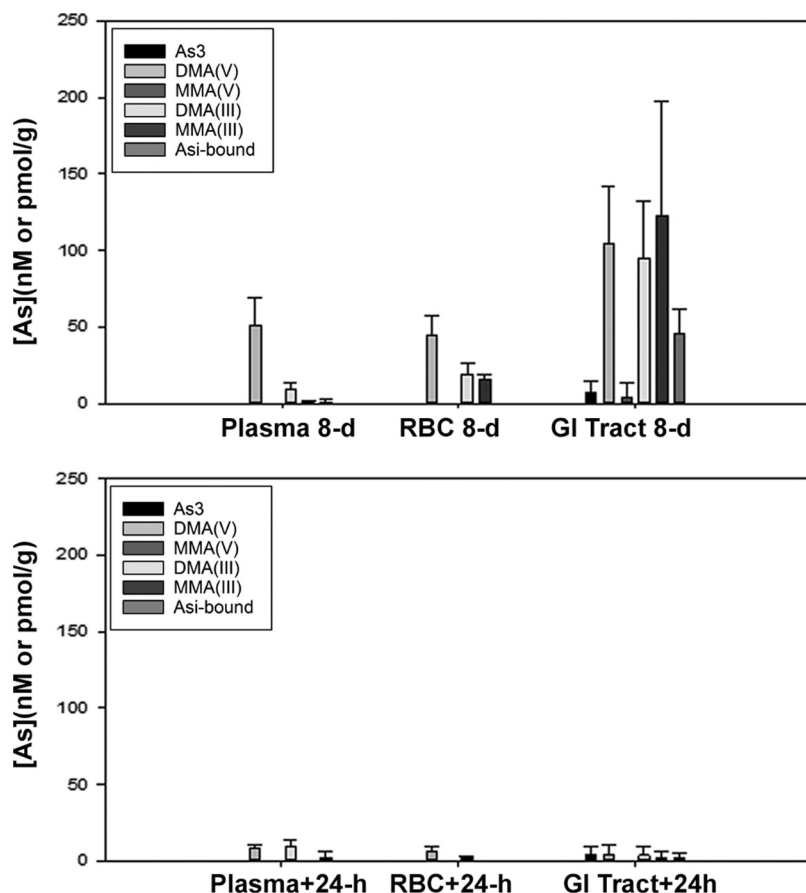


FIG 5 Arsenic speciation in the intestinal tract and blood. Pentavalent and trivalent arsenic species were quantified by using LC-ICP/MS in the ileal tissue (GI tract) as well as in plasma and erythrocytes from nonpregnant female CD-1 mice after repeated dosing in drinking water (1 mg/liter) for 8 days (top panel) and after transfer to control drinking water for 24 h (bottom panel).

the 0.05 mg/kg b.w. dose produced 870, 465, and 501 pmol/g for free As^{III}, DMA^V, and MMA^V and 400, 1,814, and 222 pmol/g for bound DMA^{III}, MMA^{III}, and As^{III}, respectively (not shown). In contrast, the repeated dosing in drinking water (1 mg/liter arsenite) produced ileal levels of free DMA^V and bound DMA^{III}, MMA^{III}, and As^{III} in the range of approximately 100 pmol/g; these metabolites were eliminated by 24 h upon cessation of arsenic dosing (Fig. 5).

Gut-associated immune status. The levels of CXC chemokine (KC) were not noticeably affected during the arsenite exposure. Only single-dose-gavaged animals (LD 24 h) showed a significant increase in the level of KC (Fig. 6A).

Among CC chemokines, secretion of eotaxin was higher in all the experimental groups than in the control; however, statistically significant differences were observed only in the cessation group and in adult LD24 (adult female exposed to a single dose of arsenite and analyzed after 24 h) (Fig. 6A to C).

All the experimental groups showed higher levels of monocyte chemoattractant protein 1 (MCP-1). In contrast, macrophage inflammatory protein 1 α (MIP-1 α) and

FIG 4 Legend (Continued)

(HD), 0.2 mg/kg b.w. Animals were sacrificed at 24 or 48 h postgavage and are designated LD24, LD48, MD24, MD48, HD24, or HD48. Another group of adult females were given continuous arsenic exposure (dosed water; 1 mg/liter) for 8 days (repeated dose [RD]), and some animals from this group were provided only water for the last 24 h (RDCE). Postnatal day 21 (PND21) female mice were exposed to a single dose of arsenic (oral gavage, 0.05 mg/kg b.w.), and their microbiome was analyzed 24 h and 48 h postexposure (PND21 24h or PND21 48h, respectively). The controls were an adult control (C) and a PND21 control (PNDC). A heat map was generated to show the alpha, beta, and gamma diversity in the animals across all the experimental groups. Panel B shows the data for the top 50 genera determined after applying ANOVA. The red arrows show the presence of distinctive bacterial species in a particular experimental group.

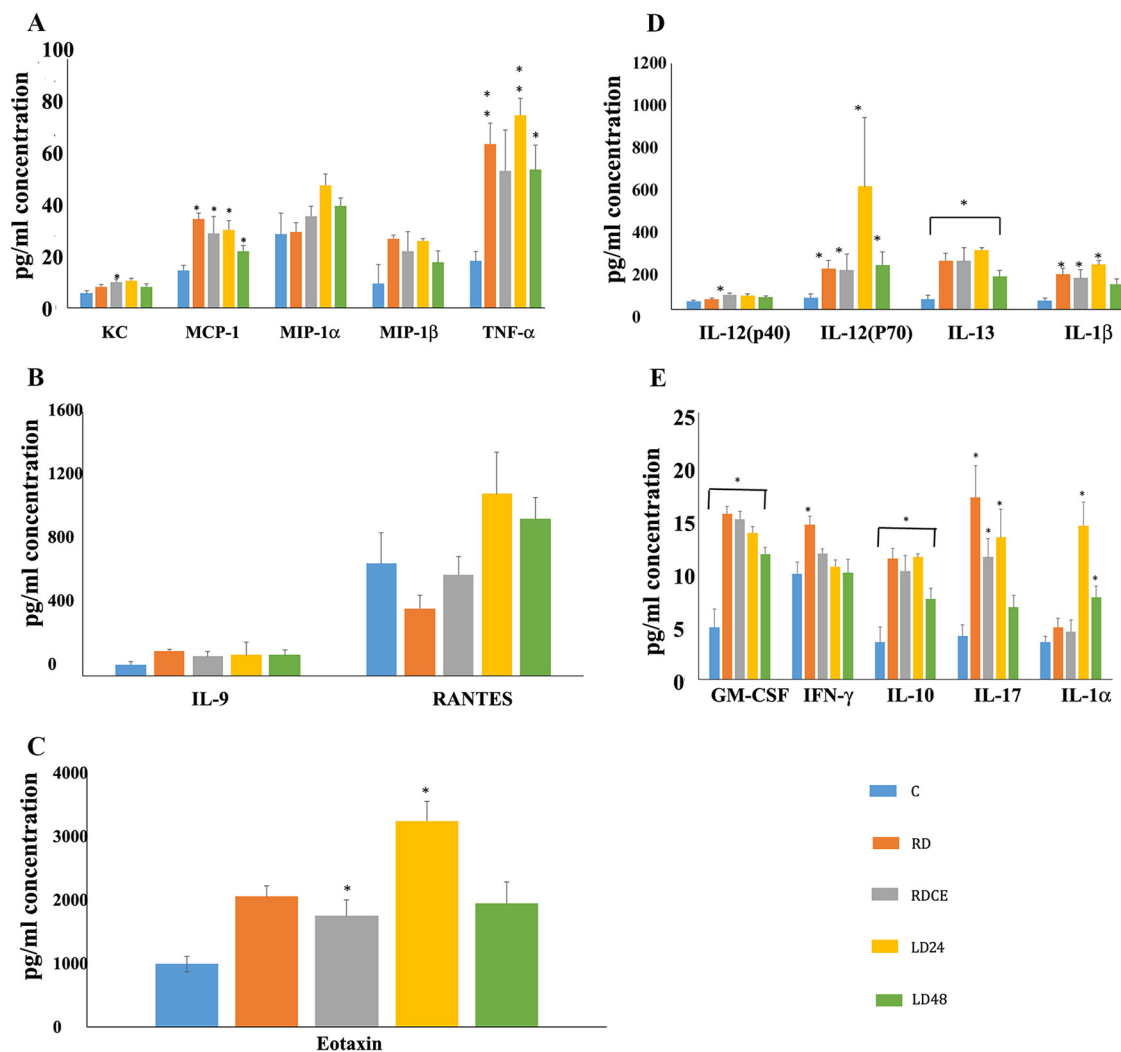


FIG 6 Effect of arsenic exposure on the gut-associated profile of cytokines and chemokines. Gut-associated mucosal chemokine (A to C) and cytokine (D and E) levels were evaluated in the tissue lysate using Bio-Plex mouse cytokine multiplex kits. The experimental groups for this comparison were as follows: adult control (C), i.e., mice treated with dosed water; 1 mg/liter (RD), i.e., repeatedly dosed mice kept on water for 24 h (RDCE); low dose 24 h (LD24); and low dose 48 h (LD48).

MIP-1 β did not show significant changes in secretion levels compared to control animals. The production of tumor necrosis factor alpha (TNF- α) was higher in all treatment groups. Interleukin-12 (IL-12) is a heterodimeric cytokine and is encoded by two separate genes, IL-12A(p35) and IL-12 β (p40). The active heterodimer IL-12(p70) and a homodimer, IL-12(p40), are formed following protein synthesis. In the present study, most of the experimental groups showed significantly higher secretion of IL-12(p70) (Fig. 6D). Furthermore, the production of IL-13 and IL-1 β was stimulated in most of the experimental groups (Fig. 6D). The acute single gavage of arsenite caused increased IL-1 α production (Fig. 6E) in adult. A significantly higher level of gamma interferon (IFN- γ) was present only in the animals exposed repeatedly to arsenite; the cessation of arsenic exposure for 24 h brought the levels of IFN- γ back to those seen with the control animals (Fig. 6E). The levels of another proinflammatory cytokine, IL-17, were higher in most of the experimental groups. Levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10 were also increased in all groups.

DISCUSSION

Our study data indicate that the activity of the microbiome-mediated adaptation mechanism against deleterious effects of arsenic probably starts at the neonatal stage

(PND21). The difference in the patterns corresponding to the recovery of bacteria at 24 and 48 h in both adults and neonatal mice may have been a consequence of the growth of the bacteria that survived the single-dose exposure of arsenic and proliferated over the next 48 h. (Please see Text S1, Fig. S1, and Fig. S2 in the supplemental material.) There have been reports of arsenic thiolation occurring during *in vitro* digestion of iAs in bioreactors simulating gastric conditions with human intestinal microbiota and in *in vivo* animal models (18, 44). In fact, the abundance of bacteria with putative thioreductase (*orf1/2*) was higher during LD arsenic exposure in our study, though large variability was observed in individual animals (see Table S2 in the supplemental material; see also Fig. S3). The transient appearance of *Clostridium sulfatireducens*, a sulfate-reducing bacterium (SRB), in LD single-gavaged animals could indicate the involvement of these bacteria in thiolation of arsenic (45). An increased abundance of bacteria that belong to the phylum *Actinobacteria* in HD-gavaged animals at 24 and 48 h postexposure as well as in the group of repeatedly dosed animals and in the animals in the cessation group could represent a detoxification mechanism (46, 47).

Repeated exposure to arsenite in adult mice led to an abundance of bacteria that belong to genera *Alistipes*, *Bilophila*, and *Lactobacillus johnsonii*. Members of genus *Bilophila* are known to be involved in intra-abdominal infections and other infections that lead to inflammatory bowel disease (IBD) (48). Furthermore, *B. wadsworthia* is an immunogenic sulfite-reducing opportunistic pathobiont and is involved in modifying conjugation states of bile acids (49, 50). The abundance of *B. wadsworthia* promoted a Th1-mediated immune response and the development of colitis in IL-10^{-/-} mice (49). Arsenic is considered one of the causative agents for IBD and ulcerative colitis (51, 52). Furthermore, several bacterial species, including *Alistipes* and *Bilophila* bacteria, had positive correlations with the formation of iAs^{III}, iAs^V, and total arsenic (TAs), which is indicative of higher arsenic resistance (19). Likewise, *L. johnsonii* has been shown to carry an arsenic resistance cassette (53). Bacterial species that belong to the phylum *Deferribacteres* are known to harbor arsenic resistance genes (54); thus, in our study it is tempting to speculate that the observed changes in the abundance of bacterial population may represent one of the mechanisms adapted by neonatal animals for surviving the toxic effects of the arsenite. In particular, the arsenic-inducible transcriptional unit (*arsD*) conferred resistance to trivalent arsenic species, with MMA^{III} being the most effective inducer, followed by As^{III} (21). Ileal tissue showed efficient conversion of arsenite into pentavalent and trivalent methylated metabolites. Methylation of arsenite (either due to bacterium-induced conversion or due to host factors) is generally viewed as a detoxification reaction because DMA^V is the predominant species excreted in urine and methylation appears to protect mice from acute toxicity of arsenite (55). However, trivalent DMA and MMA and arsenite itself are reactive species whose low-molecular-weight thiol complexes (e.g., glutathione) represent mobile forms that may be capable of transferring bound arsenic to critical thiol throughout the host tissues or the associated microbiota (44). Further studies are required to understand if the single exposure can increase the risk of disease in the animals; it would be very pertinent to assess such effects with a challenge study or to assess the immune status of single-dose-arsenic-exposed animals at a later time.

Remarkably, arsenite and its metabolic products exert a proinflammatory response *in vitro* when the dose is approximately 5 μ M (56). By comparison, the arsenic species (peak concentrations) whose levels were measured in ileal tissue following gavage dosing at 50 μ g/kg b.w. (not shown) included bound arsenic species (400, 1,814, and 222 pmol/g for As^{III}, MMA^{III}, and DMA^{III}, respectively) and free arsenic species (870, 465, and 501 pmol/g for As^{III}, MMA^V, and DMA^V, respectively); the levels were similar to the effective *in vitro* concentrations (56). It should be noted that these peak levels were transitory, since the “steady-state” levels achieved after repeated arsenite dosing through the drinking water (~200 μ g/kg b.w./day) produced concentrations in ileal tissue that were up to 10-fold lower.

The abundance of bacteria that belong to the phylum *Deferribacteres* or genera

Butyricoccus, *Phyllobacterium*, and *Parasporobacterium* could play an adaptive role against arsenic assault. Members of the phylum *Deferribacteres* are known to harbor arsenic resistance genes (54). Members of the genus *Butyricoccus* are tolerant to changing intestinal conditions, produce butyrate, and have probiotic potential (57, 58). In addition, *Butyricoccus* is known to inhibit growth of potentially harmful intestinal pathogens and protect the intestine from the formation of necrotic enteritis in broilers (59). Members of the genus *Phyllobacterium* are known to have heavy metal resistance genes. Bacteria that belong to the genus *Parasporobacterium* were shown to convert aromatic residues (gallate) into acetate and butyrate (60). *Eubacterium plexicaudatum* (61) and *Roseburia* (62) are able to produce butyric acid from fermented carbohydrates, whereas *Parasporobacterium* produces dimethyl sulfide and methanethiol from methoxy-containing aromatic compounds and sulfide (60). In the current study, all these species were predominant in PND21 animals after 48 h of exposure to arsenic. The exact role of these species during the arsenite exposure needs to be explored in further detail. Some bacterial species that belong to *Lactobacillus* and *Lactococcus* (*lactis*) were predominant in the PND21 control group. *L. lactis* subsp. *lactis* bacteria are among the early colonizers in the human infant gut (63).

Lower abundance of members of the *Intestinimonas* genus in most of the arsenite-exposed animals may result in low butyrate production. Butyrate, a metabolic product of dietary fiber digestion via intestine-microbe interaction, is a major energy source in the colon that is crucial to maintaining intestinal health (64, 65). Genus *Intestinimonas* bacteria produce butyrate from protein and Amadori products. Instead of a sole focus on butyrate generation from fibers/carbohydrate, protein fermentation by *Intestinimonas* could also contribute to the production of energy and intestinal homeostasis.

Mucispirillum schaedleri, a bacterial species that colonizes the mucus layer of the GIT, was predominant in samples from the animals exposed to a single dose of arsenic and in samples collected after 48 h in the PND21 arsenite-exposed group. *M. schaedleri* is a commensal pathobiont. The higher abundance of these bacteria at 48 h than at 24 h could be due to its tolerance of arsenic toxicity. By 48 h, the bacteria that survived the detrimental effects of arsenic proliferated more and thus resulted in higher abundance. However, the bacterial population is more diverse in adult animals and could result in the different abundances of populations of bacterial species or bacterial species that harbor an arsenic resistance gene to mitigate the arsenic toxicity. *M. schaedleri* was shown to reduce nitrate and to help in scavenging oxygen and reactive oxygen species *in vivo* (66). *M. schaedleri* harbors a type VI secretion system and putative effector proteins and can modify gene expression in mucosal tissue during inflammation (67). The close proximity of *M. schaedleri* to intestinal mucosal tissue and expansion during arsenic perturbations may produce intimate interactions with its host and possibly play a role in inflammation. In the present study, microbiome analysis was conducted in the fecal samples. Studies are ongoing to assess the adherent microbiota in the gut mucosa in the same animals. Another possibility that could not be ruled out is that exposure to arsenic might cause the intestinal mucosa to undergo a perturbation that ends in depleting the mucosal surfaces so much that these populations end up in the feces. All the experimental groups examined in the present study showed higher levels of MCP-1, a chemokine whose levels have been previously shown to be elevated during exposure of vascular smooth muscle cells to arsenic (68). However, neutrophil granulocytes may not be involved in the arsenic-induced toxicity in the GIT, as the secretion of CXC chemokine remained unchanged.

Lipopolysaccharide is known to increase the levels of IL-12 (69, 70). Enhanced production of murine IL-12 is proposed to be mediated by *Lactococcus lactis* (71), a bacterial species that was also significantly abundant in the arsenite-exposed PND21 group. In fact, trivalent arsenic species have been shown to induce changes in mRNA expression levels and in secretion levels of proinflammatory cytokines in intestinal epithelial cells. To address whether this was the case during *in vivo* exposure, we quantified the arsenic speciation in the animals that were provided arsenic in the drinking water continuously for 8 days and in those in the cessation group. Our data

support the idea of extensive metabolism of arsenic within the GIT to MMA and DMA in both the trivalent (reactive) and pentavalent states (72). This further supports the notion that metabolism of arsenite within the gut could produce diffusible species that could interact with the microbiota and host in ways that affect production of chemokine/cytokines.

Overall, this study addressed some of the knowledge gaps concerning the effects of arsenic exposure during early development as well as in the scenario where someone is exposed to arsenic only acutely, or chronically for only a short period, with an emphasis on its impact on microbiota diversity and host gut-associated immune mediators. During acute arsenic exposure, the first response observed in the animals was a defense mechanism characterized by either an increase in the bacterial population containing arsenic resistance genes or a higher abundance of bacteria that are involved in the arsenic detoxification mechanism. Bacterial species that harbor arsenic efflux systems are presumed to have evolved to purge the toxic effects of this deleterious chemical. These efflux systems include members of the multidrug resistance protein family, bacterial exchangers, and ATP-driven efflux pump (73). Interestingly, arsenite showed a negative effect on the presence/abundance of bacterial populations that are known to convert protein to butyrate, an alternative energy source. However, to compensate for that, there was an emergence of bacterial species in the arsenite-exposed animals (during repeated exposure, as well as during the developmental stages) that are proposed to be alternate producers of butyrate. This clearly corresponds to and supports the Baas Becking and Beijerinck microbe distribution hypothesis: "Everything is everywhere, but the environment selects" (74).

Furthermore, we show that the intestinal mucosal immune response pattern reflects a mechanism of protection from arsenic toxicity (Fig. 7). Altered levels of biologically relevant CC chemokines in most of the arsenite-exposed groups indicate an inflammatory response to arsenic toxicity. There was abundance of bacterial species during repeated exposure to arsenic that could contribute to dysbiosis and lead to the production of heat shock proteins (HSP). This may trigger/induce an autoimmune reaction due to the molecular analogy between HSP from bacterial origin and human HSP (40, 75, 76). Thus, it is essential to investigate the *in utero* as well as long-term effects of arsenic exposure in relation to gastrointestinal toxicity (specifically for the neoplastic transformation of epithelial cells or for susceptibility to infection) and overall homeostasis. Transplacental movements of xenobiotics are proposed to negatively interact with fetal immune stem cell maturation. Detailed studies are in progress to dissect the immune responses of developmental exposure and to assess if these are in fact sex dependent and if there is any correlation with the microbiome changes that occur during the early developmental stages. Studies are also in progress to assess if gestational exposure to arsenic has any effect on the dam and on the intestinal immune development of the fetus. Of particular interest with respect to this study is the developmental exposure that could potentially affect the immune system development and responses to diseases during adulthood.

In conclusion, our report reveals that the interactions among xenobiotics, gut microbiome, and the gut-associated immune response and the impact on the host represent a complex and dynamic process. The results warrant further investigation into arsenic developmental immunotoxicity to examine how the presence of the distinct microbial population during the early developmental stages leads to colonization of the bacterial population at the adult stage and the critical windows of dysbiosis, immunotoxicity, and differential susceptibility based on gender.

MATERIALS AND METHODS

Animal exposure strategy. All studies were approved by the Animal Care and Use Committee at the National Center for Toxicological Research, Jefferson, AR (44). The strategy for the life stage animal groups and arsenic exposure is provided in Fig. 1.

In brief, this study consisted of exposure of mice to sodium arsenite during the developmental stages of the mice (adult nonpregnant and postnatal). Female CD-1 mice (7 to 8 weeks of age) (designated the "first group" below) and pregnant CD-1 mice (unspecified age; designated the "second group" below)

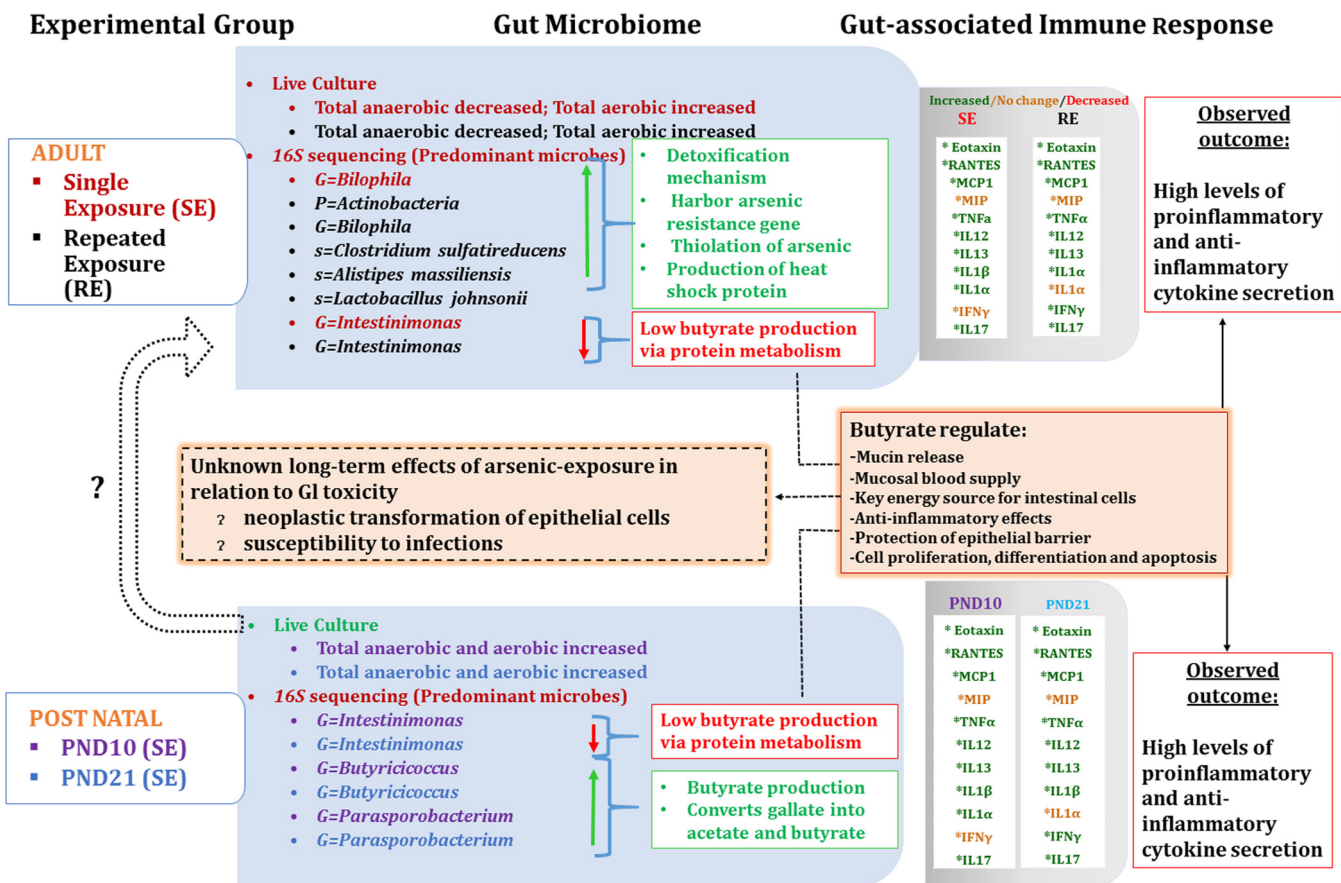


FIG 7 Summary of the effects of arsenic exposure on the gut microbiome and gut-associated immune response. This schematic diagram depicts the outcome of the exposure to arsenic at the developmental stages of CD-1 mice. The leftmost box (shaded blue) shows the adult exposure or postnatal exposure to arsenic in the form of either single exposure (SE) or repeated exposure (RE). Experimental groups are identified and matched with the individually colored fonts for the gut microbiome (sky-blue box). The outcome of the microbial population shift is summarized in the white box. The impact of the exposure to arsenic on the gut-associated immune response is shown as increased secretion (green), no change in secretion (orange), or decreased secretion (red) of cytokines within the shaded gray box. The overall effects of changes in the levels of cytokines/chemokines are indicated in the rightmost white box.

were procured from Charles River, Inc. (Wilmington, MA). The CD-1 strain of mouse was chosen for this study due to its susceptibility to the carcinogenic effects of sodium arsenite under conditions of exposure to 50 to 5,000 ppb in drinking water (77). For at least 10 days, mice were provided a common low-arsenic basal diet (5K96; Test Diets, Purina Mills, Richmond, IN) to reduce the background level of arsenic. Animal for individual experiments were housed in groups. In the first group, adult females were exposed to a single gavage consisting of three concentrations of sodium arsenite (low dose [LD], 0.05 mg/kg b.w.; medium dose [MD], 0.1 mg/kg b.w.; high dose [HD], 0.2 mg/kg b.w.; expressed as arsenic equivalents). These animals were compared to the animals that were not exposed to arsenic. Animals were sacrificed at 24 or 48 h after gavage. Mice in another group of nonpregnant adult females ($n = 6$) were given continuous exposure (dosed water; 1 mg/liter) for 8 days (repeated dose [RD]). Preparation of the dosed water was described in detail earlier (72). Based on measurements of drinking water consumption and body weight data for pregnant and nonpregnant mice, the ingested daily dose was ~200 mg/kg b.w. (range, 168 to 291; data not shown). In the RD group, the animals in one set were provided control water for an additional 24 h (ninth day) and then sacrificed (referred to here as the “repeated-dose-cessation group” [RDCE group] [$n = 3$]). All of the repeatedly dosed animals were compared to the animals that were not exposed to arsenic and were sacrificed along with single-gavaged dosed animals. The second group of animals consisted of those with postnatal exposure. In this group, both the male and female pups (postnatal day 10 [PND10] and PND21) were exposed to a single oral dose of arsenite (by gavage; 0.05 mg/kg b.w.) and the microbiome was analyzed at 24 h and 48 h postexposure and compared with the the microbiome of age-matched and sex-matched control animals.

Collection of biological samples. Animals were fasted overnight and euthanized by exposure to carbon dioxide. Verification of euthanasia by exsanguination was done prior to initiation of necropsy. Blood was collected by cardiac puncture and was immediately processed into plasma and erythrocyte fractions and stored at -60°C until analysis for arsenic speciation.

The intestine was exposed and flushed with normal saline solution, and 3-cm sections of terminal ileum were collected in individual cryo-tubes for RNA and protein extraction. These cryo-tubes were immediately flash frozen for later use.

Fecal sample collection for culture-based bacteriology and DNA extraction. Feces was collected from the colon of euthanized animals and immediately transferred to the anaerobic chamber for analysis of live bacteria (for details, please see Text S1 in the supplemental material).

Two or three pellets of feces were collected from the colon in a cryovial and immediately stored at 80°C. Fecal DNA was extracted by a bead beating and organic extraction method as described earlier (78, 79). The DNA pellet was suspended in DNase-and-RNase-free water, quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and double-stranded DNA (dsDNA) quantification by Qubit (Thermo, Fisher Scientific, Waltham, MA), and used as the template for 16S rRNA sequencing for microbial population and real-time PCR analysis of expression of arsenic resistance genes.

16S rRNA gene sequencing of bacterial population. Fecal DNA was used to perform community bacterial population analysis. The 16S rRNA gene V4 variable region was amplified using PCR primers 515/806. Pooled and purified PCR products were used to prepare an Illumina DNA library. Sequences were joined and depleted of barcodes, and then sequences of <150 bp and sequences with ambiguous base calls were removed. Sequences were denoised, operational taxonomic units (OTUs) were generated, and chimeras were removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTN against a curated database derived from RDP II and NCBI.

For statistical analysis of microbiome data, the effect of arsenic exposure on the bacteria representative of a specific phylum was calculated as the percent abundance (79). For genus-level and species-level analysis, orthogonal partial least-squares discriminant analysis (PLS-DA) was used to observe the separation between different experimental groups and to observe the similarities within an experimental group (80–82). This analysis requires $n = 3$; but there were only $n = 2$ samples available in some experimental groups (e.g., PND). This lacuna was overcome by obtaining an additional value by averaging two values, with the results depicted as a heat map for an individual sample.

Hierarchical clustering was performed with the `hclust` function in the `stat` package (83, 84). The distance was measured by applying Pearson correlation and the Ward hierarchical clustering algorithm. The outcome is presented as heat maps, which provide an inbuilt visualization of the levels of OTU abundance. Comparison of the data corresponding to each animal (in the same color box) reveals the alpha diversity. Comparison of the data corresponding to all the experimental groups reveals the change in species diversity between the experimental groups and thus the beta diversity.

Arsenic speciation in blood and ileal tissue. Pentavalent and trivalent arsenic species (DMA^{V} , MMA^{V} , As^{III} , and As^{V}) were quantified in plasma, erythrocytes, and ileal tissue homogenate in nonpregnant female CD-1 mice using ion exchange liquid chromatographic separation (LC separation or speciation), and detection/quantification using inductively coupled plasma mass spectrometry (ICP/MS) was performed as reported earlier (44).

Protein extraction from the ileal tissue. Ileal tissue protein extraction was performed using a gentleMACS dissociator (Miltenyi Biotec, Inc., Auburn, CA). The lysate was centrifuged at 4°C for 10 min at 750 rpm, and the clear homogenate was transferred to 2-ml Eppendorf tubes and centrifuged at 4°C for 15 min at 12,000 rpm. The clear supernatant was transferred into new 2-ml Eppendorf tubes, and these were stored at -80°C until cytokine profiling. The protein concentration was measured spectrophotometrically using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

Multiplex cytokine assay. Gut-associated mucosal chemokine and cytokine levels were evaluated in the tissue lysate using a Bioplex mouse cytokine 23-plex panel and Th17 plex (Bio-Rad, Hercules, CA) following the manufacturer's instructions and as previously described (85) using the multiplex Bioplex assay.

Statistical analysis for cytokine data. The Bioplex data were exported to Bioplex Manager, and comparisons of different treatment groups were performed using the Mann-Whitney test. All statistical analysis was two sided, and a P value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.01418-18>.

TEXT S1, DOC file, 0.04 MB.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.05 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, DOCX file, 0.01 MB.

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