INVESTIGATING ARSENIC-MICROBIOME INTERACTIONS IN THE GUT USING MURINE MODELS

by

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DEDICATION

> "The greatness of a community is most accurately measured by the compassionate actions of its members." – Coretta Scott King

All life is based on symbiosis and adaptation, including that of a researcher in training. This dissertation is dedicated to every mentor who has ever seen something in me that I could not, to every colleague who has given their time to help talk through problems, review drafts, or give feedback on presentation, to every friend who has invested time cultivating our relationship even when I didn't have enough to reciprocate in kind, to every teacher who has passed the torch of knowledge on to another, and to my partner Nichole and every member of my family who has encouraged my curiosities, supported my aspirations, and tolerated my absenteeism. These are some of the compartments of my own ecological community; these divisions are seldom mutually exclusive, and often indistinguishable. Over the years, all of my mentors, colleagues, friends, and teachers have contributed, in ways large and small, to my current and future success, and they may all be counted among family.

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TABLE OF CONTENTS

1. GENERAL INTRODUCTION 1

Literature Cited 4

2. THE HUMAN GUT MICROBIOME'S INFLUENCE ON ARSENIC TOXICITY 5

Contribution of Authors and Co-Authors 5
Manuscript Information Page 6
Abstract 7
Purpose of Review 7
Recent Findings 7
Summary 8
Introduction 8
Arsenic in the human environment 10
Water Contamination 10
Soils and Agriculture 11
Exposure via Food 13
Arsenic Inhalation 14
Arsenic in Medicine 15
Human Arsenic Metabolism 16
Bacterial Arsenic Metabolism 19
Arsenate reduction and arsenite efflux 19
Organoarsenic metabolism in Bacteria 22
Arsenic-microbiome interactions 23
The microbiome alters arsenic exposure 25
Arsenic perturbation of the microbiome. 27
Microbiome modulation of arsenic toxicity 31
Challenges and recommendations 31
Conclusions 34
Literature Cited 35

3. THE GUT MICROBIOME IS REQUIRED FOR FULL PROTECTION AGAINST ACUTE ARSENIC TOXICITY IN MOUSE MODELS 44

Contribution of Authors and Co-Authors 44
Manuscript Information 45
Abstract 46
Introduction 46
Results 48

TABLE OF CONTENTS CONTINUED

Microbiome Disruption Affects Arsenic Excretion/Bioaccumulation 48
As3mt and Microbiome are Required for Protection 50
Human Stool Transplantation Provides Full Protection 52
Donor-dependent Variation in Arsenic Protection 53
Individual Microbiome Members Correlate with Survival 57
Faecalibacterium Prausnitzii Provides Protection 59
Discussion 62
Methods 64
Experimental Animals 64
Human Samples 65
Chemical Reagents 66
Arsenic Exposures 66
Fecal Transplantation 67
Arsenic Quantification 68
16S rRNA Gene Sequencing 68
Gnotobiotic Mouse Experiments 70
Statistical Analyses 71
Data Availability 72
Acknowledgements 73
Additional Publisher Information 73
Supplementary Information 74
Supplementary Note 1. Arsenic dosing. 74
Supplementary Tables 77
Supplementary Figures 84
Literature Cited 89

4. SPECIATION OF EXCRETED ARSENICALS FROM GERM FREE AND CONVENTIONAL AS3MT KNOCKOUT MICE EXPOSED TO INORGANIC ARSENATE 94

Contribution of Authors and Co-Authors 94
Manuscript Information Page 95
Abstract 96
Introduction 97
Materials and Methods 100
Experimental Mice 100
Chemical Reagents 102
Experimental Exposures 103

TABLE OF CONTENTS CONTINUED

Arsenic Dosing....103
Sample Collection and Storage....103
Sample Pre-Processing at Montana State University....104
Determination of Total Arsenic Level....105
Arsenic Speciation Analyses....105
Data Transformations and Statistical Methods....106
Results....107
Microbiome Status Influences Total Arsenic Levels in Stool and Urine....107
Speciation of Excreted Arsenicals....110
Microbiome-Driven iAs^{V} Reduction....111
Methylated arsenicals....112
Discussion....114
Literature Cited....120

5. DISCUSSION AND CONCLUSIONS....123

REFERENCES CITED....128

LIST OF TABLES

Table Page

3.1. Supplementary table of dose ranges in murine arsenic exposure studies. 77

3.2. Supplementary table of As3mt-KO survival comparisons (Mantel-Cox). 78

3.3. Supplementary table of humanized mouse survival summaries. 79

3.4. Supplementary table of ANOSIM testing results. 80

3.5. Supplementary table of experimental mouse details. 81

3.6. Supplementary table of PCR primer sets. 83

4.1. Mean relative distribution of speciated arsenicals determined in the urine and stool. 112

LIST OF FIGURES

Figure Page

3.1. Total arsenic levels in the feces and host tissues of C57BL/6 mice 49

3.2. Survival of iAsV-exposed Cef-treated, Sham-treated, and GF As3mt-KO mice 51

3.3. Survival of iAs^V-exposed humanized As3mt-KO mice 54

3.4. Differences in microbiome stability during arsenic exposure 56

3.5. Specific microbiome OTUs are risk factors for survival 59

3.6. *Faecalibacterium prausnitzii* protects against arsenic toxicity 62

S3.1. The influence of cefoperazone on iAs^V exposure. 84

S3.2. Replicate exposures of As3mt-KO humanized donor groups. 85

S3.3. Rarefaction of OTUs from 16S rRNA (V4) sequence data 86

S3.4. Results of survival modeling against OTU abundance 87

S3.5. Survival in groups of GF, *E. coli* mono-colonized, and bi-colonized mice. 88

4.1. Inverse relationship between total arsenic levels in the stool and urine. 108

4.2. Speciated stool and urine arsenicals 113

ABSTRACT

Drinking water contamination with arsenic is a wide-spread public health concern, potentially affecting over 140 million people across at least 40 different countries. Current understanding of biological and behavioral factors influencing clinical outcomes is insufficient to explain the variation observed in arsenic-related disease prevalence and severity. The intestinal microbiome in humans is a dynamic and active ecosystem with demonstrated potential to mediate arsenic metabolism *in vitro* and distinct variability between individuals. This dissertation investigates arsenic-microbiome interactions, with a focus on determining how microbiome activity influences host-response and toxicity from arsenic exposures. Chapter 2 overviews common exposure routes, important metabolic pathways, and current evidence of arsenic-microbiome interactions in humans or experimental animal models. Chapter 3, the initial approach was to experimentally perturb the microbiome of common laboratory mice during arsenic exposure, measuring arsenic excretion in the stool and accumulation in host tissues. Arsenic sensitive gene-knockout mice were used to determine the microbiome's influence on subacute arsenic-induced mortality. Disrupting microbiome function—first by antibiotic treatment, then by deriving mice germ free—dramatically reduced survival times during severe arsenic exposures. Transplantation of human fecal communities into germ free mice effectively complemented the loss of function from microbiome disruption in these mice. Chapter 4 examines microbiome's impact on arsenic metabolism in germ free and conventional mice from this same arsenic-sensitive genetic background. These mice are deficient for the primary metabolic pathway involved in arsenic detoxification in both humans and mice, facilitating a more complete experimental isolation of microbiome and host metabolisms. This study provides evidence of microbiome-dependent changes in the elimination routes and metabolic transformation of ingested arsenic and provides a new experimental model for studying arsenic metabolism in the gut.

CHAPTER ONE

GENERAL INTRODUCTION

In the past 30 years, interest in the human microbiome has expanded rapidly within the scientific community and also the general public. What was once a relatively obscure field of scientific interest has emerged as a global cultural phenomenon equipped with household brand recognition and broad social appeal. Perhaps owing to its relative novelty but also intimately familiar nature, human microbiome research has become the subject of national news segments, philosophical debates, conspiracy theories, and numerous works of popular science (including a number of "fad" diet books). Even those who may not immediately recognize the terms *microbiome* or *microbiota* in casual conversation are likely aware of some fact or factoid related to the beneficial microbes living in our bodies and keeping us healthy and safe. Popular hype aside, microbiome research has led to far reaching impacts across the life science disciplines, but perhaps nowhere more so than the disciplines relating to human health (*1*).

In 2018, the terms "toxicomicrobiomics" was proposed as an emergent field of systems-based toxicology (e.g. toxicogenomics) focusing on the multidimensional interactions between toxic agents and the host-microbiome system (*2, 3*). This concept, in essence, describes the common theme underlying

this dissertation research. Establishment of causation and mechanism are two of the core challenges in microbiome research (*4, 5*). Here, systems-based research designs were applied to the study of arsenic exposure, metabolism, and toxicity; breaking down the host-microbiome system into constituent parts using novel experimental approaches.

The overarching hypothesis driving this research is that interindividual variation in the human microbiome influences health outcomes from arsenic exposure. Over the course of three manuscripts (one published, two prepared), I first frame this problem in the context of a focused literature review, then present *in vivo* evidence supporting the arguments that microbiome activity reduces acute arsenic toxicity, toxic outcomes depended on microbiome community membership variation, and that overall excretion and metabolism of arsenic is mediated by the microbiome. The first main chapter of this thesis is a review of current literature reporting arsenic-microbiome interactions and their potential health impacts. It also discusses methods used in microbiome research and highlights crucial research needed for the field moving forward. The next chapter presents a published research manuscript detailing a novel germ free mouse model using mice deficient for arsenic detoxification metabolism. This model was used to study the impacts of human and mouse microbiota on toxic outcomes from arsenic exposure *in vivo*, providing direct evidence of microbiome-dependent effects on host survival and mortality phenotypes. The last main chapter consists

of a prepared manuscript using a similar experimental system to characterize the overall impact of the native murine microbiome on arsenic metabolism and excretion *in vivo*. Together, these reports are among the first to establish a causal linking between microbiome status and host outcomes from arsenic exposure. Evidence of microbiome-dependent changes in arsenic excretion routes and metabolism are presented as a plausible mechanism for observed influences on toxic outcomes.

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CHAPTER TWO

THE HUMAN GUT MICROBIOME'S INFLUENCE ON ARSENIC TOXICITY

Contribution of Authors and Co-Authors

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Contributions: Communicated with publisher, advised formatting and organization of literature review, edited manuscript.

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Abstract

Purpose of Review

Arsenic exposure is a global public health concern of with a high degree of interindividual variability in pathologic outcomes. Arsenic metabolism is a key factor underlying toxicity. The primary purpose of this review is to summarize recent discoveries concerning the influence of the human gut microbiome on the metabolism, bioavailability, and toxicity of ingested arsenic. This review highlights the current state of knowledge along with relevant methodologies for studying these phenomena.

Recent Findings

Bacteria living in the human gut can biochemically transform arsenic-containing compounds (arsenicals). Recent publications utilizing culture-based approaches combined with analytical biochemistry and molecular genetics have helped identify several arsenical transformations by bacteria that are at least possible in the human gut and are likely to mediate arsenic toxicity to the host. Other studies that directly incubate stool samples *in vitro* also demonstrate the gut microbiome's potential to alter arsenic speciation and bioavailability. *In vivo* disruption or elimination of the microbiome has been shown to influence toxicity and body burden of arsenic through altered excretion and biotransformation of arsenicals. Currently, few clinical or epidemiological studies have investigated relationships between the gut microbiome and arsenic-related health outcomes in

humans, although current evidence provides strong rationale for this research in the future.

Summary

Human gut microbiota can metabolize arsenic, mediating redox transformations, methylation status, and thiolation status; ultimately impacting bioavailability and excretions. We discuss the current evidence and propose the microbiome be considered in future studies of human arsenic exposure.

Introduction

Arsenic is among the most widespread and dangerous environmental toxicants in the world. Most environmental arsenic comes from the natural weathering of arsenic containing minerals in the earth's crust; as such, it can become associated with human food and water as well as surface soils and particulates in the air (*6*). Anthropogenic sources of contamination can also be significant, especially in areas with mining activity and industrial arsenic applications (*7*). Acute exposure to high levels of arsenic-containing compounds (arsenicals) is toxic to most cellular life forms and chronic, low-level exposure in long-lived organisms, like humans, is also associated with disease. The International Agency for Research on Cancer (IARC) has classified arsenic and inorganic arsenicals (see below) as Group I carcinogens, finding sufficient evidence that these toxicants cause cancers of the lung, skin, and bladder (*8*).

Non-cancerous pathologies have also been linked to arsenic exposure, including skin lesions, metabolic dysregulation, diabetes mellitus, cardiovascular disease, pregnancy complications, and neurological symptoms (*9*). Importantly, the development of these pathologies in arsenic exposed populations is highly variable between individuals, even when accounting for host factors such as genetics and arsenical-specific metabolisms (*10*). Genome-wide association studies and advancements in analytical methods have led to a robust understanding of the importance of human genetic determinants of arsenical metabolism that drive chemical speciation of arsenic in mammalian cells (*11*). However, human genetic variability does not adequately explain disease penetrance among exposed populations, leaving the door open for discovering other important explanatory factors (*12, 13*).

A microbiome can be defined as the community of microorganisms occupying a defined ecosystem, and the sum total of their physical, biochemical, and ecological activities (*14*). The human microbiome is taxonomically diverse and comprised of bacteria, viruses, fungi, micro-eukaryotes, and archaea. It is now well appreciated that interactions between the microbiome, host cells, and the abiotic environment have significant impacts human health and disease (*15*). Here, we focus on microbiome activity of the human gastrointestinal tract, or gut. From an ecological perspective, the human gut is an ecosystem and provides ecosystem functions just like a forest or ocean ‘biome’. Metabolism, meaning the

biochemistry performed by microbial and human cells in the gut, is a critical function that can both directly and indirectly impact human health. Since all living organisms have to deal with arsenic to minimize its potential toxicity, it should be no surprise that members of the gut microbiome can metabolize arsenicals, thereby changing its toxicity in host tissues. On the other hand, if arsenic exposure kills certain members of the gut microbiome, their functions will be lost, which may indirectly influence host health.

In this review, we overview human exposures to arsenical compounds, known pathways of human arsenic metabolism and excretion, and the influence of arsenic-microbiome interactions on host physiology, arsenical metabolism, bioavailability, and toxicity. We also discuss evidence of arsenic-induced compositional and functional changes in the microbiome community and potential contributions to host health associated with those changes.

Arsenic In the Human Environment

Water Contamination

Inorganic arsenic (iAs) leeches from mineral deposits in the earth's crust and into underground aquifers, leading to human exposure when wells are drilled or dug to meet water needs. In fact, the most common route of arsenic exposure in humans is contaminated drinking water (*6*). Arsenic input into underground aquifers can be accelerated by anthropogenic activities, such as mining, but

surface water pollution is more often attributed to deposition from mining, smelting, and agricultural applications compared to natural leeching (*16*).

The rate of iAs leeching into the water table depends on arsenical redox state and geologic factors, such as soil type. Unlike other toxic metal(oid)s in the environment, which may form inorganic cations in solution, dissolved iAs is found as oxy-acid compounds (*17*). Under normal conditions with circumneutral pH, pentavalent arsenate (iAs^{V}) exists as an oxy-anion ($H_2AsO_4^-$, $HAsO_4^{2-}$), while reduced trivalent arsenite (iAs^{III}) is typically uncharged ($H_3AsO_3^0$) and more mobile in the subsurface (*18*). In oxygenated solution, iAs^{V} is most stable, but a shift to even mildly reducing conditions can favor the formation of the more mobile iAs^{III} in the presence of the appropriated reductants. iAs^{III} is also considered more toxic and bioavailable to human cells compared to iAs^{V}. Thus, microorganisms both directly and indirectly influence iAs cycling in soil, subsurface, and aquatic ecosystems (*19*). Collectively, this means that environmental microbiomes profoundly impact the mobility, bioavailability, and toxicity of environmental iAs to humans (*19, 20*).

Soils and Agriculture

Contaminated soils and dust have also been considered as potential sources of arsenic exposure, especially among children because of common hand-to-mouth behaviors (*21, 22*). Although arsenic ingestion from soils is much lower than from food and water, relative concentrations of arsenic in polluted soils can

sometimes reach concentrations several orders of magnitude higher than the safe drinking water standard (*23*). Pollution of soils is often a result of deposition from smelting, coal burning, and agricultural pesticides. Agricultural pesticides represent a significant source of surface soil contamination. During the late-19th and early- to mid-20th centuries, inorganic arsenates of copper, calcium, and lead were widely used as agricultural pesticides throughout the US and other countries (*24*). Despite the documented health hazards and environmental persistence of these inorganic metal(oid)s, lead arsenate ($Pb_5OH(AsO_4)_3$) became the most prominent agricultural pesticide of the time and was not officially prohibited in the United States until 1988 (*25*). This wide-spread historical use still contributes to high levels of both lead and arsenic in the soils of current and former agricultural lands (*26*), and elevated levels continue to be reported in a variety of food products (*27*).

Despite the phasing out of most inorganic arsenicals from use, organic arsenical compounds (oAs) are still widely used in agriculture and landscape management. Monosodium monomethyl arsenate (MSMA) is a commercially available arsenical herbicide used for residential lawn care and commercial weed control in golf-courses, sod farms, and highway rights-of-way (*28*). Synthetic phenyl-arsenic compounds, including Roxarsone and Nitarsone, are used in poultry farming as growth promoting feed additives, and to deter certain veterinary pathogens (*29*). While these pentavalent oAs are generally considered

non-toxic and safe at limited exposure levels, they are subject to degradation by native microbial communities, contributing to environmental arsenic pools and potential risks to public and environmental health (*29-32*). The US FDA recently withdrew approval for the use of organo-arsenical drugs as growth promoting agents in poultry feeds, but they remain commonly used outside North America and the European Union (*33*).

Exposure via Food

Detectible arsenic in foods raises concerns about the potential contribution of dietary exposures. Arsenic can accumulate in cereal grains, vegetables, and fruit crops from contaminated irrigation water or surface soil environments (*34*). Rice stands out among cereal grains as one of the world's most widely consumed stable foods. Rice and rice-based food products often contain higher fractions of pentavalent arsenicals (iAs^V; monomethylarsonic acid, MMA^V; and dimethylarsinic acid, DMA^V), compared with other grains and vegetables (*35*). The pentavalent methylated arsenicals are likely produced by microbial communities associated with the plant roots prior to uptake (*36*). Arsenic in seafood occurs predominantly as more complex forms of oAs compounds, including arsenobetaine and arsenocholine in finfish and a wide variety of arsenic-containing lipid and sugar compounds in mussels and shellfish (*37-39*). As with all routes of arsenic exposures, toxicity depends on the chemical properties of the arsenical. Depending on the sources of contamination and environmental conditions, dietary

arsenicals may have complex and diverse speciation profiles, resulting in large differences in toxicity and risk of disease. Arsenical speciation, variations in food matrices, and nutritional state of the host, likely contribute to variation in bioavailability and risk of disease (*40*). More research is necessary on food as a significant route for arsenic exposure.

Arsenic Inhalation

A number of different processes contribute to atmospheric arsenic levels, including volcanic activity, mining and industrial processes, combustion of fossil fuels, use of agricultural pesticides, and volatilization of arsine compounds. Compared with drinking water exposure, inhalation is considered a minor source of arsenic exposure for the general population (*8*), however, those living or working in proximity to emission sources may be at substantially higher risk of adverse exposure outcomes (*41*). The largest sources of atmospheric arsenic emission are metal smelting, coal combustion, and herbicides, with arsenic laden particulate matter (PM) being the major medium of atmospheric transport (*42*). Particulate matter with an aerodynamic diameter smaller than 10 μm (PM_{10}) can be inhaled, while only particles smaller than 2.5 μm ($PM_{2.5}$) penetrate into the lungs where they can be deposited onto the pulmonary epithelium (*43*). Arsenic present in coal or mineral ore evaporates during combustion and high-heat processing, then adsorbs onto finer particles of fly ash, resulting in atmospheric emissions of arsenic-containing PM_{10} and $PM_{2.5}$ (*44*). Atmospheric arsenic from

coal combustion has been cited as a major factor contributing to lung cancers in industrial regions of India and China (*8*).

Tobacco smoke is another significant source of inhaled arsenic, as arsenic-based pesticides in the soil can be taken up by tobacco plants. The risk of disease from arsenic contaminated tobacco is difficult to determine because smoking behavior carries an independent carcinogenic risk and has a synergistic effect with arsenic in food and water (*45*). Arsenic has also been detected in the synthetic fluids of electronic cigarettes and the vapors they produce (*46*). While the reported concentrations were lower than those identified in combustible tobacco products and smoke, further evaluation and quantitative risk assessment are still needed to determine whether these relatively new products represent a comparative reduction of risk.

Arsenic in Medicine

In contrast to the health problems associated with chronic incidental exposures, arsenic compounds have also been used therapeutically throughout history. In the pre-antibiotic era, drugs like arsanilic acid (Atoxyl) and arsphenamine (Salvarsan) were commonly used to treat syphilis and trypanosomiasis, among other common ailments (*47*). Before the use of arsenicals as antibiotics was curtailed and modern antibiotics (e.g. penicillin) were introduced, it was noted that both laboratory and clinical syphilis strains had evolved resistance to arsenical treatments (*48, 49*), hinting at the potential

influence of arsenic on the evolutionary dynamics of human associated bacteria, and foreshadowing contemporary struggles with antibiotic resistance.

Currently, arsenic trioxide (ATO) is still used as an anti-cancer treatment for patients with Acute Promylocytic Leukemia (APL). The exact molecular mechanisms of its anti-cancer activity are still being studied, but it has been suggested that arsenic biotransformations in the body are important to ATO's clinical efficacy (*50*). There is also growing evidence that the microbiome alters patient response and clinical outcomes of chemotherapy (*51*), leading some to speculate that targeted manipulations of the microbiome could be used to improve clinical outcomes and/or reduce toxic side-effects of current chemotherapeutics (*52*). However, this concept of "pharmacomicrobiomics" has yet to be applied to therapeutic uses of ATO and recent trials exploring orally delivered ATO as a replacement for intravenous treatments (*53*) underscore the importance of determining the microbiome's influence on arsenic metabolism in the context of human medicine.

Human Arsenic Metabolism

In humans and a number of other animals, iAs entering the body in food and water is methylated as the primary means of systemic detoxification. Methylated arsenicals are more readily excreted, leading to enhanced body clearance (*54*), and efficient arsenic methylation is associated with beneficial

long-term disease outcomes (*55*). The complete mechanism of mammalian arsenic methylation with respect to the identity of reactants and order of products is still somewhat debatable and has been extensively reviewed (*56, 57*). To summarize, ingested iAs is taken up across the epithelium via both trans-cellular and para-cellular transport pathways(*58*). Following cellular entry, iAs is transformed into a number of methylated arsenicals, with the more prominent being monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), and dimethylarsinous acid (DMA^{III}). Methylation is enzymatically catalyzed by arsenic (3+) methyltransferase (AS3MT), utilizing S-adenosylmethionine (SAM) as a methyl group donor in concert with endogenous reducing agents such as thioredoxin and glutathione (GSH) (*59, 60*). Recently, a hypothesis based on evidence from crystallography demonstrated that pentavalent intermediates are likely reduced while still bound to AS3MT, which suggests that more toxic trivalent arsenicals may be the end-products of methylation rather than the less toxic pentavalent species (*61*).

While GSH is an important reductant in the methylation pathway, glutathione-conjugated forms of arsenic are also substrates for active transport and efflux of arsenic from cells. Arsenic tri-glutathione ($As(GS)_3$) and monomethyl arsenite di-glutathione ($MMA(GS)_2$) conjugates have been detected in urine and bile of mammals (*62*). In addition to GSH conjugates, several sulfur-containing arsenicals can also be formed in the body. *In vitro* and *in vivo* studies

in a variety of organisms have demonstrated the production of thioarsenicals following arsenic exposure (*63*). For example, thioarsenicals have been detected in human urine and in urine and feces of animal models (*64, 65*). Human and rat red blood cells both have the capacity to thiolate arsenic *in vitro* (*66*), but the toxicological implications and primary mechanism(s) of arsenic thiolation in the body remain unclear. Regardless, pentavalent monothiolated arsenicals like monomethyl monothioarsonic acid ($MMMTA^V$) and dimethyl monothioarsinic acid ($DMMTA^V$), have cytotoxicity more similar to trivalent arsenicals compared to their non-thiolated counterparts (i.e. pentavalent methylated oxoarsenicals) (*67*). A number of different chemical pathways have been proposed for the formation of thioarsenicals and have been thoroughly reviewed (*56*). In general, these pathways are non-enzymatic and involve interactions between methylated arsenicals and sulfide ions or bound sulfane sulfur, resulting in one or more of the arsenic-oxygen bonds being replaced by analogous arsenic-sulfur bonds (*56*). Hydrogen sulfide (H_2S) is an important signaling molecule throughout the body and source of sulfide ions. The liver is perhaps the most well-known source of biogenic H_2S, suggesting that hepatocytes play a significant role in thiolation. This hypothesis, however, has yet to be experimentally addressed.

Bacterial Arsenic Metabolism

Bacterial arsenic metabolism has largely been identified and studied by examining microbial "resistance" to arsenic-induced effects (e.g. killing). Although metabolism and resistance often detoxify arsenicals, not all resistance pathways involve the biochemical transformation of arsenic (see below). Resistance gene clusters, dubbed *ars* operons, were first characterized in plasmids isolated from *Escherichia coli* and *Staphylococcus aureus* (*68, 69*). Bacterial *ars* genes have since been identified and characterized in a variety of clinically important pathogens including *Listeria monocytogenes, Campylobacter jejuni,* and *Yersinia* strains (*70*). The *ars* operon has also been found in known human gut symbionts like *Bacillus subtilis* (*71*) and the obligate anaerobe *Bacteroides vulgatus* (*72*). The molecular functions, distribution, and evolution of bacterial arsenic resistance have been reviewed in depth (*70, 73-75*). Here, we provide an overview of common modes of bacterial arsenic resistance and metabolism, with a focus on those described or predicted in the intestinal environment.

Arsenate Reduction and Arsenite Efflux

The 'core' function of the *ars* operon is conferred by the *arsBC* genes, encoding for an iAs^{III}-specific efflux pump (*arsB*) and a cytosolic iAs^{V} oxidoreductase (*arsC*) (*76, 77*). Resistance to iAs^{III} can be as simple as a one-step process consisting of ArsB-mediated efflux, while resistance to iAs^{V} involves at

least a two-step process via ArsC-mediated reduction to iAs^{III} followed by ArsB-mediated efflux. Most *ars* operons also contain a regulatory gene, *arsR*, that encodes a trans-acting, iAs^{III}-responsive transcriptional repressor (*78, 79*). Efflux via ArsB is complemented or augmented in some bacteria by the homologous Acr3 transporter (*80, 81*). Despite independent evolutionary origins, ArsB and Acr3 have nearly identical functions, with respect to iAs^{III}. Both function as secondary transporters coupling iAs^{III} efflux with H^+ ion exchange, and both can function as subunits in a heterodimeric, ATP-driven iAs^{III} pump in the presence of the catalytic subunit, ArsA (*82*). *arsB* and *acr3* genes are found in bacterial *ars* operons in roughly equal frequency, suggesting that iAs^{III} efflux was important in the evolution of arsenic resistance (*75*).

Arsenate reductase enzymes encoded by *arsC* genes are structurally and functionally similar to low molecular weight tyrosine phosphatases (*83*). ArsC-mediated iAs^{V} reduction is coupled with cellular thiol-disulfide exchange systems, which vary depending on the lineage of ArsC expressed (*84*). In *E. coli* and other proteobacteria, iAs^{V} reduction is commonly coupled with GSH and glutaredoxin, while thioredoxin-dependent ArsC enzymes are common in Firmicutes and Bacteroidetes phyla (*85*). A third class of ArsC enzymes, first characterized in an isolate of *Corynebacterium*, utilizes the cellular mycothiol/mycoredoxin system (*86*). This little studied redox system is so far uniquely found in Actinobacteria

but has functional similarities to the GSH/glutaredoxin systems found in Proteobacteria.

Many *ars* operons also carry extended *ars* genes that support this core functionality. Two of the most common are *arsA* and *arsD*. As mentioned above, *arsA* encodes for a catalytic ATPase that forms a heterodimeric complex with ArsB/Acr3 transporters capable of ATP-driven primary efflux of iAs^{III} (*82*). ArsD is a metallochaperone which facilitates cytosolic transport of reduced iAs^{III} to the ArsAB complex for efflux (*87*). While *arsA* and *arsD* are not essential for arsenic resistance, their expression improves the efflux efficiency and transcriptional regulation of this arsenate reduction/arsenite efflux resistance pathway.

In addition to cytosolic arsenate reduction, many prokaryotes are capable of utilizing iAs for respiration. These systems differ from ArsC-mediated iAs^{V} reduction first because they take place in the periplasm and second because they harness energy from the redox conversion between iAs^{III} and iAs^{V} in the form of electrochemical gradients and electron transport. To date, there is little evidence of respiratory arsenic metabolism in the human gut environment. This may be due to the abundance of more favorable electron donor/acceptor couples in the intestine, although it is possible that they have simply not been identified yet. Several expert reviews are available that detail the structures, mechanisms, and distribution of these energy-harvesting arsenic systems (*88, 89*).

Organoarsenic Metabolism in Bacteria

Human AS3MT, mentioned above, is a homolog of bacterial and archaeal ArsM, likely acquired through horizontal gene transfer (*90*). Bacterial ArsM catalyzes the methylation of iAs^{III} utilizing SAM as a methyl donor in a similar fashion to that of human AS3MT. Arsenic-methylating bacteria can generate significant amounts of mono-, di-, and trimethylated arsenicals from iAs^{III} (*91*). While arsenic methylation functionally detoxifies iAs into MMA^{V} and DMA^{V} under oxidizing conditions, bacterial methylation has been shown to yield more toxic trivalent forms of mono and dimethyl arsenic in in a simulated gut environment (*92*). Li et al. recently proposed that arsenic methylation would not have evolved as a mode of detoxification in the early biosphere, but as a functional secondary metabolic pathway (*93*). Similar to allelopathy in plants, arsenic-methylating bacteria may have gained a selective advantage in anoxic environments by secreting toxic MMA^{III} into their surroundings, thus inhibiting the growth of competitors. Interestingly, greater amounts of MMA^{III} are formed when arsenic methylating bacteria are grown in co-culture, whereas the less toxic MMA^{V} is the favored product of the same organisms grown in monoculture (*94*). Li et al. also discuss the evolution of multiple resistance pathways against MMA^{III} and other toxic trivalent organoarsenicals, including efflux mediated by ArsP (*95*) and ArsK (*96*), demethylation back to iAs by ArsI (*30*), and chemical oxidation to the pentavalent state (oAs^{III} to oAs^{V}) by ArsH (*97*). ArsP mediated efflux is more

specific to MMA^{III} but can also confer resistance to other trivalent organoarsenicals. ArsK is more promiscuous, conferring resistance to a variety of organic and inorganic trivalent arsenicals. ArsI is a carbon-As bond lyase capable of demethylating MMA^{III} to the less toxic iAs^{III} (*30, 98*).

Although *arsM* has been found in the genomes of different bacteria that inhabit the human gut, it is not yet clear whether arsenic methylation occurs in this environment. Similarly, ArsP, ArsH, and/or ArsI activities have not been experimentally evaluated and so it is unknown whether these play a role in human arsenic toxicity. It is worth noting that *arsI* has only been identified in aerobic bacteria, suggesting that it may not be common amongst the abundant anaerobic bacteria of the human gut (*30*). That said, there is plenty of oxygen along the gut mucosa to support facultative bacteria and so even oxygen-dependent arsenic metabolisms should be considered possible until experimentally ruled out.

Arsenic-Microbiome Interactions

Much of what is known about arsenic-microbe interactions comes from environmental microbiology and in ecosystems such as soil and the subsurface, where microbial metabolisms are the primary determinants of arsenic speciation, mobility, and toxicity (*99*). Many of the same principles used in these environmental microbiology studies of arsenic can be directly applied to

understand arsenic interactions with the human microbiome. For example, arsenic-microbiome interactions can have three general and sometimes overlapping outcomes: no noticeable effect, perturbation of microbiome taxonomic structure and function, and alteration of the pharmacological and/or toxicological properties of the toxicants (77). Several studies argue that the microbiome has little influence on toxicants like arsenic (and that these toxicants have little influence on the microbiome) because they are taken up into tissue or into circulation before such interactions can take place. As discussed below, there is now strong evidence to the contrary. Alteration of microbiome community structure/function is often part of the body's physiologic response to xenobiotic compounds, and microbiome structure-function relationships have been described for many human diseases, syndromes, and behaviors (*100*). Recovery from an altered microbiome structure depends on which members of the microbiome are affected and whether these taxa can recover. If they cannot, a "dysbiotic" state may persist that promotes deleterious health outcomes. For example, perturbation of the gut microbiome by a toxicant can influence normal host uptake, metabolism, and excretion of dietary nutrients and may feedback on uptake of the toxicant. Similarly, toxicant killing of microbiome members may alter the maintenance of the gut epithelial barrier, regulation of host inflammatory responses, and synthesis or recycling of important metabolites and co-factors involved in the host's toxic response pathways. Evidence for this type of

interaction is also discussed below. Finally, pathologic outcomes due to chemical toxicant alteration of microbiome structure-function is juxtaposed by outcomes caused by microbiome biotransformation of chemical toxicants that alter their physicochemical properties. Sometimes these alterations result from direct, enzymatic activity (e.g. ArsBC), but sometimes they result indirectly and non-enzymatically when byproducts of microbial metabolism chemically interact with toxicants. Adding to this complexity, some biotransformations may have no effect, some may ameliorate, and some may significantly increase toxicity. As with the other types of interactions, evidence for microbiome biotransformation of arsenic is discussed below.

The Microbiome Alters Arsenic Exposure

The notion that intestinal bacteria contribute to health outcomes following arsenic exposure is now more than a century old. In 1917, Puntoni reported the ability of spore-forming bacteria isolated from human stool to produce a potent garlic odor when cultivated in the presence of cacodyl arsenic (*101*). It was noted at the time that the odor was also common in people taking therapeutic arsenical compounds orally, but less common when taken subcutaneously, leading him to suspect that the gut microbiome was chemically altering arsenic. In another study, Challenger and Higginbottom (1935) identified a similar gaseous arsenical produced by *Scopulariopsis brevicaulis* (formerly *Penicillium brevicaule*) as trimethyl arsine gas (*102*). More recently, *E. coli* isolated from the cecal contents

of rats were shown to metabolize $DMAs^{V}$, producing $TMA^{V}O$ and an unidentified arsenical (*103*).

In addition to pure-culture experiments, arsenic metabolism has been studied in the context of whole microbiome communities isolated and experimentally evaluated *in vitro*. In controlled experiments, intestinal contents from rodents demonstrated a high capacity for enzymatic reduction and methylation of iAs mediated by the microbiome (*104, 105*). Furthermore, two different studies showed that human microbiota reduced and methylated iAs^{V} in a simulated human gut environment, yielding both toxic (MMA^{III} and DMA^{III}) and non-toxic (MMA^{V} and DMA^{V}) methylated arsenicals (*92, 106*). Interestingly, iAs^{V} was reduced to iAs^{III} even in autoclave sterilized experimental controls, suggesting that non-enzymatic processes contribute to this transformation in the gut.

While the above studies provide strong evidence that the microbiome has the potential to influence arsenic speciation, none of them experimentally determined the overall impact that the microbiome has on host exposure. In Coryell et al., antibiotic pretreatment of arsenic-exposed mice significantly reduced fecal arsenic excretion and increased host accumulation of arsenic in the liver and lung tissues (*107*). We speculate that microbial biomass in the gut, depleted by antibiotic exposures, is involved in mediating fecal elimination of ingested arsenic. Other studies have shown that iAs adsorbs onto extracellular polymeric substances of Gram-positive, but not Gram-negative, bacterial isolates

(*108*), providing a possible mechanism for microbial accumulation of arsenic in the gut . Some evidence from Coryell et al. also supports this mechanism, as the Gram-positive bacterium *Faecalibacterium prausnitzii* protected AS3MT-KO mice during iAs^{V} exposure, while Gram-negative *E. coli* did not (*107*).

Arsenic Perturbation of the Microbiome.

Arsenic has been found to change the taxonomic structure of the microbiome in lab animals and human populations. In mice and rats, arsenic exposure induced shifts in microbiome community composition, functional metagenome, metabolite profiles, and proteome (*109-112*). Arsenic-induced perturbations depend on the dose and duration of arsenic exposure (*112*), with significant changes reported from drinking water containing as little as 10 ppb of iAs^{III} (*113*) (i.e. the current maximum contaminant level for drinking water set by the World Health Organization and the US Environmental Protection Agency). Even though microbiome change can be quantified following arsenic exposure, it can be difficult to determine whether these changes have deleterious effects for the host. For example, lab animal studies have made efforts to identify plausible links between arsenic-induced changes in the microbiome and host physiology, including altered host nitrogen homeostasis (*113*), energy metabolism (*114*), gut immune signaling (*115*), and epithelial histology (*110*). In all of these cases, however, it remains unclear whether the observed changes in the host were caused directly by changes in the microbiota, arsenic toxicity, or interactions

between them. It is necessary to understand the difference between association and causation and complementary experiments in gnotobiotic animal models can help fill this gap.

In a cohort of US infants with low to moderate arsenic exposure, microbes representing 8 genera from the *Firmicutes* phylum were positively associated with urinary arsenic concentrations at 6 weeks of age, while 15 genera, including *Bacteroides* and *Bifidobacterium* were negatively associated. Notably, effects were stratified by sex and feeding status, with male and formula fed infants more susceptible to arsenic-related effects on the microbiome (*116*). This is in contrast to findings in CD-1 mice that females were more sensitive to arsenic-induced microbiome perturbation (*115*). In Bangladeshi children (4-6 years of age), high levels of arsenic in home drinking water was associated with a greater abundance of *Gammaproteobacteria* in the microbiome, more specifically, members of the *Enterobacteriaceae* family (*117*). Metagenomic analysis identified an overall enrichment of genes involved in antibiotic exposure and multi-drug resistance, suggesting that arsenic and antibiotic resistance may be effectively linked. This finding is supported by animal studies demonstrating co-enrichments of antibiotic and metal resistance genes in fecal metagenomes of arsenic exposed mice (*110, 118*). Arsenic resistance genes have been characterized on a number of bacterial plasmids and other mobile genetic elements that also containing antibiotic resistance determinants (*70*), and bioinformatic analyses have identified a high

degree of co-occurrence between *ars* genes, resistance to tetracycline, mercury, and copper, and a class 1 integrase gene associated with bacterial horizontal gene transfer (*119*). Thus, enrichment of antibiotic and metal resistance in the arsenic exposed microbiome may be mediated by both co-selection and mobilized genetic elements.

The effects of arsenic on the microbiome appeared to be unique when compared to other environmental metal and metalloid toxicants (*112, 120*). However, there is also little similarity in compositional microbiome shifts during arsenic exposures among different studies. For instance, in Dong et al., *Enterobacteriaceae* were effectively the only taxa enriched in the stool of arsenic-exposed Bangladeshi children (*117*), while Hoen et al. reported a strong negative association between fecal *Enterobacteriaceae* and urinary arsenic in US infants (*116*). Coryell et al. recently used humanized AS3MT-KO mice (i.e. germ free mice that received a human fecal transplant) to evaluate the effect of arsenic on microbiomes from different human donors and whether these differences influence disease outcome (mortality) (*107*). Each donor group had markedly different microbiome communities, which influenced the degree to which those communities were perturbed by arsenic exposure. Few bacterial taxa were consistently associated with a beneficial outcome (i.e. longer survival) and these belonged to some of the most common taxa found in the human gut. For example, representative taxa from *Lachnospiraceae* and *Ruminococcus* families were

significantly associated with increased survival across humanized groups. These are common and diverse groups of Bacteria associated with the human gut. Although shared by most individuals, there may be important strain level differences that lead to distinct functional outcomes (*121*). Nutritional factors may also help explain differences between studies. In mice, moderate zinc deficiency was shown to exacerbate arsenic-induced microbiome shifts (*114*), while iron supplementation reduced the effects of arsenic on the microbiome and host response (*110, 122*). Enrichments of bacterial iron acquisition pathways under arsenic exposure were also reported in rats, including the bacterial iron complex transport system (*112*) and enterochelin, a powerful iron chelator commonly found in members of *Enterobacteriaceae* (*117*).

In summary, arsenic exposure may cause compositional and functional changes in the gut microbiome, but not all reports identified consistent effects. Animal experiments using a variety of exposure conditions, delivery routes, microbiome analyses, and arsenic concentrations (10 ppb - 100 ppm), have reported arsenic-induce perturbations dependent on host sex, arsenic dose, exposure time, and dietary micronutrients. Arsenic may be an important factor in the spread and/or maintenance of antibiotic resistance genes, and further investigation is needed to determine the extent to which reservoir of antibiotic resistance genes in the gut are impacted by arsenic exposures. Both clinical and epidemiological studies examining arsenic-microbiome interactions should clarify

these and other potential links and these studies should include arsenical speciation as well as proxies for microbiome function.

Microbiome Modulation of Arsenic Toxicity

In vivo, microbiome phenotypes have been linked to altered ratios of methylated and inorganic arsenicals in the host (*123*). However, few studies have directly linked microbiome change, alteration, or absence with host health. In the study mentioned previously, Coryell et al. showed that an intact "normal" microbiome significantly delayed arsenic-induced mortality in A3mt-KO mice, compared to germ free and antibiotic treated groups proving strong evidence that the microbiome protects the host from arsenic toxicity (*107*). They also showed that engraftment of human fecal microbiota into germfree As3mt-KO conferred significant but donor-dependent protection, suggesting that interindividual community variation contributes to microbiome-mediated protection from arsenic. A better understanding of the mechanisms underlying microbiome protection from arsenic and other metal toxicity is needed. Specifically identifying which mechanisms of protection are most beneficial is a key focus of current research

Challenges and Recommendations

Terminology like "toxicomicrobiomics" and "pharmacomicrobiomics" have emerged in recent literature from increased interest in microbiome-mediated metabolism of xenobiotic compounds and its influence on physiological outcomes

(*3*). These emerging fields provide a framework for incorporating microbiome research into quantitative risk assessment, public health, and precision medicine. Despite many unknown factors, evidence discussed in this review suggests that the microbiome plays a significant role in limiting host exposure to arsenic. Further study of these functions will be needed to determine whether effects are due to direct microbial metabolism of arsenic or some other indirect mechanisms. Mechanistic research should emphasize relevant dosimetry, appropriate experimental manipulations, and seek to establish causal links to health outcomes in the host. Experimental reproducibility and generalizability are perennial challenges in health research, especially when it comes to the microbiome. Microbiome variation between different animal vivariums can contribute to unexpected experimental variation and a lack of reproducibility in results (*124*). This not only demonstrates the urgency for developing better standardizations in animal models, but also highlights the need for further investigation of natural microbiome variation between individuals and populations. Germ free and gnotobiotic models, along with transplantation of human microbiomes, represent powerful tools for assessing the effects of natural or induced variation *in vivo*. Best practices are also being proposed for standardization of experimental designs, sample collection, data analysis, and integration of microbiome data with other targeted or non-targeted data sets. More work is also needed in developing

animal models of chronic arsenic toxicity, as chronic exposure represents the greatest threat to human well-being.

So far, very few epidemiological studies have investigated arsenic-microbiome interactions. Even fewer interrogated host health as a variable. While single-timepoint observational studies may identify potential associations with microbiome composition or activity, they are limited to correlative inference. Prospective cohort studies tracking oral or fecal communities over time could help interactions between microbiome arsenic-related disease and it is important to recognize that the microbiome is highly dynamic at the strain level (*121*). Information regarding the incidence of enteric infection or antibiotic use in arsenic exposed populations could be incorporated in both cohort and case-control studies to help determine the potential influence of these known microbiome modifiers on long-term risk on arsenic-related disease. Clinical research on the use of ATO in medical treatment of AML could also benefit from integration of microbiome analyses into empirical case reports and mechanistic studies. Given the evidence in animal models, it is plausible that antibiotic use or other microbiome perturbations may influence the kinetics and efficacy of ATO administered orally. Current evidence of the microbiome's influence on arsenic uptake and excretion may or may not translate to ATO administration, as alternate chemical sources of arsenicals are more often common in animal models of arsenic toxicity.

Conclusions

The science of arsenic toxicology underwent a paradigm shift with the development of technologies and methods allowing for rapid and accurate speciation of arsenical compounds. Similarly, advancements in DNA sequencing and meta-omics technologies have changed our understanding of microbial interactions in human health. We reviewed and summarized recent insights into the influence of the microbiome on arsenic metabolism, excretion, and toxicity and discussed the influence of microbiome perturbations on arsenic exposure in both animal models and humans. The microbiome clearly has the potential to alter host arsenic metabolism and disease outcomes in mice. However, more research is needed to quantify microbial metabolism of arsenic, *in vivo*, and to identify underlying mechanisms influencing host uptake, metabolism, and excretion. Animal research is currently limited by availability of adequate animal models for arsenic-induced disease, especially with regards to health effects from chronic arsenic exposures, which represent perhaps, one of the greatest threats to human health. Further exploration and application of germ free and gnotobiotic animal models may help identify causal arsenic-microbiome links relevant to clinical practices and interventions.

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CHAPTER THREE

THE GUT MICROBIOME IS REQUIRED FOR FULL PROTECTION AGAINST ACUTE ARSENIC TOXICITY IN MOUSE MODELS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Collected data, mouse designed methods

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Abstract

Arsenic poisons an estimated 200 million people worldwide through contaminated food and drinking water. Confusingly, the gut microbiome has been suggested to both mitigate and exacerbate arsenic toxicity. Here, we show that the microbiome protects mice from arsenic-induced mortality. Both antibiotic-treated and germ free mice excrete less arsenic in stool and accumulate more arsenic in organs compared to control mice. Mice lacking the primary arsenic detoxification enzyme (As3mt) are hypersensitive to arsenic after antibiotic treatment or when derived germ free, compared to wild-type and/or conventional counterparts. Human microbiome (stool) transplants protect germ free As3mt-KO mice from arsenic-induced mortality, but protection depends on microbiome stability and the presence of specific bacteria, including *Faecalibacterium*. Our results demonstrate that both a functional As3mt and specific microbiome members are required for protection against acute arsenic toxicity in mouse models. We anticipate that the gut microbiome will become an important explanatory factor of disease (arsenicosis) penetrance in humans, and a novel target for prevention and treatment strategies.

Introduction

Arsenic is a toxic metalloid and human carcinogen, ranking first on the US Agency for Toxic Substances and Disease Registry and the US Environmental

Protection Agency Priority List of Hazardous Substances for the past 20 years (since 1997) (*125*). Arsenic toxicity varies widely depending on the chemical composition of arsenic-containing compounds (arsenicals). Trivalent species are more toxic than pentavalent species, although the toxicity of the latter can increase depending on whether and to the extent it becomes thiolated. The microbiomes of humans and mice were shown to metabolize arsenic when cultured *in vitro* (*106, 126, 127*). Similarly, mice with different microbiome compositions were found to excrete different types of arsenicals following exposure to arsenic (*123, 128*). However, no study has provided direct *in vivo* evidence that the mammalian microbiome either exacerbates or protects the host from arsenic-related pathology or disease.

In a series of experiments utilizing antibiotic-treated, transgenic, germ free (GF), and gnotobiotic mouse models, we illustrate the critical contribution of the gut microbiome in protecting the host from acute arsenic toxicity. We provide evidence that microbiome stability during arsenic exposure is a key determinant of survival to arsenic exposure; link important bacterial taxa from the human microbiome with protection; and show that the human gut commensal, *Faecalibacterium prausnitzii*, is sufficient for at least some protection against arsenic. The overall conclusion from this study is that the gut microbiome is a significant factor underlying survival to acute arsenic toxicity.

Results

Microbiome Disruption Affects Arsenic Excretion/Bioaccumulation

We initially defined the microbiome's role in arsenic detoxification *in vivo* by experimentally disrupting the bacterial community of laboratory-reared, wild-type (WT) mice with the cephalosporin antibiotic, cefoperazone (Cef). This drug decreases total bacterial load in mice by ~3 orders of magnitude and significantly alters taxonomic composition (*129*). Cef was administered in drinking water for 2 days prior to and throughout a 14-day exposure to inorganic sodium arsenate (iAs^V), the most common arsenic species in human drinking water. Groups of mice were exposed to 25 and 100 ppm iAs^V based on (i) an established dose-equivalent equation that adjusts for surface area differences between mice and humans (*130*); (ii) the use of similar exposures in studies addressing arsenic cytotoxicity in mice (*131-134*); and (iii) documented human drinking water exposures (*135*) (Supplementary Note 1 and Supplementary Table 3.1). In short, our approach was designed to evaluate arsenic toxicity in the context of high, acute exposure.

Significantly less arsenic was excreted in the stool of Cef-treated mice (87 ± 6% less in 25 ppm group and 93 ± 3% less in 100 ppm group) compared to Sham-treated counterparts (Figure 3.1a). These results suggest that more arsenic was retained in Cef-treated mice, and evaluation of organs confirmed an overall arsenic accumulation ($p < 0.0001$, 25 ppm; $p = 0.0003$, 100 ppm; two-way ANOVA). The experimental design was not powered to determine whether

specific organs were more likely to accumulate arsenic, but we did observe significant accumulation in lung ($p < 0.0001$) and liver ($p = 0.025$) after correcting for multiple comparisons (Figure 3.1b). Together, these results support the hypothesis that the microbiome mitigates host exposure and uptake of ingested iAs^V through fecal excretion.

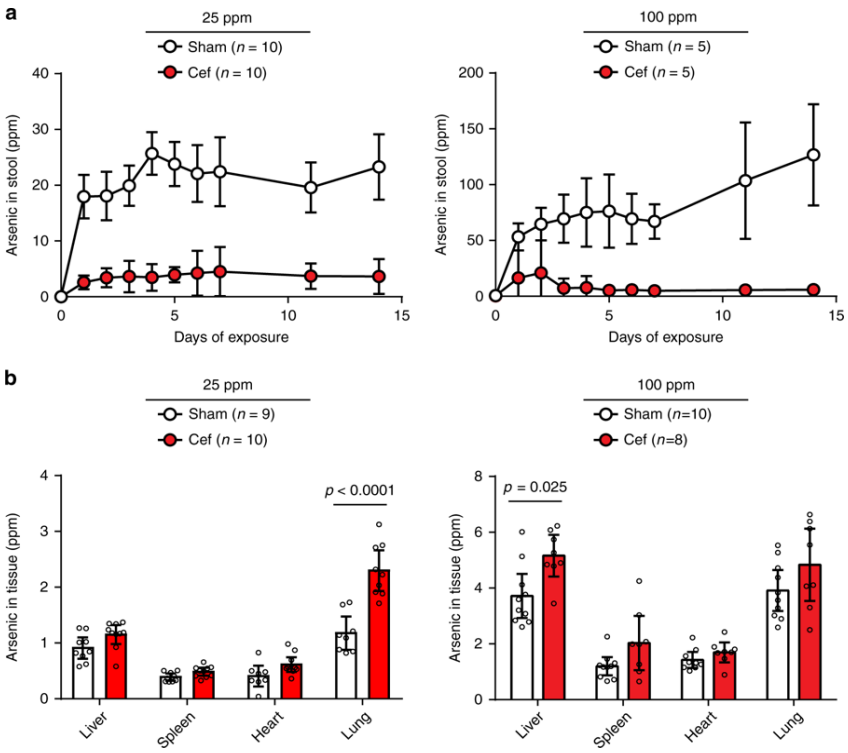


Figure 3.1. Total arsenic levels in the feces and host tissues of C57BL/6 mice. Cefoperazone (Cef) and Sham treatments were initiated 48 h prior to inorganic sodium arsenate (iAs^V) exposure in drinking water at either 25 ppm (left) or 100 ppm (right). Group-wise means are shown in all graphs and error bars represent 95% confidence interval of the mean. **a** Significantly lower levels of arsenic were excreted in feces of Cef-treated mice beginning 24 h after exposure at both doses ($p < 0.0001$, two-way ANOVA after log transformation). **b** Significantly higher levels of arsenic accumulated in organs of Cef-treated mice after 14 days of exposure (25 ppm, $p < 0.0001$; 100 ppm, $p = 0.0003$, two-way ANOVA after square root transformation) with the greatest differences in lung (25 ppm) and liver (100 ppm). *p*-values in **b** were adjusted for multiple comparisons (Sidak's multiple comparisons test).

As3mt and Microbiome are Required for Protection

The above mice showed few signs of disease with only two exceptions (two Cef-treated mice in the 100 ppm group were found dead). This is consistent with at least two other studies of WT mice, where no mortality was observed during exposure to 25 and 100 ppm iAs^{V} (*132, 136*) or to the more toxic, trivalent arsenite (iAs^{III}) for at least 4 weeks (*132*). In contrast to WT mice, arsenic (+3 oxidation state) methyltransferase-deficient mice (As3mt-KO) are dysfunctional in arsenic methylation (*137*), accumulate more intracellular inclusions in urothelial tissue during arsenic exposure (*132*), and have increased overall arsenic-induced cytotoxicity (*131, 134*). We hypothesized that As3mt-KO mice would be more sensitive to high doses of arsenic compared to WT mice, and that toxicity would increase following microbiome perturbation. iAs^{V}-induced mortality in As3mt-KO mice at 25 ppm iAs^{V} exposure has not been reported, and as expected, no toxicity was observed in Sham-treated As3mt-KO mice exposed to 25 ppm iAs^{V} for up to 22 days. However, the same level of exposure was lethal in half of Cef-treated As3mt-KO mice by day 12 (Figure 3.2a). At 100 ppm iAs^{V}, mortality was observed in both Sham- and Cef-treated groups (Figure 3.2b) but was more rapid in mice with a disrupted microbiome ($p = 0.003$, Mantel–Cox test).

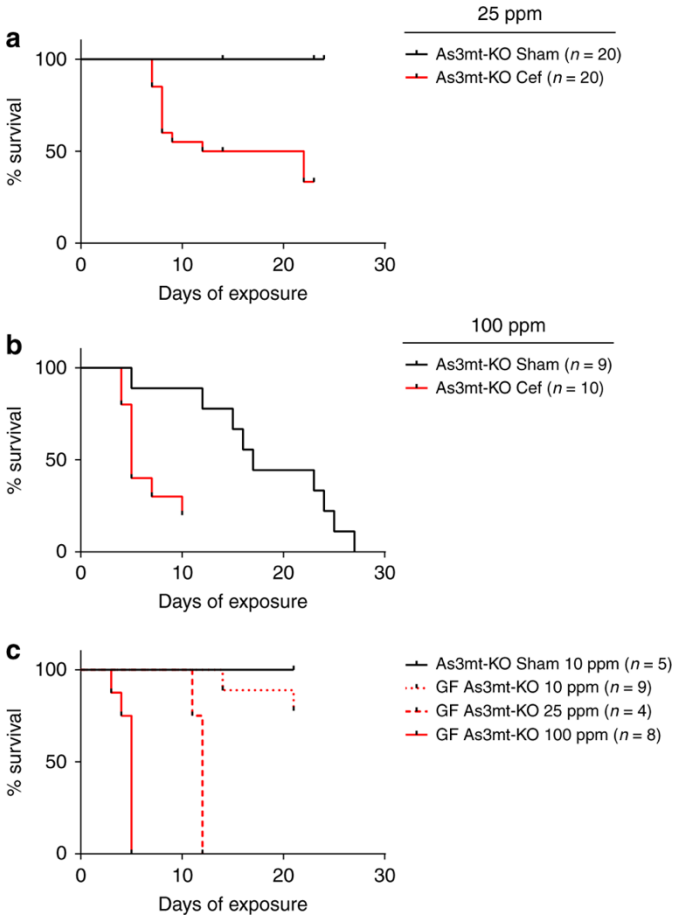


Figure 3.2. Survival of iAsV-exposed Cef-treated, Sham-treated, and GF As3mt-KO mice. Cef and Sham treatments were initiated 48 h prior to iAs^{v} exposure in drinking water at either 25 ppm (**a**) or 100 ppm (**b**). Survival of Cef-treated mice was significantly lower compared to Sham-treated mice at both 25 ppm ($p < 0.0001$, Mantel–Cox test) and 100 ppm ($p = 0.0032$, Mantel–Cox test) exposures. For GF As3mt-KO mice (**c**), three groups were exposed to increasing levels of iAs^{v} along with a negative control group of conventional As3mt-KO mice. Dose-dependent survival was observed among these groups. Although mortality was observed in GF As3mt-KO mice exposed to 10 ppm, survival was not significantly different compared to the conventional, Sham-treated As3mt-KO mice exposed to 10 ppm ($p = 0.2274$, Mantel–Cox test). Survival was significantly lower in GF As3mt-KO mice exposed to both 25 ppm ($p = 0.0050$, Mantel–Cox test) and 100 ppm ($p = 0.0014$, Mantel–Cox test) compared to conventional, Sham-treated As3mt-KO mice. Survival was also significantly lower in GF As3mt-KO mice exposed to 100 ppm compared to 25 ppm ($p = 0.0031$, Mantel–Cox test)

To quantify survival in the absence of a microbiome, a GF line of As3mt-KO mice was derived and were exposed to increasing amounts of iAs^V. Dose-dependent mortality was observed in mice exposed to 10, 25, and 100 ppm iAs^V (Figure 3.2c), suggesting toxicity increased with increased exposure. For comparison, a GF line of WT mice was exposed to iAs^V (25 and 100 ppm). Mortality was observed in only 2 of 22 WT mice exposed to 100 ppm iAs^V out to 40 days (one each on days 19 and 38). Therefore, both As3mt *and* an intact gut microbiome are necessary for full protection against arsenic toxicity in this mouse model. Finally, we tested the hypothesis that Cef treatment somehow increased iAs^V toxicity. The timing of antibiotic pre-treatment in conventional As3mt-KO mice was changed, and the survival of GF As3mt-KO mice was quantified with and without Cef treatment (Figure S3.1). There was no evidence that Cef increased iAs^V toxicity, and so iAs^V toxicity was likely the sole determinant of mortality.

Human Stool Transplantation Provides Full Protection

Bacteria in human stool possess genes encoding arsenic-active enzymes (*138*) that can biochemically transform arsenic *in vitro*(*106, 127, 139*), and genes encoding arsenic-active enzymes are present in human gut bacterial genome databases(*138*). Beyond such direct enzyme–arsenic interactions, bacteria might also provide indirect benefits to the host during arsenic exposure by producing metabolites that otherwise enable excretion and tissue repair. However, the ability of the human gut microbiome to provide protection to a

mammalian host has never been quantified. Given their hypersensitivity to arsenic, GF As3mt-KO mice were used to determine whether a human microbiome could provide protection from arsenic. Stool from a healthy human donor was transplanted into these mice prior to iAs^V exposure. The human donor was not knowingly exposed to arsenic and drank water from a regulated municipal source in the US. The transplanted stool was established in GF recipient As3mt-KO mice (referred to as F0) for 10 days prior to arsenic exposure. In parallel, the same stool was transplanted into gravid, GF As3mt-KO dams, so that offspring (F1) could develop with this microbiome from birth. We hypothesized that F1 mice would have increased protection against arsenic due to early-life development in the presence of a human microbiome. In experiments where F1 mice were reared to approximately the same age at exposure as F0 mice, transplanted human stool provided full protection against 100 ppm iAs^V, regardless of the timing of microbiome acquisition (Figure 3.3a) and the median survival of both F0 and F1 groups was not significantly different from conventional As3mt-KO mice ($p = 0.3335$, Mantel–Cox test). These results establish *in vivo* evidence that a human gut microbiome provides protection against arsenic-induced mortality.

Donor-dependent Variation in Arsenic Protection

Human gut microbiomes vary greatly in species composition between people. We therefore tested whether host protection varies as a function of inter-individual microbiome composition using five groups of iAs^V-exposed GF As3mt-

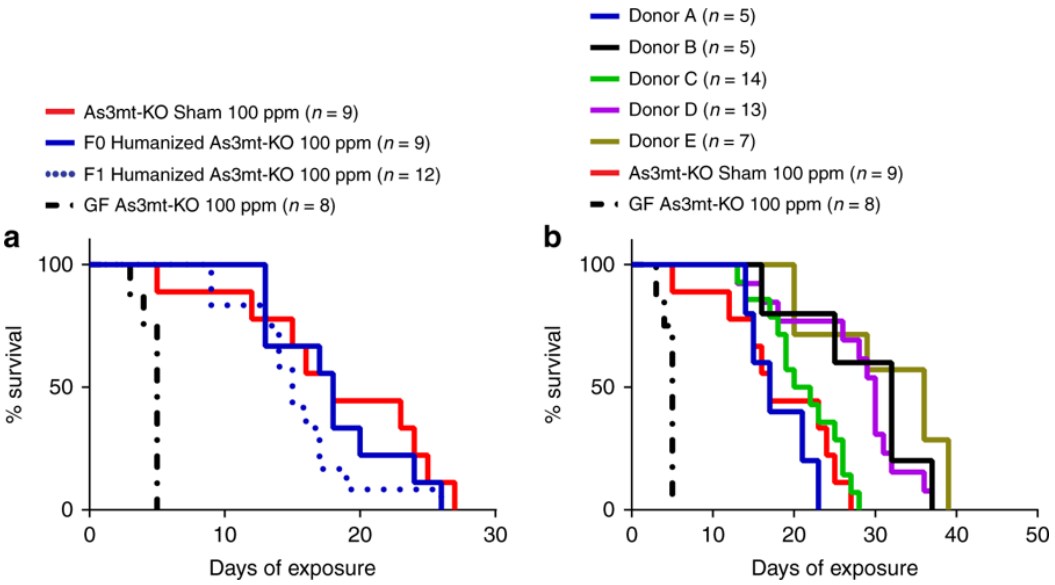


Figure 3.3. Survival of iAs^{v}-exposed humanized As3mt-KO mice. Groups of As3mt-KO mice received a fecal transplant from a healthy human donor (humanized) either 10 days prior to iAs^{v} exposure (F0) or at birth from a humanized dam (F1). **a** Survival of F0 and F1 groups was not significantly different compared to conventional, Sham-treated As3mt-KO mice following exposure to 100 ppm iAs^{v} (F0, $p = 0.6500$; F1, $p = 0.1887$; Mantel–Cox test), but survival in both groups was significantly greater than GF As3mt-KO ($p < 0.0001$, Mantel–Cox test). Five groups of humanized As3mt-KO mice, each representing a unique human microbiome donor, were exposed to 100 ppm iAs^{v} (two to four replicate cages per group). **b** Median survival in all donor groups was significantly greater than that of GF As3mt-KO mice ($p \leq 0.0014$, pairwise Mantel–Cox), and equivalent to or greater than that of Sham-treated conventional mice. There were also significant differences in survival among different humanized groups ($p = 0.0030$, Mantel–Cox). Pairwise comparisons (Mantel–Cox test) are shown in Supplementary Table 3.4.

KO mice that received stool transplants from different healthy adult donors. GF As3mt-KO mice were humanized and exposed to 100 ppm iAs^{V} as described above (i.e. F0 mice). All humanized microbiome groups survived significantly longer than the GF As3mt-KO group ($p \leq 0.0014$, Mantel–Cox test, Table 3.2). However,

significantly different survival was observed between humanized groups of mice ($p < 0.0001$, Mantel–Cox test) with median survival times ranging from 17 to 36 days (Figure 3.3b; Supplementary Table 3.3). Independent (replicate) experiments were performed using two donor groups; one that provided better protection than the other. Although median survival varied somewhat (Figure S3.2), the overall effect was reproducible (i.e., median survival was greater in one group in both replicates; aggregate data for replicates were used for all analyses). Thus, human stool transplantation revealed inter-individual differences in protection from arsenic toxicity.

To identify patterns of bacterial diversity underlying survival, we conducted 16S rRNA gene sequencing on stool samples from humanized mice at the outset of arsenic exposure (day 0) and again at day 7 prior to any mortality. Rarefaction analysis indicated that reasonable sequencing coverage was obtained in all samples (Figure S3.3). Humanized mouse microbiomes were significantly more similar within donor groups (i.e., mice sharing the same donor) than between donor groups ($p = 0.001$, ANOSIM; Figure 3.4a). Despite these donor-specific patterns, significant changes in microbiome structure (i.e., presence–absence and relative abundance of taxa) was observed in all groups between days 0 and 7 of arsenic exposure ($p \leq 0.013$, ANOSIM; Supplementary Table 3.4). These results support previous studies in mice (*109, 123, 128*) that arsenic perturbs the gut microbiome; however, the extent of perturbation between groups

was significantly different ($p < 0.0001$, Kruskal–Wallis test), suggesting that perturbation is dependent on the starting microbiome (Figure 3.4b). The magnitude of perturbation between days 0 and 7 in each donor group can be considered a measure of community stability (i.e., stability—little change).

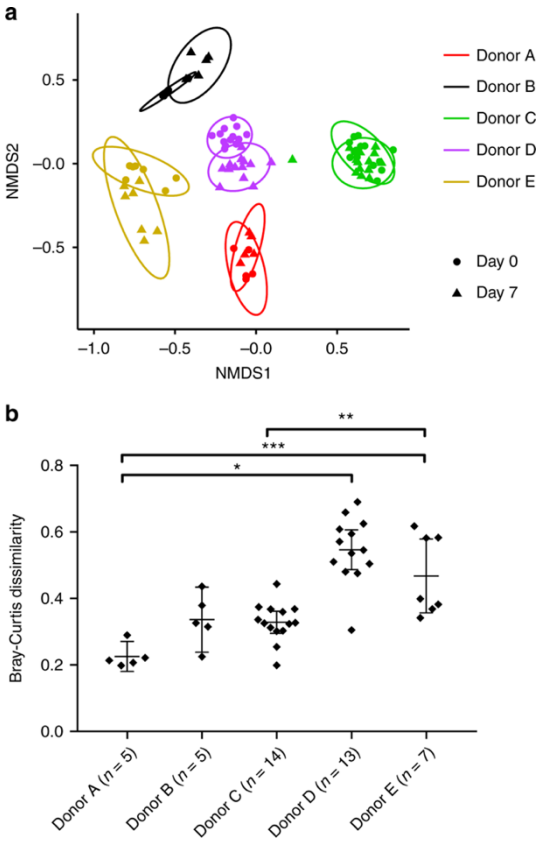


Figure 3.4. Differences in microbiome stability during arsenic exposure. **a** Non-metric multidimensional scaling of Bray–Curtis dissimilarity illustrates both within and between group as well as within and between exposure time changes in microbiome diversity. Each point represents a single humanized mouse microbiome according to donor (color) and time-point (symbol). Ellipses represent 95% confidence. Microbiomes were more similar within donor groups than between ($p = 0.001$, ANOSIM), and in all donor groups, day 7 microbiomes were significantly different from baseline (day 0) microbiomes ($p \leq 0.009$, ANOSIM; Supplementary Table 3.5). **b** Stability of humanized mouse microbiomes during iAs^{v} exposure (mean and 95% confidence interval) was significantly different between groups as shown (*$p < 0.01$; **$p < 0.005$, ***$p < 0.0001$, Kruskal–Wallis test)

In survival models (Cox Proportional Hazards; CPH) accounting for differences within donor groups, an increase in alpha diversity (both Shannon and inverse Simpson diversity) was significantly correlated with longer survival (Shannon, $p = 0.0447$; inverse Simpson, $p = 0.0155$). Likewise, stability significantly correlated with survival ($p = 0.0325$, Wald test). Also, these covariate models performed significantly better than the reduced model (i.e., donor group only, $p < 0.05$, ANOVA), indicating the importance of such diversity estimates within donor groups. Overall, these results suggest that the ability of the gut microbiome to maintain taxonomic integrity under arsenic stress is important for host survival.

Individual Microbiome Members Correlate with Survival

Gut bacteria that metabolize arsenic into less toxic arsenicals or provide some other detoxification properties could potentially be beneficial during exposure. We attempted to identify these bacteria with survival modeling of the 100 most abundant operational taxonomic units (OTUs) observed among all humanized mice. Considering OTU presence/absence at days 0 and 7 and controlling for false discoveries ($q < 0.1$), 48 unique OTUs were identified as being associated with survival either negatively or positively (Figure 3.5). Of these 48 taxa, 22 were significantly associated with survival at both days 0 and 7 (shared), 21 were only associated at day 0 (Figure 3.5a), and 5 were only associated with day 7 (Figure 3.5b). All OTU-survival associations shared on both

days were in agreement with respect to coefficient sign (i.e., positive or negative), supporting a consistent direction of their influence. A complementary analysis was performed using log-transformed OTU abundance data for days 0 and 7 with similar results (Figure S3.4). This analysis identified 53 significant associations between OTU abundance and host survival, with 30 being shared (i.e., significantly on both days). A total of 38 (72%) of the OTUs associated with survival using relative abundance data were also identified in the presence/absence analysis, including 17 of the 22 (77%) shared OTUs. These results suggest that both the presence/absence and relative abundance of specific microbiome members influence survival during arsenic exposure.

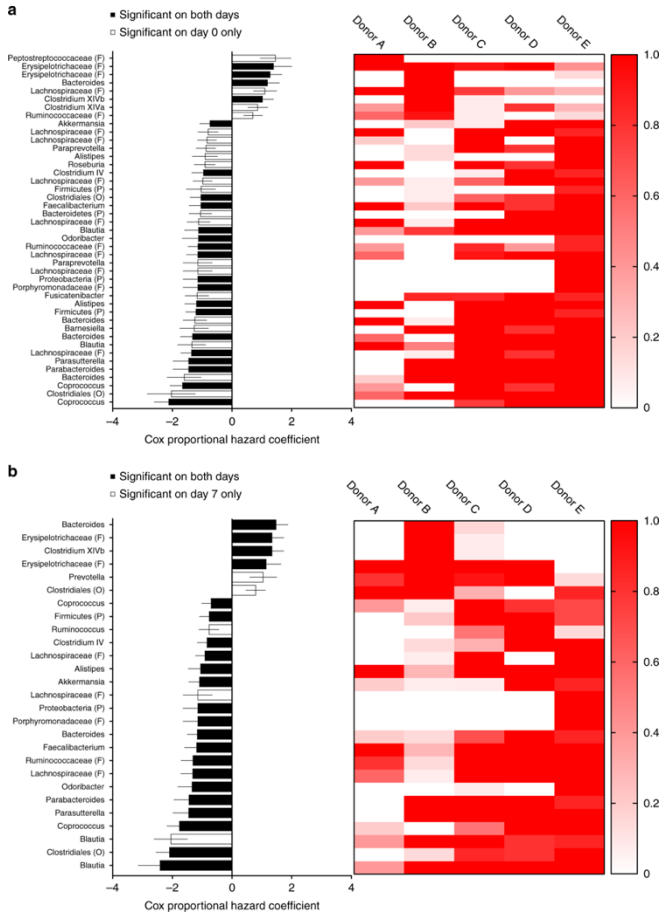


Figure 3.5. Specific microbiome OTUs are risk factors for survival. Controlling for false discovery rate (<0.1), significant beta coefficients (with standard error) for microbiome members at day 0 (**a**, left panel) and day 7 (**b**, left panel) are shown along with the group-wise prevalence of each OTU displayed as heat maps (**a**, **b**, right panels). Significant OTUs found on both sampling days (0 and 7) are shown with black bars and significant OTUs found to be unique on one day or the other are shown in open bars Taxonomic levels above genera are indicated in parentheses (F family, O order, P phylum).

Faecalibacterium Prausnitzii Provides Protection

Interpreting the above results is somewhat difficult due to co-evolutionary relationships that lead to the co-occurrence of microbiome members within and between donor groups. In other words, such analyses only establish correlative

relationships, and remain insufficient to resolve causality between specific taxa and *in vivo* function. To determine causality for at least one microbiome member and to establish whether our model of arsenic toxicity is sensitive enough to detect the influence of a single bacterium, gnotobiotic experiments with *F. prausnitzii* in As3mt-KO mice were conducted. The rationale for selecting this particular taxon is as follows: *Faecalibacterium* is one of the most abundant and commonly identified taxa in the gut microbiome of healthy humans, of which *F. prausnitzii* is the only named species. *F. prausnitzii* contributes to butyrate and other short-chain fatty acid production in the gut(*140*), and a reduction in *F. prausnitzii* has been associated with a wide range of gastrointestinal diseases, including inflammatory bowel disease (*141*), colorectal cancer (*142, 143*), and *Clostridium difficile* infection (*144*). Analysis of *F. prausnitzii* genomes (NCBI) revealed the presence of an iAs^{III} S-adenosyl methyltransferase gene, *arsM*, in at least some genomes, which encodes a well-known methyltransferase involved in microbial arsenic detoxification (*145*). A *Faecalibacterium* OTU was detected in the microbiome of 36 out of the 44 humanized mice; it was present at both days (0 and 7) across all donor groups; and was abundant, ranging from 0.01% to 5.3% of normalized reads. Survival modeling (both presence/abundance and abundance analyses) identified this OTU to be significantly associated with survival at both days 0 and 7 (Figure 3.5, Figure S3.4). Finally, *F. prausnitzii* is already marketed as a human probiotic, thus providing a clear connection to

human health. The combination of results from previous studies, results from humanized mice, the potential for production of an arsenic-active enzyme(s), and current marketability led us to hypothesize that *F. prausnitzii* provides protection against arsenic toxicity.

To test the above hypothesis, gnotobiotic mouse experiments were conducted using *F. prausnitzii* strain, A2-165, that was originally isolated from a healthy human. Miquel *et al.* previously found that A2-165 did not readily colonize GF mice when administered alone, but colonized at a high level ($\sim 10^8$ CFU per gram of stool) when administered in combination with *Escherichia coli* (*146*). This was also observed in the current study; i.e., A2-165 could not be detected in stool of GF mice 48 h post-gavage, whereas it became abundant in stool of *E. coli* mono-colonized mice. In a series of experiments, mono-colonization of GF mice with *E. coli* strains K-12 (W3110) or B (BL21) did not provide significant protection during 25 ppm iAs^V exposure (Figure S3.5). However, *E. coli–F. prausnitzii* bi-colonization significantly increased survival compared to both GF ($p = 0.0035$) and *E. coli* mono-associated mice ($p = 0.0024$, Figure 3.6). These results support the hypothesis that *F. prausnitzii* is sufficient for at least partial protection against arsenic toxicity, and also suggest that the GF As3mt-KO model of arsenic exposure is highly sensitive to detect these effects. Future studies are now needed to determine whether this effect is due to *in vivo* production of active arsenic enzymes (i.e., metabolism), production of non-

arsenic-related metabolites (e.g., short-chain fatty acids), or some other host–microbiome factor(s).

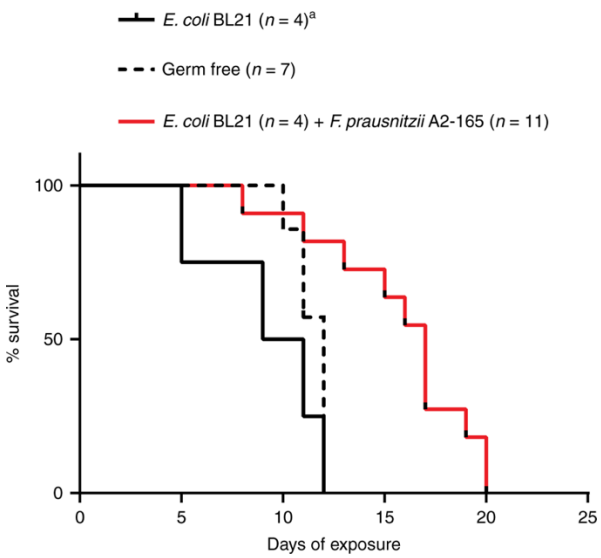


Figure 3.6. *Faecalibacterium prausnitzii* protects against arsenic toxicity. Germ free, E. coli mono-colonized (one cage of four mice), and E. coli + F. prausnitzii bi-colonized (three replicate cages of 3 or 5 mice each) As3mt-KO mice were exposed to 25 ppm iAsV. Survival was not significantly different between GF and E. coli mono-colonized groups (p = 0.1759, Mantel–Cox test), whereas mice bi-colonized with E. coli + F. prausnitzii survived significantly longer than both GF (p = 0.0035, Mantel–Cox test) and E. coli mono-colonized mice (0.0024, Mantel–Cox test).

Discussion

Mammalian cells and many microbes detoxify arsenic, but the *in vivo* role of the human microbiome in detoxifying arsenic has not been addressed. Experiments summarized herein show that either microbiome perturbation or absence increases host arsenic bioaccumulation and toxicity. Further, GF As3mt-

KO mice were shown to be a useful, hypersensitive arsenicosis model and that human stool transplantation restores protection in these mice. Protection was at least partially due to microbiome composition and stability, providing the first *in vivo* evidence that the human microbiome protects against arsenic toxicity. Finally, *F. prausnitzii* appears to be a useful correlate of microbiome stability during arsenic exposure in humans, and also provides some protection in the GF As3mt-KO murine model. Although microbes can enzymatically transform arsenical compounds, they can also bioaccumulate this toxin (*147-151*), which may facilitate excretion and help limit host exposure. Thus, future research is needed to quantify the net influence of these two potentially important mechanisms for arsenic detoxification in the human body. Based on these results, we propose that the microbiome should be a target for arsenicosis prevention and treatment strategies, and considered a plausible explanatory factor in epidemiologic studies that attempt to account for the often observed variability in disease penetrance among similarly exposed individuals (*13*). Given the scale and scope of global arsenicosis, probiotics with active arsenic metabolisms capable of mitigating arsenic toxicity may represent feasible, low-cost therapeutics.

Methods

Experimental Animals

Animal experiments were approved by the Montana State University Institutional Animal Care and Use Committee. All mice were bred and maintained at an American Association for the Accreditation of Laboratory Animal Care accredited facility at Montana State University. Conventional mice were housed under specific pathogen-free conditions (including murine norovirus) in individually ventilated cages with sterilized bedding. GF mice were housed in hermetically sealed and HEPA-filter ventilated, vinyl isolators and received autoclaved water and food (LabDiet, St. Louis, MO). All food and water for GF mice were quarantined, monitored, and tested for contamination prior to introduction. No statistical methods were used to select *a priori* sample sizes, no randomization techniques were used, and no investigator blinding was done.

Only C57BL/6 mice were used, being either wild-type or deficient for the murine *as3mt* gene (As3mt-KO). Breeding pairs of As3mt-KO mice were obtained from Drs. L. Arnold and S. M. Cohen (University of Nebraska Medical Center) and Drs. C. Douillet and M. Styblo (University of North Carolina), and used to establish a breeding colony at Montana State University. These mice were originally derived at the Environmental Protection Agency (*137*). GF colonies of both WT and As3mt-KO mice were derived from conventional mice. Briefly, conventional and GF surrogate (Swiss-Webster) females were gestationally

synchronized so that litters were born within 12 h of each other. At precisely full term, gravid conventional dams were euthanized, and a sterile hysterectomy was performed. Neonatal pups were quickly removed, revived, and transferred into a GF isolator where they nursed on surrogate GF Swiss-Webster dams. GF status was monitored regularly by attempting aerobic and anaerobic culture techniques on rich medium (Mueller–Hinton broth and agar plates) and by periodic PCRs (Supplementary Table 3.6) targeting the bacterial 16S rRNA encoding gene with DNA extracted from stool serving as a template.

Human Samples

Human fecal samples collected and cryopreserved as part of an unrelated study were used for humanization experiments to test for inter- individual variation in the protective effect of human microbiome communities in GF As3mt-KO mice. A total of nine volunteers were originally recruited according to a research protocol approved by the Institutional Review Board of Montana State University in Bozeman, MT, USA. All participants were enrolled with informed consent, and the only inclusion criterion was that the individual was ≥21 years of age. No information regarding health or medical status of volunteers was collected. Fecal samples were self-collected using a disposable Commode Specimen Collection System (Fisherbrand), transferred into 50 ml screw-cap tubes (~5 g) using a sterile tongue depressor, refrigerated at 4 °C for up to 2 h before processing. Under anaerobic conditions (anaerobic chamber, Coy Laboratories),

~5 g of fresh donor stool sample was mixed with ~30 ml sterile, pre-reduced PBS, mixed with 15% glycerol (final concentration), aliquoted into sterile 1.5 ml cryogenic gasket-cap vials (Neptune Scientific), and stored at −80 °C prior to use in humanization experiments. Archived samples were used from 6 donors (5 male, 1 female) ranging in age from 24 to 40 at the time of sample collection.

Chemical Reagents

Cefoperazone (cefoperazone sodium salt) was purchased from Chem-Impex International Inc. (Wood Dale, IL) and stored with desiccant at 4 °C. iAs^{V} was ACS grade (≥98% pure) sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_20$, Sigma-Aldrich, St. Louis, MO). All chemicals were used as received with no additional purity analysis.

Arsenic Exposures

Except where indicated (see Supplementary Table 3.5), age-matched mice (7–13 weeks old) of both sexes were used. In rare cases, the decision to use mice >13 weeks of age was based solely on mouse availability. To disrupt the microbiome of mice, cefoperazone (Cef, 0.5 mg per ml) was added to drinking water 48 h prior to and throughout arsenic exposure. Sham-treated groups received drinking water containing an identical concentration of Cef that was denatured by boiling for 5 min and cooled to room temperature before use. Denaturation was confirmed using a bioassay with a sensitive *E. coli* strain

(MG1655) in LB broth. Fresh water was made for both Cef- and Sham-treated groups every 3–4 days.

All arsenic exposures were as inorganic sodium arsenate (iAs^V at either 25 or 100 ppm). Fecal pellets were collected daily and frozen at −80 °C for quantification of total arsenic. After 14 days of iAs^V exposure, surviving mice were humanly euthanized by isoflurane overdose and tissue was collected for arsenic quantification. GF WT and As3mt-KO mice were exposed to 10, 25, or 100 ppm iAs^V in drinking water. Most exposures were continued until complete mortality or when mice appeared severely moribund at which point, they were humanely euthanized.

Fecal Transplantation

GF As3mt-KO recipient mice received a fecal transplant from human donors prior to 100 ppm iAs^V exposure via drinking water. We chose this model because it was found that mortality occurred gradually after 14 days in these mice, thus allowing for statistical comparisons of median survival. Frozen human donor stool samples (see above) were thawed inside an anaerobic chamber, suspended in sterile, pre-reduced PBS at approximately a 1:1 weight to volume ratio, and 100 μL of this slurry was used to inoculate GF mice via oral gavage. Humanized mice were allowed to equilibrate for 10–14 days prior to iAs^V exposure. In addition to humanizing naïve adult GF mice (F0), we created a cohort of As3mt-KO mice that were humanized from birth (F1) by humanizing GF

As3mt-KO dams prior to breeding. All experiments with humanized mice were conducted inside sterile, vinyl isolators.

Arsenic Quantification

Arsenic concentrations in biological samples were determined using an Agilent 7500 ICP-MS. Mouse tissue and fecal pellet samples were weighed and digested in a 70% solution of trace-metal grade nitric acid (VWR International, Radner, PA) using heat and pressure (115 °C, 29.7 PSI for 30 min). Digested samples were diluted in ultra-pure water to achieve a final nitric acid concentration of 5%. Final dilutions were centrifuged at ~2000 rcf (Beckman GS-6R centrifuge) for 10 min to remove particulates, and the supernatant was collected for analysis. Samples were injected via a constant-flow-rate peristaltic auto-sampler and the results quantified against an external standard curve using Agilent's ChemStation software package.

16S rRNA Gene Sequencing

Groups of up to 3–5 mice were co-housed in the same cage during exposure studies. Cages were assigned to treatment groups, while attempting to control for sex as a biological variable. Fecal pellets were collected by holding mice above the cage and collecting pellets directly into sterile Eppendorf tubes (i.e., mice reproducibly defecate when held). Bulk DNA was extracted from fecal pellets using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) and stored

at −20 °C. DNA was shipped overnight on dry ice to the University of Michigan, Center for Microbial Systems, for Illumina MiSeq amplicon sequencing of the 16S V4 variable region. After DNA quantification, V4 amplicon libraries were generated with dual-index barcoded primers (Supplementary Table 3.6), followed by library purification, pooling, and MiSeq paired-end sequencing using 2 × 250 base-pair chemistry.

Raw sequencing reads were processed and curated using the mothur (v. 1.39.5) software package (*152*), following the mothur MiSeq standard operating procedure (*153*) accessed on May 17, 2017 (http://www.mothur.org/wiki/MiSeq_SOP). Paired-end reads were assembled into contigs and screened for length and quality (minimum 253 bp and no ambiguous base calls). The remaining contigs were aligned to coordinates 6427 through 25319 of the Silva ribosomal RNA gene reference database, release 128 (*154, 155*). Potentially chimeric sequences were identified and removed using the Uchime (v. 4.2.40) algorithm via mothur (*156*). Taxonomic classifications were assigned using a Bayesian classifier at the Ribosomal Database Project (*157*) (v. 14) implemented in mothur, using training set version 16(*158*). Reads classifying as non-target organisms/organelles (mitochondria, chloroplast, Eukaryota, and sequences unclassified at the domain level) were removed. Operational taxonomic units (OTUs) were assigned in mothur using the VSEARCH (v. 2.3.4) distance-based greedy clustering algorithm at the 97% sequence

similarity threshold (*159*), and an OTU based data matrix was built. Rare OTUs (represented by less than 100 total reads after identification and removal of chimeras) were removed to minimize the influence of spurious OTUs. Finally, representative OTU sequences were classified to the genus level using the Bayesian classifier at the Ribosomal Database Project (*157*).

Gnotobiotic Mouse Experiments

F. prausnitzii, strain A2-165, was obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ) and cultured in YCFAG broth (*160*). *E. coli*, strains W3110 and BL21, were obtained from Dr. Harry Mobley (University of Michigan) and New England Biolabs, Inc., respectively, and cultured in LB broth. GF As3mt-KO recipient mice received either 10^8 colony forming units (CFUs) of *E. coli* alone or in combination with 10^8 CFUs of *F. prausnitzii* (bi-colonization) via oral gavage. For bi-colonization, *E. coli* was administered to mice 24 h prior to *F. prausnitzii*. A second gavage of *F. prausnitzii* was administered 24 h after the first. To increase F. prausnitzii establishment, sodium bicarbonate (0.1 M) was administered just prior to *F. prausnitzii* gavage. Colonization by both *E. coli* and *F. prausnitzii* was confirmed by direct plating from stool onto either MacConkey (to inhibit *F. prausnitzii*) or YCFAG agar containing nalidixic acid (15 µg per ml; to inhibit *E. coli*).

Colony PCR was performed on *F. prausnitzii* isolates using species-specific primers (*161*) to confirm taxonomic identification (Supplementary Table 3.6). In

rare cases, where no *F. prausnitzii* was detected in individual mouse pellets collected 48 h after the initial gavage, a third gavage of sodium bicarbonate and *F. prausnitzii* culture were performed. Colonization was confirmed for at least 72 h prior to arsenic exposure.

Statistical Analyses

Arsenic levels in feces and organs of mice were either log or square root transformed, respectively, prior to statistical testing and passed the Shapiro–Wilk normality test (GraphPad Prism 7.03 for Windows, GraphPad Software, La Jolla, CA, USA; www.graphpad.com). A two-way repeated measures ANOVA was used to test for group-wise differences in fecal excretion throughout arsenic exposure. An ordinary two-way ANOVA was used to test for group-wise differences in organ arsenic content. Mouse survival between groups during arsenic exposure was tested using the Mantel–Cox test (GraphPad Prism). Rarefaction, alpha diversity estimates (Shannon and Inverse Simpson), comparisons of beta diversity (Bray–Curtis dissimilarity), and ordinations (e.g., NMDS plots) of 16S rRNA gene sequences were conducted in R (*162*) (v. 3.3.3) using R packages *vegan (163)* (v. 2.4-5) and *LabDSV (164)* v. 1.8-0 as implemented in RStudio (*165*) v 1.1.383. Statistical analyses and graphs were generated using R package *ggplot2(166)* (v. 2.2.1) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA; www.graphpad.com).

For the purposes of this analysis, Bray–Curtis distance between individual mouse microbiome communities sampled on either day 0 or day 7 of arsenic exposure was used as a proxy for microbiome stability or community change (with more stable communities having smaller BC distance). Differences in microbiome stability between groups of mice were tested using the Kruskal—Wallis test (GraphPad Prism). Analysis of Similarities (ANOSIM), as implemented in the R package vegan (*163*), was used to test for differences in microbiome communities between donor groups and different time points within donor groups. Statistical models testing covariate associations with survival were performed using CPH modeling, implemented using the *survival* package (*167*) (v. 2.38) in R. This technique models predictor variables against a function of proportional hazard instead of a survival function. Therefore, a negative association (beta coefficient < 0) indicates increased protection or survival in the subjects (lower hazard = greater expected survival). The significance of model coefficients was tested using the Wald test, while over model significance was tested using the Likelihood Ratio (LR) test. All p-values represent 2-tailed tests unless otherwise noted in text or figure legends.

Data Availability

All 16S rRNA sequencing reads were deposited in the National Center for Biotechnology Information (NCBI) BioProject database with accession code

PRJNA486373. The authors declare that all other data supporting the findings of the study are available in this article and its Supplementary Information files, or from the corresponding author upon request.

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Supplementary Information

Supplementary Note 1. Arsenic dosing.

Selection of the dosing levels in this study (10, 25, and 100 ppm) was based on three criteria. First, for reasons highlighted below, acute arsenic toxicity studies in mice typically use high doses of inorganic arsenate (iAs^{V}) or arsenite (iAs^{III}), even though most humans are naturally and chronically exposed to much lower levels. The range of iAs^{V} dosing considered here was intentionally consistent with other studies using C57BL/6 WT and As3mt-KO mice (see Table 1 below). We also draw attention to the fact that a similar range of dosing in mice (1-50 ppm) was used to develop a *human* pharmacokinetic (PK) and pharmacodynamic modeling framework at the 2007 Annual Meeting of the Society of

Toxicology(*168*). Consequently, in terms of dosing *per se*, the levels we used are superimposable to those historically used.

Second, if the focus of a study is on toxicity, the level of exposure should be above the lowest-observed-adverse-effect-level (LOAEL) and somewhat lower than the immediate lethal dose (LD_{50}). Unfortunately, there is considerable variability in LOAEL and LD_{50} estimates for humans, but according to the ASTDR and EPA's toxicological profile on arsenic(*169*), the LOAEL for acute, oral exposure (Appendix A in profile) is approximately 0.05 mg iAs per kg body weight per day. Assuming (as recommended by ASTDR(*169*)) a 55 kg person drinking 4.5 L of water per day and a 0.002 mg iAs per kg per day daily food intake, this level of exposure equates to 611 ppb arsenic in drinking water. Also, according to the ASTDR, death in oral arsenic exposures in drinking water >60,000 ppb can result in death. Thus, exposures between 0.6 - 60 ppm should be appropriate for studying acute toxicity.

Third, an allometric conversion must be done to normalize dosages between humans and animals. For example, the FDA suggests(*170*) that a human equivalent dose (HED) in drug exposure studies is:

$$\text{HED } \left(\frac{\text{mg}}{\text{kg}}\right) = \text{Animal dose } \left(\frac{\text{mg}}{\text{kg}}\right) \text{multiplied by } \frac{Animal\ Km}{Human\ Km}$$

Equation S3.1:

where *Km* values are the ratio of body weight to surface area (mouse Km = 3, human Km = 37)(*171*). Using this equation, 10, 25, and 100 ppm exposures in

mice correspond to HEDs of 811, 2,027, and 8,108 ppb in humans. All three of these exposures are well within the ASTDR toxicity range described above. In addition, the first level (811 ppb) is actually lower than that of drinking water recently reported for an arsenic rich part of Chile(*172*). The middle and upper exposure levels represent doses where we expected to see increasing (dose-dependent) levels of toxicity.

Supplementary Tables

Table 3.1. Supplementary table of dose ranges in murine arsenic exposure studies.

Arsenical	Mouse Line[a]	Dose (ppm)[b]	Reference
iAs^{V}	A/J	1, 10, 100	Cui et al. (*173*)
iAs^{III}	WT	0.01, 0.25	Dheer et al. (*105*)
iAs^{III}	WT	18.75, 37.5, 62.5	Garcia-Montalvo et al. (*174*)
iAs^{III}	WT	10	Lu et al. (*128*)
iAs^{III}	WT, IL10-KO	10	Lu et al. (*123*)
iAs^{III}	WT	10	Lu et al. (*109*)
iAs^{III}	CD1	6, 12, 24	Tokar et al. (*175*)
iAs^{III}	CD1	0.05, 0.5, 5	Waalkes et al. (*176*)
iAs^{III}, iAs^{V}	WT, As3mt-KO	25, 100	Dodmane et al. (*132*)
iAs^{V}	WT, As3mt-KO	3.125	Naranmandura et al. (*177*)
iAs^{V}	WT, As3mt-KO	3.125	Drobna et al. (*137*)
iAs^{V}	WT, As3mt-KO	3.125	Hughes et al. (*178*)
iAs^{III}	WT, As3mt-KO	25	Arnold et al. (*131*)
iAs^{III}	WT, As3mt-KO	1, 10, 25, 50	Yokohira et al. (*134*)
iAs^{III}	WT, As3mt-KO	50, 100, 150	Yokohira et al. (*133*)

[b]WT refers to C57BL/6 mice with the wildtype As3mk-KO gene.

[a]Exposures reported by Garcia-Montalvo et al, Naranmandura et al, Drobna et al, and Hughes et al were converted from mg As per kg body weight per day to ppm in water based on a 20 gram mouse drinking 3.2 mL per day(*174*).

Table 3.2. Supplementary table of As3mt-KO survival comparisons (Mantel-Cox).

Mantel-Cox Comparison	p-value
GF vs. Sham	0.0004
GF vs. Donor A	0.0014
GF vs. Donor B	0.0014
GF vs. Donor C	>0.0001
GF vs. Donor D	>0.0001
GF vs. Donor E	0.0003
Sham vs. Donor A	0.3830
Sham vs. Donor B	0.0189
Sham vs. Donor C	0.3509
Sham vs. Donor D	0.0006
Sham vs. Donor E	0.0040
Donor A vs. B	0.0197
Donor A vs. C	0.1257
Donor A vs. D	0.0030
Donor A vs. E	0.0088
Donor B vs. C	0.0240
Donor B vs. D	0.4382
Donor B vs. E	0.3079
Donor C vs. D	0.0009
Donor C vs. E	0.0024
Donor D vs. E	0.0946

Table 3.3. Supplementary table of humanized mouse survival summaries.

Group	Subjects (N)	Deaths	Median survival (days)
Germ free	8	8	5
Sham	9	9	17
Donor A	5	5	17
Donor B	5	5	32
Donor C	14	14	21
Donor D	13	13	30
Donor E	7	7	36

Table 3.4. Supplementary table of ANOSIM testing results.

Pairwise donor-group comparisons		
Comparison	ANOSIM R	p-value
Global donor effect	0.8314	0.001
Donor A vs. B	1	0.001
Donor A vs. C	0.9916	0.001
Donor A vs. D	0.5571	0.001
Donor A vs. E	0.9348	0.001
Donor B vs. C	0.9941	0.001
Donor B vs. D	0.7776	0.001
Donor B vs. E	0.9354	0.001
Donor C vs. D	0.997	0.001
Donor C vs. E	0.997	0.001
Donor D vs. E	0.0995	0.001
Time-point comparisons (Day 0 vs. Day 7)		
Comparison	ANOSIM R	p-value
Global time-point effect	0.1926	0.001
Donor A	0.536	0.009
Donor B	0.0792	0.008
Donor C	0.5849	0.001
Donor D	0.9807	0.001
Donor E	0.5933	0.002

Table 3.5. Supplementary table of experimental mouse details.

Figure	Panel	Genotype	Treatment	iAs^V (ppm)	N	Sex	Age (weeks)
1	a	WT	Cef	25	5	Male	10
1	a	WT	Cef	25	5	Female	10
1	a	WT	Sham	25	5	Male	10
1	a	WT	Sham	25	5	Female	10
1	a	WT	Cef	100	5	Female	7
1	a	WT	Sham	100	5	Female	7
1[1]	b	WT	Cef	25	5	Male	10
1[1]	b	WT	Cef	25	5	Female	10
1[1]	b	WT	Sham	25	4	Male	10
1[1]	b	WT	Sham	25	5	Female	10
1	b	WT	Cef	100	3	Male	8
1[2]	b	WT	Cef	100	5	Female	7
1	b	WT	Sham	100	5	Male	8
1[2]	b	WT	Sham	100	5	Female	7
2	a	As3mt	Cef	25	5	Male	13
2	a	As3mt	Cef	25	5	Female	12
2	a	As3mt	Cef	25	5	Male	12
2	a	As3mt	Cef	25	5	Female	13
2	a	As3mt	Sham	25	5	Male	11
2	a	As3mt	Sham	25	5	Female	13
2	a	As3mt	Sham	25	5	Male	12
2	a	As3mt	Sham	25	5	Female	15
2	b	As3mt	Cef	100	5	Male	9
2	b	As3mt	Cef	100	5	Female	11
2	b	As3mt	Sham	100	4	Male	10
2	b	As3mt	Sham	100	5	Female	10
2	c	As3mt	Sham	10	5	Female	11
2	c	As3mt	GF	10	4	Female	11
2	c	As3mt	GF	10	2	Female	10
2	c	As3mt	GF	10	3	Female	10
2	c	As3mt	GF	25	2	Male	7
2	c	As3mt	GF	25	2	Female	7
2	c	As3mt	GF	100	2	Male	12
2	c	As3mt	GF	100	3	Female	12
2	c	As3mt	GF	100	3	Female	17

Figure	Panel	Genotype	Treatment	iAs^V (ppm)	N	Sex	Age (weeks)
3[3]	a	As3mt	Sham	100	4	Male	10
3[3]	a	As3mt	Sham	100	5	Female	10
3	a	As3mt	GF-F0	100	5	Male	14
3	a	As3mt	GF-F0	100	4	Female	8
3	a	As3mt	GF-F1	100	2	Male	7
3	a	As3mt	GF-F1	100	5	Female	7
3	a	As3mt	GF-F1	100	5	Female	7
3[3]	a	As3mt	GF	100	2	Male	12
3[3]	a	As3mt	GF	100	3	Female	12
3[3]	a	As3mt	GF	100	3	Female	17
3	b	As3mt	GF-Donor A	100	2	Male	7
3	b	As3mt	GF-Donor A	100	3	Female	7
3	b	As3mt	GF-Donor B	100	2	Male	7
3	b	As3mt	GF-Donor B	100	3	Female	7
3	b	As3mt	GF-Donor C	100	2	Male	8
3	b	As3mt	GF-Donor C	100	5	Female	8
3	b	As3mt	GF-Donor C	100	4	Male	9
3	b	As3mt	GF-Donor C	100	3	Female	9
3	b	As3mt	GF-Donor D	100	4	Male	6
3	b	As3mt	GF-Donor D	100	2	Female	6
3	b	As3mt	GF-Donor D	100	3	Male	9
3	b	As3mt	GF-Donor D	100	4	Female	9
3	b	As3mt	GF-Donor E	100	4	Male	8
3	b	As3mt	GF-Donor E	100	3	Female	8
3[3]	b	As3mt	Sham	100	4	Male	10
3[3]	b	As3mt	Sham	100	5	Female	10
3[3]	b	As3mt	GF	100	2	Male	12
3[3]	b	As3mt	GF	100	3	Female	12
3[3]	b	As3mt	GF	100	3	Female	17
6	a	As3mt	*E. coli*	25	4	Female	17
6	a	As3mt	*F. prausnitzii*	25	3	Male	17
6	a	As3mt	*F. prausnitzii*	25	3	Female	17
6	a	As3mt	*F. prausnitzii*	25	5	Female	9
6	a	As3mt	GF	25	2	Male	7
6	a	As3mt	GF	25	2	Female	7
6	a	As3mt	GF	25	3	Female	15

[1]These mice were used to quantify both fecal and tissue levels of arsenic, with the exception of tissue for one mouse in the Sham-treated group. Tissue samples for this mouse were not collected.

[2]These mice were used to quantify both fecal and tissue levels of arsenic. A separate experiment was conducted using males (3 Cef-treated, 5 Sham-treated) to add data for tissue levels of arsenic.

[3]Survival of groups in figure 3.2b (i.e. As3mt, Sham-treated, 100 ppm) and 2c (i.e. As3mt, GF, 100 ppm) was included in figure 3.3 (a and b) for comparison.

Table 3.6. Supplementary table of PCR primer sets.

Target	Primer	Sequence 5' - 3'	Reference
Universal Bacterial 16S gene	27 F	AGAGTTTGATCCTGGCTCAG	Schmidt and Relman
	1492 R	GGTTACCTTGTTACGACTT	
F. prausnitzii (16S)	Fpra428 F	TGTAAACTCCTGTTGTTGAGG AAGATAA	Lopez-Siles et al.
	Fpra583 R	GCGCTCCCTTTACACCCA	
Bacterial 16S V4 (Ilumina)	515 F	GTGCCAGCMGCCGCGGTAA	Caporaso et al.
	806R	GGACTACHVGGGTWTCTAAT	

Supplementary Figures

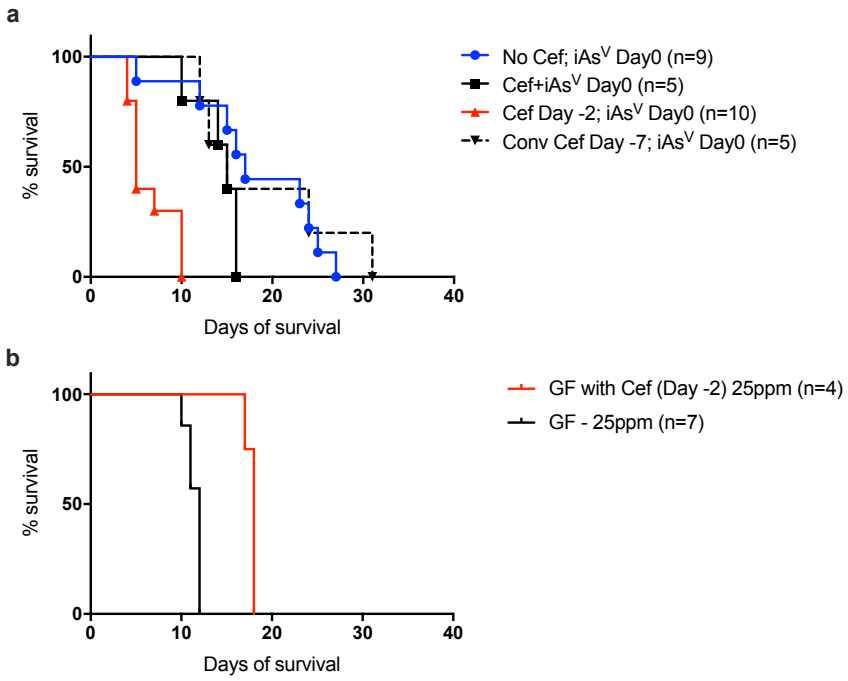


Figure S3.1. The influence of cefoperazone on iAs^V exposure. Combined cefoperazone (Cef, 0.5 mg per mL) and iAs^V (25 ppm) treatment in conventional and germ free (GF) As3mt-KO mice was evaluated in two different experiments. First, survival in conventional As3mt-KO mice during iAs^V exposure was compared between mice pretreated with Cef for 7 (Day -7) or 2 (Day -2) days, mice given Cef on the same day of iAs^V exposure (Day 0), and mice that did not received Cef (**a**). Single or replicate experiments were conducted using four or five mice per experiment as indicated in the legend. Survival in the Day -2 Cef treatment group was significantly shorter compared to mice that did not receive Cef ($p=0.0002$, Mantel-Cox test), whereas survival in the other Cef treatment groups was not significantly different (Day 7, $p=0.7192$; Day 0, $p=0.0894$; Mantel-Cox test). These results in conventional mice suggest that the influence of Cef is greatest when administered 2 days prior to iAs^V exposure at the point in which the microbiome is significantly altered. In a second experiment (**b**), survival in GF As3mt-KO mice (one cage of four mice) was compared between mice that received the Day -2 Cef treatment and mice that did not receive Cef (two cages of three or four mice). Survival was significantly shorter in mice that did not receive Cef ($p=0.0043$, Mantel-Cox test), suggesting that Cef pretreatment did not significantly increase toxicity. Collectively, these results provide evidence that there was minimal additive synergistic effects on toxicity when Cef and iAs^V were co-administered in this murine model.

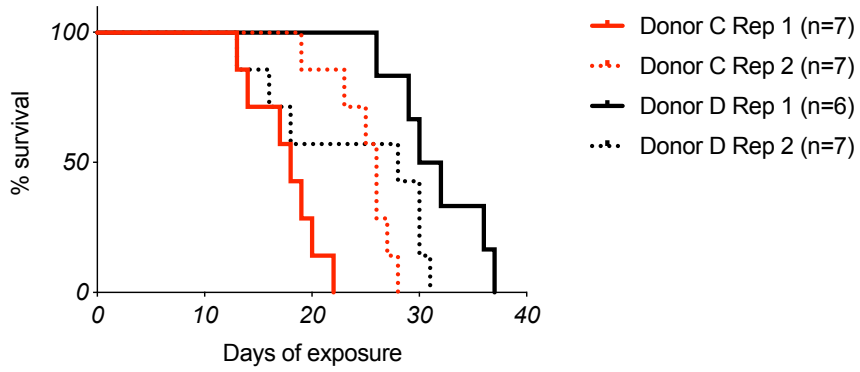


Figure S3.2. Replicate exposures of As3mt-KO humanized donor groups. In the first experiment (Rep 1), median survival of As3mt-KO mice receiving stool transplantation from donor D was significantly longer following arsenic exposure (iAs^V, 100 ppm) compared to mice receiving stool transplantation from donor C ($p=0.0004$, Mantel-Cox test). Repeating the experiment (Rep 2) and doubling the overall number of mice in the comparison (aggregate data) did not change this result ($p=0.0009$, Mantel-Cox test), even though the median survival of group C mice was significantly longer in the second experiment ($p=0.0009$, Rep 1 vs. Rep 2, Mantel-Cox test), and was not significantly different from group D mice in the second experiment ($p=0.2268$, Group C Rep 2 vs. Group D Rep 2, Mantel-Cox test).

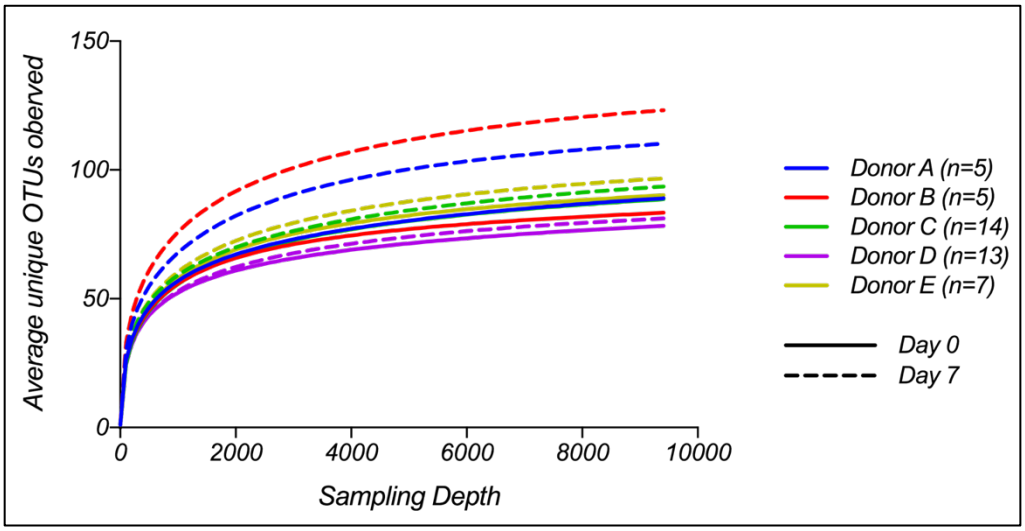


Figure S3.3. Rarefaction of OTUs from 16S rRNA (V4) sequence data. Rarefaction analysis of the fecal microbiome of humanized mice on days 0 (solid lines) and 7 (hashed lines) generated by random subsampling to a depth of 9408. Adequate coverage was obtained for each sample (>99% Good's coverage estimates). Observed species richness ranges from 64 to 128, with ACE richness estimates between 66 and 128.

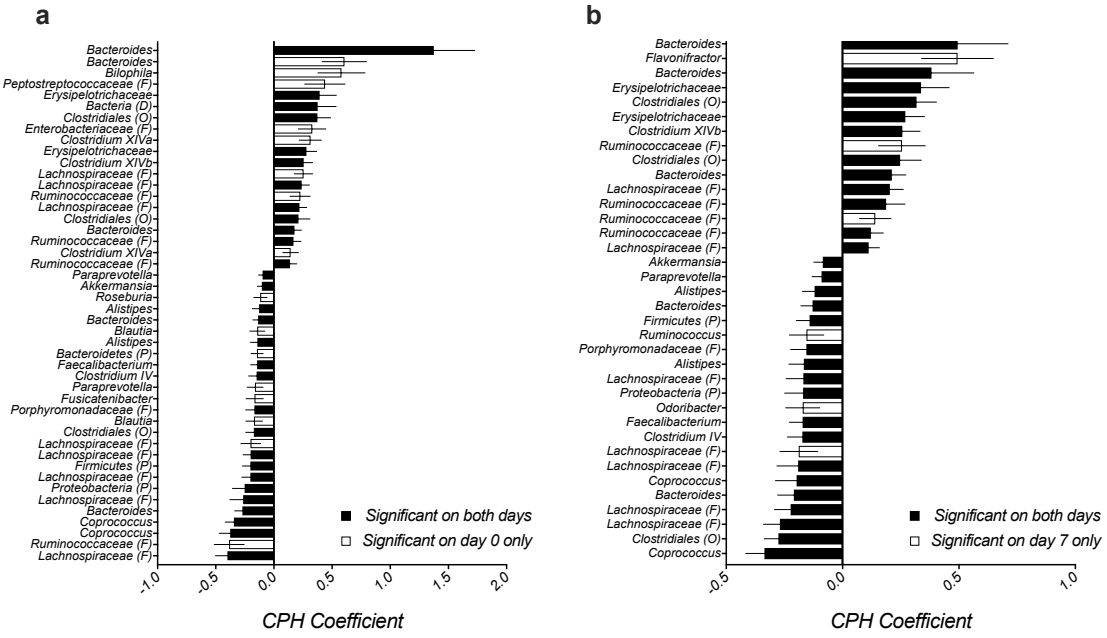


Figure S3.4. Results of survival modeling against OTU abundance. Log transformed abundance of the 100 most abundant OTUs were fit to univariate survival models (Cox proportional hazards). Controlling for false discovery rate (<0.1), significant correlations between OTU abundance and survival are represented here. Bars represent the correlation (beta) coefficient estimate with standard error represented. Correlations identified on both day 0 and day 7 are presented. Black bars were correlated at both time points. Negative coefficients are associated with increased survival (decreased hazard).

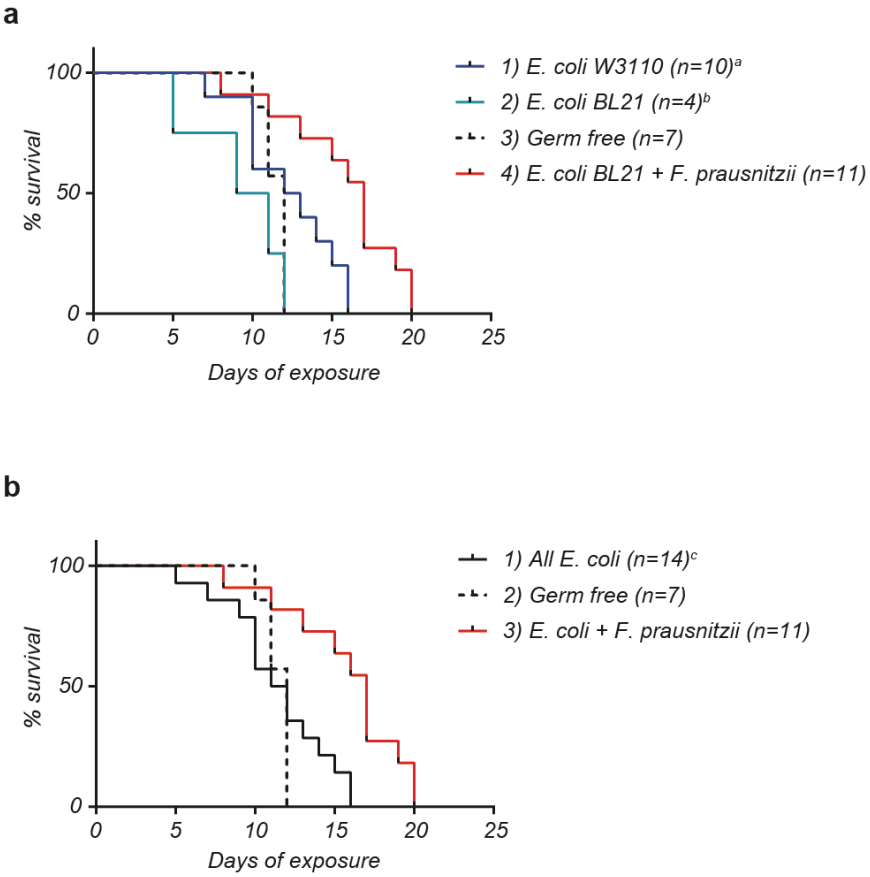


Figure S3.5 Survival in groups of GF, *E. coli* mono-colonized, and bi-colonized mice. Survival of mice mono-colonized with two different *E. coli* strains (W3110 and BL21) are shown separately in **a**, or as pooled data in **b**. Survival of both *E. coli* mono-colonized groups of mice in **a** was not significantly different from GF mice (p=0.2102, W3110; p=0.1759, BL21; Mantel-Cox test). Survival of pooled *E. coli* mono-colonized mice in **b** was not different from GF mice (p=0.5791, Mantel-Cox test). Survival of *E. coli* + *F. prausnitzii* bi-colonized mice was longer compared to GF mice (p=0.0035, same groups shown in **a** and **b**; Mantel-Cox test) and *E. coli* mono-colonized mice (p=0.0080, W3110 in **a**; p=0.0024, BL21 in **a**; p=0.0018, all *E. coli* in **b**; Mantel-Cox test). Results reported in the main body of the manuscript (Figure 3.4 e) represent data from *E. coli* BL21 only. [a]W3110 carried the empty cloning vector, pMal-c5x, and was maintained in mice with ampicillin (100 μg per mL) in drinking water. [b]BL21 carried the empty cloning vector, pET29a, and was maintained in mice with kanamycin (50 μg per mL) in drinking water. [c]All *E. coli* refers to pooled W3110 and BL21 data from panel a.

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CHAPTER FOUR

SPECIATION OF EXCRETED ARSENICALS FROM GERM FREE AND CONVENTIONAL AS3MT KNOCKOUT MICE EXPOSED TO INORGANIC ARSENATE

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Designed research approach, conducted experiments, analyzed data, made figures, wrote manuscript.

Co-Author: M. Yoshinaga

Contributions: Advised methods, analyzed samples, collected data.

Co-Author: T.R. McDermott

Contributions: Conceived of project, advised research.

Co-Author: S.T. Walk

Contributions: Conceived of project, directed research,

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____ Accepted by a peer-reviewed journal
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Abstract

Arsenic is a ubiquitous environmental toxicant and known human carcinogen. *In vitro*, gut-associated microbiota of humans and mice have a high capacity for enzymatic reduction and methylation of inorganic arsenic. Antibiotic disruption of the microbiome *in vivo* has been shown to alter the disposition and metabolism of ingested arsenic. Despite this demonstrated influence on arsenic toxicity, there is little understanding of the specific metabolic transformations of arsenic mediated by the gut microbiota, or the degree to which these processes influence overall disposition of ingested arsenic. In this study, germ free, conventional, and antibiotic-treated mice deficient for host arsenic methylation were given a single dose of inorganic arsenate, and their excretions were collected over the following 24 hours for arsenic quantification. Germ free and antibiotic treated mice excreted less arsenic via stool, and more via urine compared with conventional mice. Arsenical speciation was notably influenced by microbiome status, especially in the stool. Notably, the stool of germ free mice contained more inorganic arsenate and less inorganic arsenite (proportionally) compared with conventional mice. Additionally, small amounts of monomethyl arsenic were detected in the urine and/or stool of all treatment groups except germ free mice. Together, these results show that microbiome-mediated arsenic metabolism contributes to the chemical reduction and methylation of arsenate *in vivo*.

Keywords: [Arsenic, methylation, microbiome, germ free, axenic]

Introduction

Arsenic occurs naturally in nearly every environment on earth in varying concentrations and in a diversity of chemical compounds (*84*). Chronic arsenic exposure causes cancers of the lung, skin, and bladder in humans and is implicated in non-cancerous health outcomes including skin lesions, diabetes, obesity, cardiovascular disease, and complications during pregnancy (*169, 179*). Natural groundwater contamination is the most pervasive route of human arsenic exposure, with an estimated 150 million people at risk for exposure to unsafe concentrations (*16*). This makes arsenic one of the most dangerous environmental toxicants in the world and, as such, it ranks first on the Agency for Toxic Substance and Disease Registry priority list of hazardous substances (*125*).

Arsenic occurs in a variety of different organic (oAs) and inorganic (iAs) compounds with varying degrees of environmental mobility, bioavailability, and toxicity. In contaminated ground and surface waters, arsenic mostly occurs in one of two common inorganic species, pentavalent arsenate (iAs^{V}) or trivalent arsenite (iAs^{III}). iAs^{III} is generally more mobile in surface and subsurface water flows and also more bioavailable and toxic. In humans, the primary metabolic pathway for detoxifying ingested arsenic is arsenic methylation, mediated by the arsenic (3+) methyltransferase (AS3MT) enzyme. Methylated arsenicals (mAs) are the simplest

organic forms of arsenic. In humans and animal models, most arsenicals excreted in urine following arsenic exposure are methylated (*180*). Experimental disruption of the *as3mt* gene in mice severely impairs arsenic methylation, resulting in decreased arsenic excretion and a corresponding increase in the systemic half-life of ingested arsenic (*137*). These methylation deficient as3mt knockout mice (As3mt-KO) mice are also highly sensitive to severe systemic toxicity and mortality from iAs exposures (*107, 133*).

Despite lacking a functional As3mt enzyme, multiple studies have detected methylated arsenicals in arsenic-exposed As3mt-KO mice (*137, 177, 181*). While the levels of methylated arsenicals were low compared to As3mt-competent (WT) mice, these results suggest that other pathways are involved in arsenic methylation in mammals. The biological origins of this residual arsenic methylation remain largely speculative in the literature. Mammalian gut microbiota have a high capacity for enzymatic reduction and methylation of iAs *in vitro* (*105, 106*). However, a number of studies have suggested that microbes are unlikely to provide meaningful contributions to arsenic metabolism in a living host because ingested arsenic is rapidly taken up across the intestinal epithelium. Thus, it is unclear whether ingested arsenic would be in contact with the microbiome long enough for substantial arsenic metabolism to occur.

Despite these contrasting views, there is compelling evidence that arsenic-microbiome interactions influencing toxicity in the host. Coryell et al. 2018

recently provided the first direct evidence of microbiome-mediated influences on arsenic toxicity in As3mt-KO mice, showing that antibiotic treatment and germ free status were associated with significantly greater arsenic-induced mortality compared to mice with a conventional microbiome (*107*). This study showed that transplanting human fecal communities into germ free (GF) As3mt-KO mice extended survival times during arsenic exposure in a donor-dependent fashion. It also showed that antibiotic disruption of the conventional mouse microbiome decreased fecal excretion and increased the levels of arsenic in host tissues. This finding was supported recently in a study in which antibiotic perturbation led to decreased fecal excretion and increased urinary excretion, with modified methylation patterns in excreted arsenicals (*182*). Together, these studies suggest that the microbiome alters arsenic toxicity by modulating fecal excretion and overall methylation profiles through currently uncharacterized mechanisms. Given these recent insights into the microbiome's influences on arsenic toxicity and elimination, I wanted to better understand how microbiome interactions influence arsenic metabolism in a living host.

The aim of this study was to characterize the effect of microbiome status on arsenic metabolism and excretion in conventional and germfree As3mt-KO mice. High-performance liquid chromatography (HPLC) and inductively coupled mass spectrometry (ICP-MS) were used to quantify total and speciated arsenic levels in stool and urine collected from individual mice over 24 hours following

administration of a single dose of inorganic arsenate. Microbiome status was experimentally controlled to measure the effects of germ free status, as well as antibiotic disruption and recovery on arsenic metabolism compared to conventional As3ml-KO mice. Here, I report microbiome-associated differences in the relative excretion routes of ingested arsenic, inorganic arsenate reduction rates, and the production of monomethyl arsenic. These results provide more direct evidence that the host and microbiome work together to mitigate the deleterious effects of arsenic.

Materials and Methods

Experimental Mice

Animal experiments were approved by the Montana State University Institutional Animal Care and Use Committee. All mice were bred and maintained at an American Association for the Accreditation of Laboratory Animal Care accredited facility at Montana State University. Conventional mice were housed under specific pathogen free conditions in individually ventilated cages stocked with sterilized bedding, irradiated complete nutrition chow (LabDiet 5053), and acidified water purified by reverse osmosis (RO). Germ free mice were housed in hermetically sealed and Hepa-filter ventilated vinyl isolators and received autoclave sterilized water and chow (LabDiet 5013, St. Louis, MO). All food and water for GF mice was quarantined, monitored, and tested for contamination

prior to introduction. During experimental collections, mice were singly housed in free-standing, wire-floor metabolic cages designed for complete collection and separation of urine and stool (Techniplast USA, Inc.). During this time, mice had free access to pulverized chow and filter-sterilized RO water.

All mice used in this study came from a C57BL/6 gene knockout background deficient for the murine *As3mt* gene (As3mt-KO). These mice were originally derived at the Environmental Protection Agency (*137*). Breeding pairs of As3mt-KO mice were obtained from Drs. C. Douillet and M. Styblo (University of North Carolina), and used to establish a breeding colony at Montana State University. Mouse colonies were derived GF from conventional mice as described in previous work (*107*). Briefly, conventional As3mt-KO and GF surrogate (Albino Swiss-Webster) females were gestationally synchronized so that litters were born within 12 hours of each other. At precisely full term, gravid conventional dams were euthanized, and a sterile hysterectomy was performed. Neonatal pups were quickly removed, revived, and transferred into a GF isolator where they nursed on surrogate GF Swiss-Webster dams. GF status was monitored regularly by attempting aerobic and anaerobic culture techniques on rich medium (Mueller-Hinton broth and agar plates) and by periodic PCRs targeting the bacterial 16S rRNA encoding gene with DNA extracted from stool serving as template.

Chemical Reagents

Montana State University. Cefoperazone (cefoperazone sodium salt) was purchased from Chem-Impex International Inc. (Wood Dale, IL) and stored with desiccant at 4°C. For mouse exposures, iAsV was ACS grade (≥98% pure) sodium arsenate dibasic heptahydrate (Na2HAsO4 · 7H20, Sigma-Aldrich, St. Louis, MO). Nitric acid (VWR Chemicals BDH®, Aristar® plus grade) and hydrochloric acid (Fischer Scientific™, TraceMetal™ grade) were purchased through the Montana State University chem stores. Pepsin enzyme (1:10,000 Biotechnology grade) was purchased from VWR® Life Sciences. All chemicals were used as received with no additional purity analysis.

Florida International University. All chemicals were of analytical or better grade from Millipore-Sigma (Burlington, MA). For arsenic speciation analysis, Sodium (meta)arsenite, sodium arsenate, disodium methylarsonate hexahydrate and sodium cacodylate trihydrate were used as chemical standards for arsenite iAs^{III}, arsenate iAs^{V}, monomethyl arsenate MMA^{V} and dimethyl arsenate DMA^{V}. The trivalent form of methyl arsenic, methyl arsenite, (MMA^{III}) was prepared by chemical reduction of MMA^{V} as previously described (*183*). Briefly, 0.2 mM MMA^{V} was mixed with 27 mM $Na_2S_2O_3$, 66 mM $Na_2S_2O_5$, and 82 mM H_2SO_4, following which the pH was adjusted to 6 with NaOH.

Experimental Exposures

Prior to arsenic dosing, conventional male and female mice were randomly assorted into three treatment groups in equal numbers, representing the conventional, antibiotic-treated (AT), and antibiotic recovered (AR) groups. AT and AR groups received cefoperazone sodium (0.5 mg per mL) ad libitum in their drinking water for 7 days, during which they were monitored daily for signs of distress or weight change. AT mice were dosed with iAs^V immediately following cefoperazone exposure. AR mice were place back on standard drinking water for an additional 2 weeks prior to iAs^V dosing.

Arsenic Dosing

A dose of 0.5mg iAs^V per kg body weight was selected based on previous literature quantifying arsenic speciation in As3mt-KO and C57 Bl/6 wild-type mice (*177*). A stock solution of sterile filtered sodium arsenate dibasic heptahydrate in DI water (0.075 mg iAs^V per mL) was used, adjusting gavage volume to individual mouse bodyweight at the time of gavage. To ensure consistent dosing across experimental subjects, excess aliquots from the same stock solution were frozen and stored at -20° C for the duration of the experiments, reducing the potential for technical or experimental variation.

Sample Collection and Storage

Immediately following gavage of iAs^V (0.5mg per kg-body weight) mice were placed in metabolic cages with ad libitum access to pulverized chow and

autoclave sterilized RO water. After 24 hours, mice were euthanized via exsanguination under isofluorane anesthesia. Livers were collected and immediately transferred to -80° C for storage. Urine and fecal collection vessels were removed from metabolic cages, and contents transferred to clean, sterile collection tubes. Urine collection cones were rinsed with DI water to collect any residual urine. Collected urine and rinsate were stored at -80° for later analysis. Fecal collections were weighed fresh and stored at -20 °C.

Sample Pre-Processing at Montana State University

Individual urine and cage rinsates were combined, diluted to a uniform volume of 8ml with deionized (DI) water, centrifuged for 20 minutes at 2000 rcf to remove particulate matter, and the supernatants were collected for analysis. Whole stool collections were homogenized in a slurry with DI water (4-parts water per 1-part stool by weight) resulting in a slurry of approximately 0.2 g-stool per mL. For total arsenic determination, slurried stool aliquots (0.5 mL) were dried to a constant weight in a benchtop vacuum centrifuge evaporator (Jouan Precision, RC10-10). Dried samples were digested in ~10 volumes of 70% nitric acid by heating to 70° C for 4-5 hours, until clear. Digests were diluted to achieve a final nitric acid concentration of 6%. For arsenic speciation analysis in the stool, a pepsin-assisted extraction protocol was adapted from Peng et al. (*184*). Stool slurry aliquots (1ml) were diluted with an equal volume of 2x extraction solution to working digestion mixture containing 20 mg per mL pepsin powder and 0.5%

hydrochloric acid. Samples were homogenized on ice for 15 minutes by emersion bath sonic disruption (Bioruptor UCD-200, Diagenode Inc.). Sonicated samples were incubated overnight at 37° C under constant shaking. After incubation, samples were sonicated an additional 5 minutes, centrifuged (15 minutes at 2000 rcf), and the supernatants were collected for arsenical speciation. No additional extraction procedures were performed prior to shipping urine samples for arsenic analyses. All samples were stored at -20° C after preparation and shipped overnight on dry ice for analysis at Florida International University (FIU).

Determination of Total Arsenic Level

At FIU, urine samples were mixed with equal amount of 70% nitric acid and incubated at 70° C for 30 min. The digestion solution was cooled to room temperature and 17.5-times diluted with deionized water. Stool and liver digestion solutions were 5-times diluted with deionized water. Arsenic level in each sample was quantified by inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e; PerkinElmer, Waltham, MA) according to an arsenic standard curve prepared with standard solutions in the range of 1-50 ppb in 2% nitric acid using an arsenic standard (Ultra Scientific, N. Kingstown, RI).

Arsenic Speciation Analyses

All samples were filtered by using Amicon Ultra Centrifugal Filters with a 3K membrane (Millipore). Arsenicals were separated via HPLC (Series 2000;

Perkin-Elmer) using Thermo Fisher Scientific BioBasic™ 18 LC column (250 mm × 4.6 mm, 5 µm, 300 Å) isocratically eluted with a mobile phase consisting of 3 mM malonic acid and 5% methanol (v/v) (pH 5.95 adjusted by tetrabutylammonium hydroxide) with a flow rate of 1 mL min^{-1} at 25 °C (20 μL injection volume) and detected via inline ICP-MS. Each of the indicated arsenic species was quantified from the corresponding peak area using Chromera Chromatography Data System version 2.1 (Perkin Elmer) according to standard curves prepared with standard solutions in the range of 0.1-1 μM in water.

Data Transformations and Statistical Methods

Total arsenic values measured in excreta (urine and stool) were normalized to the estimated total amount of excreted arsenic based on the weight of sample digested for determination and the total sample weight (stool) or volume (urine). Measurements were reported in relation to the amount of arsenic recovered as a percentage of the total or theoretical (baseline mice only) arsenic dose of 0.5 mg-iAs^{V} per kg body weight. Baseline values from unexposed mice were not included in statistical analyses but are presented in some figures for reference.

For arsenic speciation analysis, arsenical values were reported relative to their sum total (percentages), after subtraction of digestion blank values (Figure 4.2). A detection limit cutoff of 0.5 ppb was used, with measurements reported below this limit treated as 0 ppb. Mean differences in the relative proportions of

MMA levels were reported as combined MMA^{III} and MMA^{V} values. Other reported combined values include total methylated arsenic, or mAs (MMA^{III} + MMA^{V} + DMA^{V}), and total iAs (iAs^{V} + iAs^{III}).

Group means were compared using ordinary one-way ANOVA (total arsenic values) or mixed-effects ANOVA (speciated arsenicals), using Dunnett's test for multiple comparisons correction, treating the conventional group as a control against which all other group means were compared. Some outlier values were excluded from total arsenic comparisons based on observations made during experimental procedures, including inadequate gavage delivery, technical malfunctions with collection apparatus, and observed abnormality in mouse condition or behavior. All descriptive statistics, significance testing, correlation analyses, and data visualizations were performed in Prism 8 for macOS (v. 8.2.0, GraphPad Software, Inc.).

Results

Microbiome Status Influences Total Arsenic Levels in Stool and Urine

To understand the influence of microbiome status on arsenic excretion, total arsenic concentrations were quantified in stool and urine collected from individual mice housed for 24 hours in metabolic cages following oral dosing with iAs^{V} (0.5mg per kg). Total arsenic concentrations, measured by ICP-MS, were normalized to collected sample size and expressed as a percentage of the

administered dose (Figure 4.1). Microbiome status was significantly associated with the mean proportion of excreted arsenic in both stool (one-way ANOVA, $p > 0.0001$) and urine (one-way ANOVA, $p = 0.004$). In conventional mice, urine and fecal arsenic accounted for 36.9% ± 6.2% and 9.3% ± 2.3% of the administered arsenic dose, respectively.

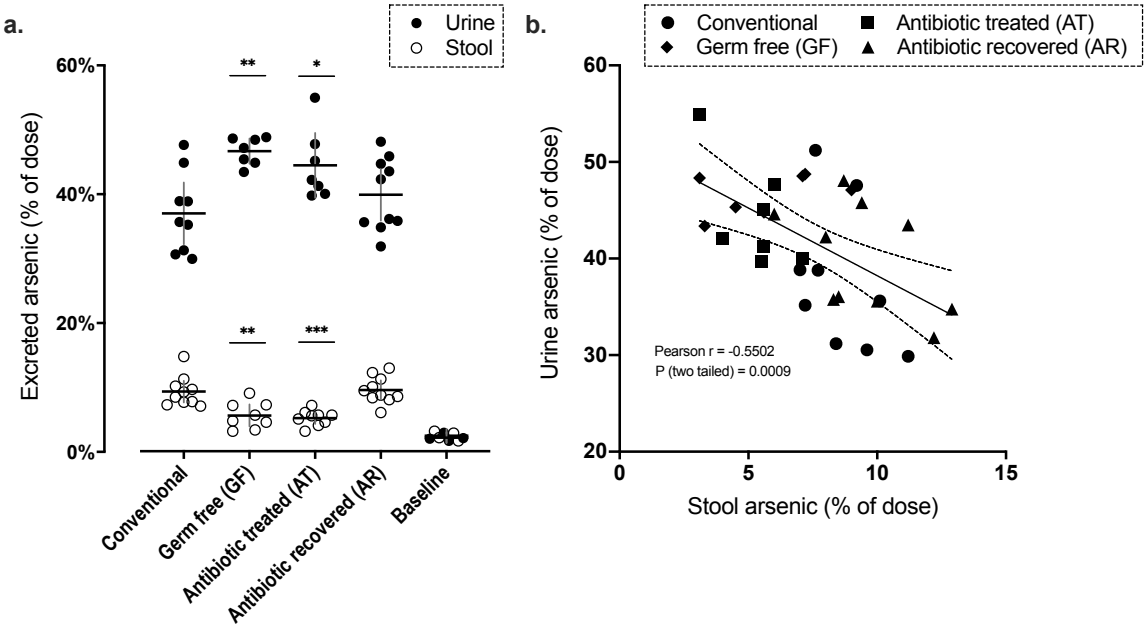


Figure 4.1 Inverse correlation between total arsenic levels in the stool and urine. Total arsenic levels (**a**) in the stool and urine, normalized to the percentage of administered dose recovered with group means and 95% confidence intervals. The correlation between stool and urine arsenic (**b**), with trend line and 95% confidence bands.

In germ free (GF) mice, fecal excretion was significantly decreased, accounting for 5.5% ± 2.0% of the administered dose (Dunnett adjusted $p = 0.0041$) with a reciprocal increase in urinary excretion (46.6% ± 5.5; Dunnett adjusted $p = 0.0029$), suggesting the microbiome limits systemic arsenic in the host by mediating fecal excretion. A similar trend was observed in antibiotic-

treated (AT) mice, where on average, stool (Dunnett adjusted $p = 0.0002$) and urine (Dunnett adjusted $p = 0.024$) accounted for 5.2% (± 1.2) and 44.4% (± 5.5) of the administered dose, respectively. However, after a 2-week recovery period following AT, stool and urine levels were not significantly different from baseline, conventional mice (39.8% ± 5.6 and 9.5% ± 2.1, respectively; Dunnett adjusted $p = 0.98$, stool; $p = 0.51$, urine). While the total percentage of excreted arsenic (stool + urine) was elevated in GF and AT groups compared to the other groups, these differences did not reach statistical significance (one-way ANOVA, $p = 0.073$ on data not shown), meaning that approximately the same amount of arsenic was excreted by mice in all groups despite microbiome-dependent changes in the route of excretion. Across all treatment groups, an average of 7.4 times as much arsenic was excreted via urine compared to stool, and there was a significant negative correlation between paired arsenic levels in the stool and urine (Figure 4.1b.). Together, these results support a significant negative influence on fecal arsenic excretion when the microbiome is removed (GF) or disrupted (AT), but that fecal excretion is effectively recovered within two weeks of disruption.

Notably, baseline control mice receiving a vehicle control gavage without arsenic also had detectable levels of arsenic in their stool and urine. While small by comparison, arsenic levels in baseline stool and urine collections, respectively, represented 2.4% (± 0.54) and 2.1% (± 0.54) of the theoretical dose these mice

would have received (0.5 mg-iAs^V per kg body weight). This level of *background* arsenic could represent a majority of fecal arsenic detected in some GF and AT mice. Since approximately equal amounts were excreted in the stool and urine, background exposure was less bioavailable or bioaccessible than the iAs^V used for experimental exposure, suggesting a dietary origin in the chow. It is also possible that these measurements resulted from trace contamination in the vehicle gavage or the sample collection apparatus. Despite thorough cleaning and decontamination between uses, the hydrophobic coating used on metabolic cages precluded the application of mineral acid solutions (e.g. nitric acid) commonly used for decontamination of labware.

Speciation of Excreted Arsenicals

To better understand the microbiome's influence on *in vivo* arsenic metabolism, HPLC-ICP-MS was used to quantify inorganic (iAs^{III} and iAs^V) and methylated (MMA^{III}, MMA^V, and DMA^V) arsenicals from the same sample materials that were analyzed for total arsenic levels above. This analytical approach is a rapid and repeatable method for determining the methylation status and oxidation state of arsenicals including iAs^{III}, iAs^V, MMA^{III}, MMA^V, and DMA^V with a detection limit of approximately 0.5 parts per billion (ppb). Values below this limit or below the sample blank concentrations were treated as zero for analyses. Because trivalent methylated arsenicals are prone to oxidation, MMA^{III}

and MMA^{V} were combined and reported simply as total MMA, unless otherwise noted in figures or text.

Microbiome-Driven iAs^{V} Reduction

As expected from previous studies with As3mt-KO mice, inorganic arsenicals represented the majority of excreted arsenic in both stool and urine collections (Figure 4.1). The dominant arsenical observed in both urine and stool was iAs^{III}, accounting on average for 66% ± 6.6% of stool arsenicals and 64% ± 2.1% of urinary arsenicals. The second most prominent arsenical in the analysis, iAs^{V}, accounted for 26% ± 7.6% of fecal and 32.6 ± 2.2% of urinary arsenicals (Note: these percentages are relative total arsenical species detected, not normalized to dose). Importantly, the mean percentages of iAs^{III} and iAs^{V} were significantly altered in the stool of GF mice compared to conventional mice (Table 4.1, Figure 4.2). On average, iAs^{III} made up a significantly smaller percentage of arsenicals in GF stools compared to conventional stools (Dunnett-adjusted $p = 0.021$), while iAs^{V} accounted for significantly more of the arsenicals in GF compared to conventional stools (Dunnett-adjusted $p = 0.0056$). Decreased iAs^{III} in the stool of GF mice suggests that arsenate reduction is at least partially microbiome dependent. In the urine, there were no significant iAs difference between treatments groups, suggesting that host metabolism compensates for this loss of microbiome function.

Table 4.1. Mean relative distribution of speciated arsenicals determined in the urine and stool.

	Stool arsenicals (mean %, SD)			
Microbiome Status	% iAs^{III}	% iAs^{V}	% MMA^{III+V}	% DMA^{V}
Conventional	68.1(5.3)	24.8(6.05)	4.0(1.4)	3.2 (1.3)
Germ free (GF)	58.4(7.3)	36.7(7.10)	ND	4.9 (1.1)
Antibiotic Treated (AT)	64.3(7.8)	23.9(8.70)	ND	11.8 (3.8)
Antibiotic Recovered (AR)	74.1(7.2)	18.6(8.66)	3.9(1.7)	3.3 (0.80)
	Urine arsenicals (mean %, SD)			
	% iAs^{III}	% iAs^{V}	% MMA^{III+V}	% DMA^{V}
Conventional	64.0(6.9)	33.0(7.8)	0.11(0.1)	2.9(1.0)
Germ free (GF)	64.9(7.0)	33.9(7.1)	ND	1.2(0.28)
Antibiotic Treated (AT)	68.0(4.1)	29.4(4.5)	0.21(0.2)	2.5(0.74)
Antibiotic Recovered (AR)	63.9(6.3)	33.6(6.7)	0.11(0.1)	2.4(0.58)

Methylated arsenicals

In the urine, methylated arsenicals only accounted 2.4% ± 0.81% of all arsenic species, compared to 7.8% ± 2.9% in the stool. MMA species were detected in stool of both conventional and AR groups, accounting for 4.0% ± 1.4% and 3.9% ± 1.7% of arsenic species, respectively (Table 4.1), but were not detected in the GF or AT mice, suggesting that MMA is a product of microbial metabolism in the gut (Figure 4.2a). In the urine, MMA was a minor component of detected arsenicals in conventional, AT, and AR groups (averages ≤ 0.21%), but was not detected in GF urine, supporting a possibly microbial origin of this metabolite.

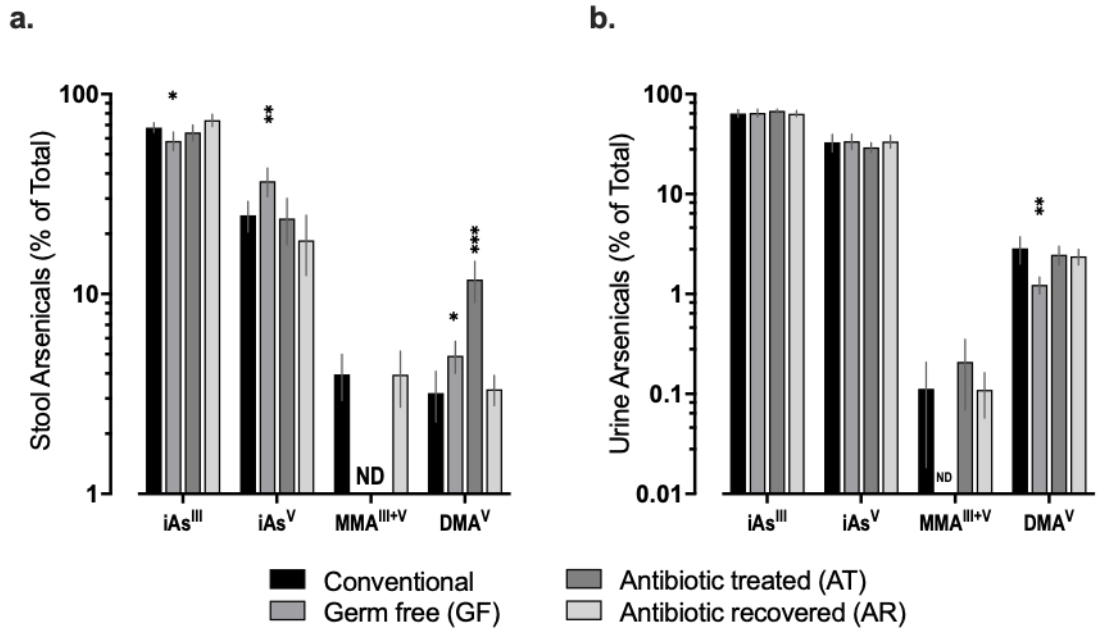


Figure 4.2. Speciated stool (**a**) and urine(**b**) arsenicals. Bars represent group mean percentages with 95% CI shown. Note: $\log_{10}$ scale y-axes were used for visual emphasis.

Trace amounts of DMA^V were detected in the stool and urine across all treatment groups, accounting, on average, for 5.8% ± 4.1 % of fecal arsenicals and 2.3% ± 0.73% of urinary arsenicals. Compared to conventional mice, DMA percentages were elevated in stool of GF and AT mice (Dunnett-adjusted $p = 0.018$ and $p < 0.0001$, respectively) and lower in the urine of GF mice (Dunnett-adjusted $p = 0.002$). While these differences appear significant, measured DMA concentrations were mostly outside of those used in standard-curve calculation and may or may not represent true difference.

Discussion

Prior to this study, there have been few reports characterizing arsenic metabolism in GF mice of any variety, and the handful of studies examining the influence of gut microbiome on arsenic speciation have largely relied on *in vitro* experiments. These studies demonstrate the metabolic potential of gut microbes to reduce and methylate iAs (*104-106*), but metabolic potential *in vitro* may not always predict the metabolic functions of microbial communities in their native environment. My previous work showed that both human and murine intestinal microbiota have a significant protective effect against acute arsenic toxicity in As3mt-KO mice, and that fecal elimination of arsenic was impaired by disrupting the microbiome of C57 Bl/6 mice (*107*). The present study builds on this work, providing strong evidence of microbiome-dependent arsenic metabolism *in vivo*.

These results show that germ free status and antibiotic treatment are associated with decreased fecal elimination of arsenic and with a reciprocal increase the urinary output, suggesting that native microbiota help decrease systemic arsenic exposure by augmenting excretion via stool. Though antibiotic induced microbiome disruption altered total arsenic levels in the urine and stool, these changes returned to normal status when arsenic dosing was separated from antibiotic exposures by a two-week period of recovery. This supports previous work suggesting that antibiotic use may be a risk factor during arsenic exposure

(*182*), but suggests that relevant microbiome functions are quick to recover following antibiotic perturbation.

Arsenical speciation analysis identified oxidation-state specific differences in the fecal excretion of iAs, with higher relative levels of iAs^{V} and lower iAs^{III} excreted in the stool of GF mice. This analysis also found small but statistically significant differences in the percentages of methylated arsenicals excreted by GF and AT mice, compared with conventional mice. More notably, MMA was present in the stool of conventional and AR mice but was not detected in the stool of either GF or AT mice. MMA is a common methylated metabolite of inorganic arsenic associated with increased disease risk in humans (*11, 55, 185*). These findings suggest that microbiome activity is required for the formation of MMA in the absence of host arsenic methylation activity. As both GF and AT mice also has significantly reduced levels of total arsenic in the stool, the possibility remains that non-detection was an artifact of overall reduction in fecal elimination. If this were the case, reciprocally elevated MMA would be expected in the urine, as was observed in total arsenic levels. While MMA was detected in the urine of AT mice, it was not apparently elevated above conventional mouse levels, and none was detected in the urine of GF mice. Thus, these findings support a microbial origin of MMA in these mice, however the role of antibiotic perturbation and MMA production is unclear.

DMA^V was detected in both stool and urine in roughly equal amounts across all treatment groups. The relative consistency across treatment groups, including GF mice indicates that this is probably not a product of *in vivo* iAs methylation in this instance. Besides As3mt, there are no known pathways capable of methylating iAs in mice. Were this a product of host metabolism, one would also expect to detect MMA as an intermediate metabolite, even in GF mice. Given the relatively high levels of total arsenic determined in stool slurries, it is likely that trace contamination in the food or drinking water supplies is contributing to observed arsenical profiles, possibly including DMA.

DMA is poorly absorbed compared to iAs (*186*), potentially explaining the approximately equal amounts of arsenic excreted in the urine and stool of baseline mice. In addition to documented occurrence in number of different food products, DMA in the urine can also occur as a metabolite of ingested arsenosugars and arsenolipids, which are present in several dietary sources (*37*). It is possible that commercial chows used in the vivarium in which the mice were housed could contain low levels of arsenical contamination which would not be detected using the current screening protocol. Some of the listed ingredients which could potentially contribute to background arsenic levels include fishmeal, animal fat, wheat and soy derivatives, and ferrous iron (*37, 187-190*). Based on the total arsenic concentrations determined in urine and fecal samples collected from baseline mice over the 24 hour collection period (average 0.02 mg-As per kg

body weight), assuming steady-state exposure/elimination rates and 100% extraction efficiency, average background exposure would be approximately 0.02 mg-Arsenic per kg per day. An adult mouse consuming 5g of chow daily would reach or exceed this estimated level of background exposure from as little as 130 ppb of total arsenic in the chow. This is approximately an order of magnitude below the maximum arsenic concentration the manufacturer is able to guarantee in their certified chow products (1 ppm). To be clear, this study did not include any systematic analysis of commercial products and is not intended to make any statements or suggestions regarding the quality or purity of said products. This was presented merely as a plausible, though speculative, explanation for how the background arsenic levels observed in this study could easily result from levels that would remain undetected under the protocols used.

Determining the speciation of excreted arsenicals has become common place in assessments of arsenic exposure and metabolism in human populations and is essential for understanding arsenic biotransformations occurring *in vivo* (*12, 191, 192*). In this experimental system, the decision to use As3mt-KO mice was made in an effort to minimize the influence of host metabolism on arsenic speciation, with the thought that difference between GF and conventional mice would be explained as an effect of the microbiome. Altered ratios of iAs^{III} and iAs^{V} in the stool of GF mice are presumably due to the absence of microbial arsenate reduction activity in the GF gut. However, given that antibiotic treatment did not

produce a similar effect, this result could also be explained by differences in the physiochemical parameters of germ free mice. For example, the conventional mouse gut is a reducing environment, with cecal E_h values circa -200 mV, while the GF mouse cecum has a highly positive E_h around +200 mV (*193*). This more oxidizing gut environment could slow the reduction of iAs^V in the body, resulting in redox-dependent changes in overall arsenic metabolism. The independent influences of gut redox chemistry and bacterial arsenate reductase activity could be studied in using GF As3mt-KO mice as a model system for gnotobiotic manipulation, as gut E_h and pH differences in GF mice can be successively normalized to varying degrees by gnotobiotic colonization (*193*).

The lack of detectable MMA in the excretions of GF As3mt-KO mice can be taken as strong evidence that MMA production is a result of—or somehow enabled by microbiome activity. However, it is not clear whether these minor amounts of MMA would have biologically meaningful consequences in an arsenic-exposed host. In consideration of this, it is worth noting that gut transit takes substantially longer in people than in mice (*194*). Thus, a relatively small microbiome effect in mice could be amplified in humans due to this prolonged gut residency time. Regardless of its origin, the presence of DMA^V in all treatment groups makes it unclear whether MMA is produced by the methylation of iAs, or the degradation/demethylation of DMA^V. Both methylation and demethylation

have been demonstrated in and animal exposure models (*195*) and as a result of microbial metabolism *in vitro* (*105, 196*).

Fecal arsenic is rarely measured in studies characterizing systemic metabolism or elimination of arsenic. This study shows that nearly 10% of single gavaged dose of iAs^V can be eliminated over 24 hours via the stool in conventional As3mt-KO mice, and that fecal elimination is microbiome-dependent. While this methylation-deficient mouse line has well documented deficiencies in overall metabolism and excretion rates, this finding alone justifies further research into microbial interactions with arsenic in the gut related to fecal elimination. Further experiments incorporating functional gene complementation in bacterial strains gnotobiotically associated with GF As3mt-KO mice could be used to help to shore up current knowledge gaps regarding *in vivo* reduction, methylation, or demethylation of ingested arsenic by the microbiome, broadening the depth of understanding around of arsenic-microbiome interactions occurring in the gut.

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CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

These research projects were focused on identifying and addressing knowledge gaps to help define causal relationships between microbiome activity and arsenic toxicity or other host phenotypes. There is some emerging evidence in human populations of a link between microbiome variation and arsenic-related outcomes. For example, microbial taxa in the stool were associated with interactive effects between arsenic exposure and markers for cardiovascular disease in a Bangladeshi population (*197*). Such studies are still rare in the literature, and need further verification. Largely using a top-down approach, the experiments in this dissertation were designed to establish high-level causal relationships between microbiome status and arsenic toxicity (mortality) or metabolism. Because these findings were based on small experimental numbers, high exposures to arsenic, and broad host outcomes, more work will be needed to confidently determine the generalizability and relevance to human exposures. However, these findings do demonstrate a direct relationship between microbiome status and toxic outcomes from arsenic, providing strong justification for future investigations in this field.

Evidence reported in chapter 3 shows that microbiome status directly influences host survival phenotypes in As3mt-KO mice, and that human

microbiota have similar properties in this mortality model of arsenic exposure. Donor-dependent variation in survival time also supports a key part of the underlying hypotheses for this research project—that interindividual microbiome variation contributes to health outcomes from arsenic exposure. Chapter 3 also demonstrates that this experimental model is amenable to gnotobiotic experimentation, and that relatively small differences in survival can be detected at lower exposure levels. It is important to understand that these experiments were designed to test for a causal relationship between the gut microbiome/microbiota and toxic response to arsenic in the host. An interesting follow-up would be to successively add representative members to gnotobiotic communities, to determine if a point of protection can be reached that is equivalent to a complex (undefined) microbiome community. This approach could also be used to characterize the relative influence of specific strains or species of gut bacteria.

The results in chapter 4 demonstrate that arsenic uptake, excretion, and overall metabolism are mediated by microbiome activity in As3mt-KO mice. There was strong evidence of a microbiome effect in the relative elimination routes of arsenic, with fecal excretion depleted by germ free status and antibiotic disruption. The strongest evidence of a microbial influence on metabolism was in the relative percentages of inorganic arsenicals in stool, where germ free status favored a greater amount of arsenate. Interestingly, arsenate should have greater

affinity for organic matter in the gut, such as digested food matter (*198*). Despite this, the overall level of arsenic in germ free stool was reduced. This suggests that the overall presence of bacteria in the gut may be more important than specific metabolic activity, with regard to fecal elimination.

Together, these two studies strongly support the hypothesis that microbiome function has an overall protective effect during arsenic exposure, and that the microbiome augments fecal elimination of arsenic, which may be an overlooked factor in many exposure assessments. While top-down experimental approaches to studying the microbiome (e.g. antibiotic perturbation, humanization of germ free animals) support stronger inferential conclusions at the whole community level, they often lack the experimental control necessary to identify specific organisms or molecular mechanisms responsible for outcomes with any degree certainty. Given this trade-off, my own research findings should be complemented with more controlled, bottom-up experimental designs. One such approach is the gnotobiotic colonization of germ free mice, but this approach is not without limitations. Laboratory strains of bacteria like *E. coli K12* are often well characterized and amenable to functional genetic manipulations. Cloning genes from wild organisms into *E. coli* or other experimental organisms is a tried and true approach characterizing gene function in organisms that are difficult to culture or manipulate. However, it is important to account for other factors when applying this approach to microbiome function and arsenic metabolism. For

instance, gene function may be evolutionarily adapted to a specific environment, and thus less functionally active in the gut. Similarly, while some functions may be mediated by a single gene or small cluster of genes, metabolic pathways involving arsenic often rely on specific cellular pathways, like glutathione or thioredoxin, for functionality. Animal research is time and resource intensive compared to most benchwork; thus care should be taken to ensure constructed bacterial strains reproduce the desired functions in culture experiments prior to proceeding with animal experimentation. Also, there is evidence to suggest that Gram-negative and Gram-positive bacteria interact differently with arsenic. Because native microbiome communities are complex and robust with both Gram positive and negative organisms, consideration should be given the application of similar methods using a variety of different model bacterial organisms.

Another approach to gnotobiotic experimentation is the use of defined consortia. Even a small consortium can more completely normalize the physiochemical conditions of the germ free gut environment to more closely approximate the gut of conventional mice. Some defined microbial consortia can stably colonize mice even across generational timelines. Such a system would allow for a similar degree of experimental control, while also yielding more robust experimental results. One drawback would be the additional upkeep needed to maintain such conditions over time. Additionally, some conclusions may also be less mechanistically defined when working in a community culture, compared to a

single organism, as the number of metabolic interactions increases exponentially each additional community member in a microbial ecosystem. While mechanistic certainty would require more robust controls in such a system, single-gene or single-organism effects could also be more broadly interpreted and generalizable. I hope that these considerations will help guide my fellow researchers towards robust and insightful findings on path of knowledge.

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