Lead (Pb), the Gut Microbiota, and Colonization by Antibiotic Resistant Bacteria (ARB).

By

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Abstract

Imbalance of good and bad bacteria within the gut microbiota has been associated with many adverse health outcomes including infection. Antibiotic resistant bacteria (ARB) infections are a major cause of global morbidity and mortality and effective treatment options are disappearing as antibiotic resistance proliferates. Avoiding imbalance of the gut microbiota may help prevent colonization and subsequent infection by ARB and other pathogens. Environmental lead (Pb) exposure can change gut microbial composition in animal models, potentially leading to an unhealthy imbalance. Pb exposure can also select for bacterial resistance to antibiotics, and reduce immune function in humans. All of these mechanisms suggest that exposure to Pb may increase risk of ARB infection. This study investigates the association between Pb exposure, and water filter use as a potential exposure intervention, and gut microbial composition. Furthermore, it examines the potential mediating effect of the gut microbiota on the association between Pb exposure and ARB colonization. This study uses survey data and biological samples including stored urine and stool samples collected by the ongoing Survey of the Health of Wisconsin and its ancillary microbiome study. This complex relationship is examined using a combination of molecular sequencing techniques and culture based microbiological methods, within a cross-sectional epidemiologic study framework. Data analysis consists of various established and relatively novel analytical approaches. Evidence from this study suggests that Pb and water filter use alter the composition of the gut microbiota, and Pb may be associated with ARB colonization in some subsets of the population. These findings support the need for further investigation of theses associations, and could be used to support policy and practice to prevent gut microbial dysbiosis and ARB colonization and infection.

Chapter 1. Introduction

1.1. OVERVIEW

Having a balanced gut microbiota is crucial to maintaining health, as the gut microbiome has been shown to play an important role in metabolism, nutrition, immune function, and nervous system signaling.[1] Given its association with these varying biological mechanisms, imbalance, or dysbiosis, of the gut microbiota has been linked with many adverse health effects including infection, obesity, diabetes, inflammatory bowel disease, allergic disease, and mental health conditions.[2] There is no single definition of a healthy gut microbiota, however, typically, the more diverse the microbiota, the better, especially in the case of infection.[1,3] Research is just beginning to identify signatures of a "healthy" vs. "un-healthy" gut microbiota, and many questions remain, particularly with respect to what level of diversity and which clusters of bacteria are most protective and necessary for maintaining health. Further, the role that external factors, including diet and environmental toxicants (xenobiotics), play in altering these organisms and increasing risk of disease needs further examination.

Although many have postulated that the environment and geography may affect the composition of the gut microbiota, research into the mechanisms by which these factors influence the gut microbiota is in its infancy. Xenobiotics can influence gut microbiota in several ways. They are potentially toxic to the bacteria in our gut, and can cause dysbiosis by altering the composition of the gut microbiota through several mechanisms.[4] Likewise, the bacteria in the gut can change the metabolism of xenobiotics and potentially mediate the toxic effect of an internal dose on the body. [4] Lead (Pb) is one such xenobiotic. It is a pervasive environmental contaminant that plays no known necessary biological function for humans and most bacteria.[5] Pb has been causally linked to myriad detrimental health effects in children and adults including altered brain development and reduced immune function. While Pb has been a known toxicant for centuries, it is only recently that regulators and scientists agree there is no “safe” threshold of Pb exposure.[6] Pb also plays a toxic role for many microbes, thus when bacterial communities, including the gut microbiota, are exposed there is potential to cause a significant shift in the bacterial community composition.[7–9] In the environment, Pb has been shown to alter microbial communities of water and soil.[10,11] Animal studies have shown shifts in gut microbiomes change over time and these shifts have impact on important inflammatory and immune metabolic pathways.[12–15] Only one study in children has explore adverse effects of Pb exposure and child gut microbiome and found significant differences in abundance of *Succinivibrionaceae* and *Gammaproteobacteria.*[9] Despite this emerging evidence, few empirical and observational studies exist and no study to date has examined the relationship between lead exposure and the microbiota of adults.

A healthy and diverse microbiota has been linked to reduced risk for infections.[3,16–18] Of greatest concern are antibiotic resistant bacteria (ARB), many of which are not susceptible to most forms of existing antibiotic treatment. In the United States infections by ARB cause more than 23,000 deaths annually.[19] Bacterial genes for metal resistance and antibiotic resistance are often linked.[20] Environmental and animal studies show that exposing bacteria to Pb leads to increased prevalence of antibiotic resistant bacteria.[21–24] Pb’s ability to co-select for antibiotic resistance in bacteria, disrupt the gut microbiota, and reduce immune function, make it highly plausible that Pb exposure leads to increased risk of ARB infection, yet few studies have been done to examine this association in humans.

Drinking water is an important possible source of both Pb exposure and bacteria with potential antibiotic resistance. Both groundwater and surface water can be contaminated with Pb and bacteria. Although municipal water sources are treated before distribution, there is not regulation for monitoring and treatment of private wells. Well owners are responsible for the maintenance of their own water quality, yet most do not test their water regularly.[25] However, even for those whose water has been treated, Pb and microbes from the distribution system can contaminate water before it reaches the home.[26,27] Household water filtration is a modifiable approach that has the potential to alter water quality, reducing risk of exposure to both ARB and heavy metals. This change in xenobiotic and biotic exposure likely affects composition of the human microbiota, although this association has yet to be examine.

The overarching goal of this dissertation is to gain further insight into the relationships between environmental Pb exposure, household water treatment, and the gut microbiota, including colonization by ARB. Understanding these relationships in a real world setting sets a foundation for further examination of Pb and other environmental exposures in relation to the gut microbiota and disease, and may lead to clinical and policy recommendations for preventing microbial dysbiosis and associated health outcomes.

1.2. LITERATURE REVIEW

1.2.1. The Human Microbiota

The study of the many microorganisms that colonize the human body has been going on for many years. In the past, research was limited to the microbes that could be cultured and isolated in the lab. Now there are molecular methods, including fluorescence in situ hybridization (FISH) and rapid metagenomic sequencing that allow us to get a more accurate picture of all of the organisms in a given sample. Since the advent of these techniques, there has been a boom in research characterizing the microbiome and its effect on human health.[28–30]

Trillions of microorganisms colonize the human body, and we have varying forms of relationships with these microbes. Most of our microbial colonizers are symbiotic or commensal organisms, meaning that we play either mutually beneficial roles, or we benefit the bacteria and they cause us no harm or benefit.[31] When there is a disruption in the normal balance of our microbiota, or dysbiosis, some of our normally commensal bacteria can grow unchecked and lead to an opportunistic infection. Other bacteria, known as pathogens, cause infection whenever they reach a certain bacterial load. Having a balanced group of microbiota can help prevent infection by opportunists and pathogens.

Most of what we know about the healthy human microbiota comes from two large-scale microbiota studies, the U.S. based Human Microbiome Project (HMP) funded by the NIH[32] and the Metagenomics of the Human Intestinal Tract project (MetaHIT) a consortium based project by the European Union.[33] Results from the HMP and other microbiome studies show that the dominant phyla in adults are Bacteroidetes and Firmicutes,[34] however, there is no single species of bacteria found in all humans.[1,29] Given the vast intra-individual diversity of microorganisms even within healthy individuals, there is no consensus on exactly what a healthy microbiome should contain, which can make comparisons of microbiome composition challenging.

There are many different ways of characterizing microbiota diversity, most of which come from the field of ecology. Diversity is a combination of richness, the number of different species or operational taxonomic units (OTUs), and abundance, the number of organisms present within each OTU. Once bacterial samples have been collected and the bacteria present have been identified, microbiota studies typically examine the α-diversity, or the amount of diversity within an individual ecosystem, or in most cases an individual’s gastrointestinal (GI) tract. β-diversity, or the difference in diversity of bacterial taxa between groups, is another measure of diversity commonly used when comparing different groups of exposures or outcomes. Another way of looking at diversity is to look at the presence or absence, and abundance of specific bacterial taxa, with known biological processes.[35,36]

Many different factors, some intrinsic and some modifiable, are known to influence the composition of the human gut microbiota. Humans are first colonized at birth via the birth canal, and the ecology of the gut is primarily established within the first three years of life, in which the diversity greatly increases with time.[37,38] After the age of 3, the level of diversity stays relatively constant, however, individual species and their relative abundances may change due to other factors.[37] Gender appears to play a part in microbiota composition, as well as shaping the role of the microbiota in downstream health effects.[39,40] The link between genetics and the microbiome is an ongoing area of investigation, but studies suggest that genetics and environment both play a key role in defining the gut microbiota.[41–44]

Some modifiable determinants of gut microbiota composition include birth mode, direct manipulation, diet, environment, smoking, cohabitation, and animal contact. Some of these factors show more acute effects, while some lead to long term shifts. Birth mode is a key early determinant in microbiota composition and its effects tend to last throughout the life-course. Babies born via Cesarean section generally have less gut microbial diversity, the predominant microbes being *Staphylococcus* and *Corynebacterium*, typically found on the skin, and show delayed gut colonization by Bacteroidetes, a typically predominant species in the human gut.[38,45] Forms of direct manipulation include the use of antibiotics, probiotics and prebiotics. These interventions tend to have acute effects, but can lead to long-term effects if exposure is chronic or repeated.[46,47] Antibiotics that are used to treat infections can also cause widespread reductions in commensal (mutualistic or beneficial) bacteria. Probiotics composed of beneficial bacteria, typically *Lactobacillus* and *Bifidobacterium*, can be taken alone or in combination with prebiotics, poorly digested complex carbohydrates, to directly promote diversity and colonization of health-promoting bacteria within the gut.[48] The nuances of dietary impacts are still being examined, however, several studies have shown the fiber and fat content of the diet can have especially dramatic effects on the microbiota.[39,49–56] The environment affecting gut microbiota include a wide range of environmental contaminants, as well as aspects of the built environment that dictate what surfaces and bacteria we come in contact with on a daily basis.[57–59] Diet and environmental effects can be acute if the exposure is acute, but prolonged and repeated exposures tend to have lasting effects. However, early exposures to dietary and environmental exposures while the gut microbiota is first being established, can have lasting effects even without prolonged exposure.[8,30] There are marked differences in the respiratory and gut microbiota of smokers and non-smokers, and cessation causes drastic changes as well.[60,61] The gut microbiota composition is also closely related to those of cohabitating family members and domestic animals.[62]

1.2.2. The Gut Microbiota and Health

The gut microbiota takes on a host of functions within human health, and microbial dysbiosis has been implicated in many acute and chronic health conditions. Our microbes mediate digestion of food and other chemicals that enter our system.[63] They create a variety of metabolites that affect processes within the gut and throughout the body, and can biotransform xenobiotics, including pharmaceuticals, into non-bioavailable forms.[64,65] The microbiota are so involved in the interface with xenobiotics in our bodies, that experts are starting to consider the microbiota as a biomarker between xenobiotic exposure and health outcome.[66] They can also affect energy harvest by changing the rate at which our cells store fat.[67] Our microbiota also helps prevent colonization and infection by pathogens and opportunistic commensals. One mechanism for this is competitive inhibition, whereby the commensal microbes compete for the same resources and mucosal binding sites as the infectious bacteria and limit their growth.[68] Another mechanism is that growth of commensal bacteria along the mucosal lining of the GI tract helps to strengthen the epithelial barriers by promoting certain growth factors in the actin filaments that increase the strength of the tight junctions between epithelial cells. This strengthening helps reduce the ability of pathogenic bacteria to enter our cells and blood stream and cause infection.[69]

Our microbiota play a large role in the development of the immune system, and continue to interact with the immune system to maintain homeostasis throughout our lives.[70–72] Having a healthy microbiota throughout the life-course helps the gut immune cells to develop differently from other immune cells. By maintaining a health bacterial load within the gut, the immune cells in the gut are essentially desensitized, having fewer receptors, making them less sensitive to the presence of microbes, and producing fewer pro-inflammatory cytokines when stimulated, helping to reduce inflammation throughout the life-course.[72,73] Beneficial bacteria within the microbiota produce cytokines, short and long chain fatty acids, and other signaling molecules that affect mucus production, and epithelial barriers, as well as increasing Type 1 T helper cell (Th1) response.[70–72,74] This increase in Th1 response leads to increased destruction of pathogenic bacteria via increased Interferon gamma (IFN-γ) production and increased lysozyme and major histocompatibility complex class II (MHCII) expression.[70]

In summary, the gut plays an important role in health, while much of the gut microbiota is established early in life, exposures throughout the lifecourse can alter gut composition over time, therefore, it is an important area of ongoing investigation both for reducing disease burden as well as identifying potential treatments.

1.2.3. Environmental Metal Exposure

People living and working in different spaces are exposed to different levels of environmental toxicants, and the study of those environmental exposures and disease has been on-going for hundreds of years. However, the formal establishment of the US EPA and several regulatory and policy decisions, including removal of lead from gasoline have shown dramatic decline in overall burden of environmental exposures and efficacy of environmental policies over time. [75,76] Sources of environmental hazards are very diverse and can vary on large (city or state) to small scales (housing, neighborhood, etc.) leading to within and between individual variability in exposure based on daily living and lifestyle patterns. Heavy metal exposure has been of particular importance and some of the first classes of chemicals examined, including mercury and Pb, were both found to be neurotoxic in early examination of occupational hazards. Heavy metal exposure is known to vary differentially due to industrial activity, governmental regulations, and home and consumer practices.

Pb is among the many heavy metals that are naturally occurring elements in the environment. Some natural phenomena like volcanic eruption and soil erosion can lead to higher metal exposure levels than normal. Human activity has caused higher levels of exposure, due to activities like mining, and the use of metals in industry, agriculture, and technology.[77] Some metals are essential nutrients for biological processes; however, many metals are considered non-essential, as they serve no biologically useful purpose. Non-essential metals can cause a wide variety of health effects due to their varying biological mechanisms.[6,77] Some of the adverse health outcomes include developmental effects, cancer, cardiovascular disease, diabetes, neurotoxicity, and intellectual disability.[78,79] Literature is just starting to be published on the effects of metals on the microbiome, however, early animal and epidemiological studies show changes in microbial composition caused by several metals including arsenic, mercury, and Pb.[7–9,12–15,80–84]

1.2.4. Environmental Pb

Like many other heavy metals, Pb is a naturally occurring element but human activity has greatly increased the level of human exposure.[6] Pb in the environment comes from many sources including mining for Pb and other metals, and industrial plants that use or manufacture Pb, Pb compounds, and Pb alloys. Pb is often used in the production of batteries, many of which end up in landfills. Pb was often used in gasoline between 1920 and 1975, until the introduction of unleaded gasoline, although Pb was not banned for use in gasoline by the EPA until 1996, the burning of which was the main source of lead in the environment during that time period, with Pb concentrations in soil particularly high near roadways.[85] Pb is still used as a component in many brake pads, which add to the accumulation of Pb in soil near roadways. Pb can also be released into the air from combustion of oil, charcoal, and other waste.[6] Pb concentration in air is still used as a metric of air quality as part of the National Ambient Air Quality Standards.[86] Pesticides used before 1950 on many orchards also contained Pb.[6] Pb has been an important element for building the nation’s water infrastructure and many of our municipal water supplies have service lines throughout their distribution systems.[87] Before its ban in 1978 by the Consumer Protection and Safety Commission, [88] Pb was used in paints and pigments, many of which are still a major source of Pb exposure today, particularly for children. Pb from these various sources ends up in soil by falling from the air in rain, chipping in paint from buildings, roadway emissions, or direct contamination from waste and industrial sources. Pb binds strongly to soil particles and remains for many years. Groundwater can also be contaminated by small amounts of Pb from soil, urban runoff, and industrial waste.[6]

1.2.5. Pb and Health

The health effects of Pb exposure are known to be greater in children because exposure to Pb is often higher and its effects within the body are more severe in children than adults. Pb is more readily absorbed into the bloodstream of children than adults, and exposure to Pb is higher per body weight for children. Children can be exposed in utero during pregnancy and, after birth, are also more likely to be exposed through soil and dust because they more frequently play on the ground and put foreign objects in their mouths.[6,89] Once absorbed into the blood stream, Pb can pass through the blood-brain-barrier of children more easily than in adults, particularly in children 5 years old and younger, having more detrimental effects on a still developing brain.[90]

While Pb is often considered a children's environmental health issue, Pb exposure can also contribute to a wide array of health effects for both children and adults.[6] Adverse health effects of Pb in children include anemia, kidney damage, muscle weakness, brain damage, colic, stunted mental and physical development, and death. Effects of Pb in adults are similar, including neurotoxicity, reduced kidney function, anemia, joint weakness, hypertension, reduced immune function, and reproductive complications including miscarriage and reduced sperm production.[6] The main treatment for Pb exposure is to eliminate the source of exposure, however, if Pb levels are high enough it can be treated to reduce the biological load through chelation and ethylenediaminetetraacetic acid (EDTA) therapies.[6] While limiting Pb in the body helps to reduce the adverse effects of Pb, not all effects can be reversed upon treatment.[6]

Pb's neurotoxicity is primarily based upon its ability to substitute for calcium in the body.[90] It uses this ability to pass through the blood-brain-barrier via Ca^{2+} pumps. Once in the brain, Pb alters calcium homeostasis within cells, often impairing mitochondrial function and resulting in cell death.[90] Pb also substitutes for calcium ions in activating second messengers, damaging potassium channels, and reducing the functionality of Protein Kinase C, which affects neuronal proliferation, differentiation, disrupts cellular functions involved in learning and long-term memory, and reduces synaptic ability. Pb also disrupts the dopamine system in the brain, can cause anemia, and damage the blood-brain-barrier which can lead to swelling and ischemia.[90]

One of the subtler health effects of Pb, is reduced immune function, which can happen even at low levels of Pb exposure.[91] Pb has been shown to affect almost every aspect of immune function, however, certain immune effects are considered trademarks of Pb exposure. The most prominent of these is a shift toward Type 2 T helper Cell (Th2) response and increased Interleukin-4 secretion, reducing the Th1 response, the primary mechanism for fighting bacteria and viruses. Other distinct changes in immune response include increased IgE production (autoimmune antibodies), suppression of Delayed Type Hypersensitivity, reduced resistance functions of macrophages, including nitric oxide production and new macrophage generation, and increases in pro-inflammatory cytokines and reactive oxygen intermediates.[91] All of these changes in immune function reduce the body's ability to fight infection, and epidemiologic studies using data from the National Health and Nutrition Examination Survey (NHANES) show a significant association between Pb exposure and colonization by methicillin resistant

Staphylococcus aureus (MRSA),[92] as well as several chronic infections including *Helicobacter pylori* (which can be antibiotic resistant), *Toxoplasma gondii*, and Hepatitis B virus (HBV).[93]

1.2.6. Antibiotic Resistant Bacteria

Antibiotic resistance is an international health crisis. Infections by ARB are becoming increasingly common, and effective treatment options are decreasing rapidly. Infections by these organisms are often very serious, leading to increased medical care usage and even death. Lacking effective treatment options for these infections also endangers the outcomes of other medical treatments including surgery and cancer treatment.[94] ARB are often transmitted in health care settings, but can also be acquired through the community.[95–97] Not all ARB are pathogenic, however, asymptomatic colonization by an ARB can be a strong predictor of future infection.[98–101]

Vancomycin-Resistant *Enterococci* (VRE), Multi-Drug-Resistant Gram Negative Bacilli (RGNB), and MRSA are three types of ARB with the capacity to cause seriously detrimental health effects.[19,102] [102101]*Enterococci* are Gram positive cocci that are normally commensal, often found in the GI tract, the female genital tract, and in the environment. Types of *Enterococci* that can become resistant to vancomycin include *Enterococcus (E.) faecalis*, *E. faecium*, and others. VRE often causes infections associated with hospitalization, including urinary, bloodstream, catheter and surgical wound infections.[103] RGNB are defined as Gram-negative rods, resistant to one or more antibiotics. Bacteria that can become RGNB include *Klebsiella*, *Acinetobacter*, *Pseudomonas aeruginosa*, *Escherichia coli*, and others. RGNB can cause pneumonia, sepsis, meningitis, and surgical site infections.[104] *Staphylococcus* (*S.*) *aureus* is carried by approximately 30% of the U.S. population, while MRSA is carried by about 1%.[105–107] *S. aureus* carriage can be commensal, but can also cause opportunistic infections. MRSA is a Gram-positive cocci that is often resistant to more than just methicillin.[24]

1.2.7. Pb and Bacteria

Heavy metals primarily affect bacteria by entering through essential metal transport proteins and denaturing proteins and nucleic acids within the cell.[5,108] Lead [Pb(II)] specifically affects microbes by altering nucleic acids and proteins, hindering enzyme activity, inhibiting membrane functions, and changing osmotic balance.[5] All of these effects can be toxic to bacteria and have the potential to change the microbial ecology within an ecosystem. Studies in animal models have shown changes in gut microbial composition with increased Pb exposure,[7,8,12–15] and one study in a small sample of human children found significant association between high blood Pb levels and presence of specific bacteria (*Succinivibrionaceae* and *Gammaproteobacteria*).[9]

There is evidence suggesting that metal exposure is not only changing the composition of the gut microbiota, but leading to increased metal- and antibiotic-resistance as well.[20–24,82,109–114] Metal exposure kills susceptible bacteria, leaving metal-resistant bacteria behind. There are several mechanisms by which microbes can resist the toxic effects of Pb. Bacteria can use components of their outer cell wall to bind extracellular Pb and prevent it from entering the cell. They can precipitate Pb into phosphate salts in and outside the cell, and bind Pb using proteins making it unavailable for disrupting cellular mechanisms. Bacteria can also biotransform Pb into volatile forms, or most effectively, efflux Pb to the extracellular environment.[5] Many bacteria that are resistant to metal are also resistant to antibiotics via several mechanisms. Co-resistance happens when microbial metal-resistance and antibiotic-resistance genes are physically linked on a genetic unit such as a plasmid. Cross-resistance occurs where one microbial gene produces a set of proteins that confers resistance to both metals and antibiotics. Co-regulatory resistance is when separate metal-resistance and antibiotic-resistance genes are linked by transcriptional regulation, whereby a stimulus for either gene leads to transcription of both.[20] In-vitro and animal studies have found metal and antibiotic resistance to be linked to each other, and associated with Pb exposure.[21–24]

Many different bacterial species have been identified as having Pb resistance, including *Staphylococcus* sp., *Bacillus cereus*, *Bacillus megaterium*, *Arthrobacter* sp., *Corynebacterium* sp., *Pseudomonas marginalis*, *Pseudomonas vesicularis*, *Enterobacter* sp, and *Alcaligenes* sp, however, not every individual within each species carries Pb resistance. Metal and antibiotic resistance genes occur naturally in many bacteria, but over time without a stimulus to maintain them, these genes can be lost. Introducing metal to the environment gives a selective advantage to maintaining and proliferating these

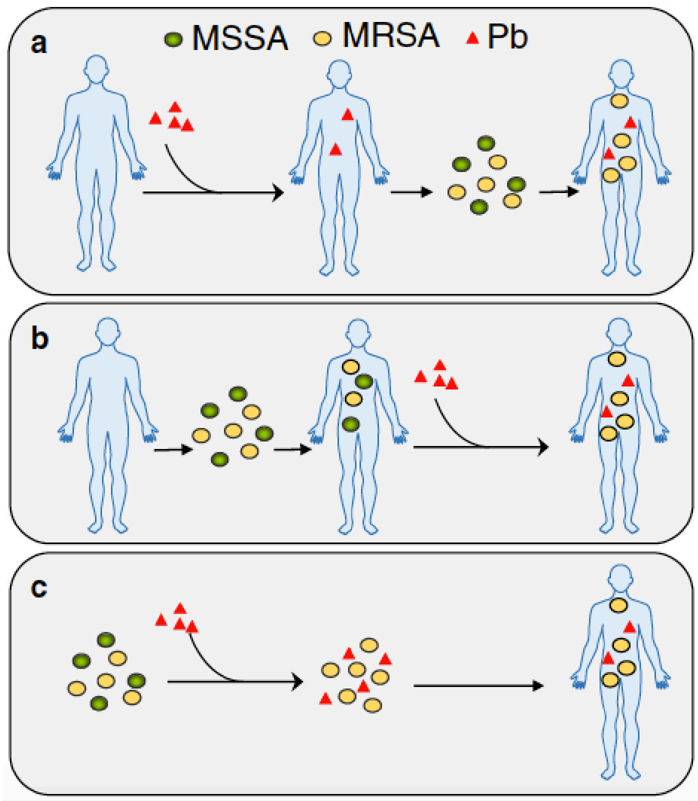

Figure 1.1. (Taken from previous publication[92])The natural history of Pb exposure and selection for antibiotic resistance in *Staphylococcus aureus* that colonize individuals could work in several ways. a. The individual is exposed to Pb first, and is then exposed to MRSA and MSSA. The Pb prevents colonization by MSSA, but not MRSA. b. The individual is colonized by MRSA and/or MSSA first, and is then exposed to Pb. The Pb selects for antibiotic resistance by killing MSSA and leaving MRSA behind. C. MRSA and/or MSSA is exposed to Pb in the environment. The Pb exposure selects for antibiotic resistance in the environment. The individual is then exposed to both MRSA and Pb from the same source.

resistance genes. The genes are then transferred to the next generation of bacteria through cell replication, but they can also be transferred horizontally to otherwise susceptible bacteria. Mechanisms of horizontal gene transfer include conjugation – transfer of a plasmid from cell to cell, transformation – incorporating foreign DNA taken up from the environment, and transduction – transfer of DNA via bacteriophage. These mechanisms of gene transfer can happen in the environment or *in vivo*. Therefore, the natural history of colonization by ARB and Pb exposure may work in multiple ways. The first mechanism could be that a person is exposed to Pb and then later exposed to an ARB (and other antibiotic susceptible bacteria) (Figure 1.1.a). In that case the Pb could prevent colonization by the susceptible bacteria but not the ARB. Alternatively, a person may be exposed to an ARB first, and then to Pb (Figure 1.1.b); the Pb would then reduce the number of susceptible bacteria, and select for the ARB's proliferation. A third scenario is that the ARB is exposed to Pb in the environment, where it selects for resistance, and later the individual is exposed to both the Pb and ARB through the same source (Figure 1.1.c).[92]

1.2.8 Water Quality and Treatment

For those using municipal water sources, some of which use groundwater as their source water, and some use surface water (lakes and rivers), the water treatment process typically removes Pb from these sources, however, outdated municipal water infrastructures often use Pb pipes and solder, which can contaminate the water after the decontamination process, particularly when the water is acidic or "soft."[6] In the case of Flint, Michigan, the overall change in acidic levels of the water led to higher levels of corrosiveness in the water and lead to chemical interactions within the distribution system that released increased iron and Pb into the water supply post-treatment. Further, the chlorine used to initially treat for bacteria was no longer effective because it interacted with iron in the water making the water toxic from both chemical contamination and high levels of bacteria.

While the introduction of Pb into the environment has been greatly reduced in the US since the regulations on its use in paint and gasoline, Pb in the environment persists. Due to the extensive use of Pb based paints in housing, roadways, and industrial sites, states like Wisconsin, in the Midwestern and Northern regions of the US, are at higher risk of Pb exposure than other regions.[115] The recent Pb contamination of water in Flint, Michigan has drawn attention to the outdated municipal water infrastructure still used throughout the country, including in Milwaukee, Wisconsin.[116] The municipal water delivery system in Milwaukee is made of three main components, the water main and the service line up to the curb stop, which are owned by the city, and the service line from the curb stop to the housing unit, which is owned by the property owner.[117] The water main does not contain Pb, but pipes and solder used in the service lines may contain Pb. The map shown in Figure 1.2, displays each residence using municipally owned Pb service lines, however, there may be more residences not displayed here that are using Pb in the privately owned sections of service line.[117] The use of Pb in the water infrastructure and its extensive use in housing in the Milwaukee and Racine areas resulted in recommendations from the Wisconsin Department of Health to screen all children for Pb poisoning in Milwaukee and Racine three times before the age of three.[118] This screening and surveillance effort has shown a steady decrease in childhood Pb poisoning since 1996.[118]

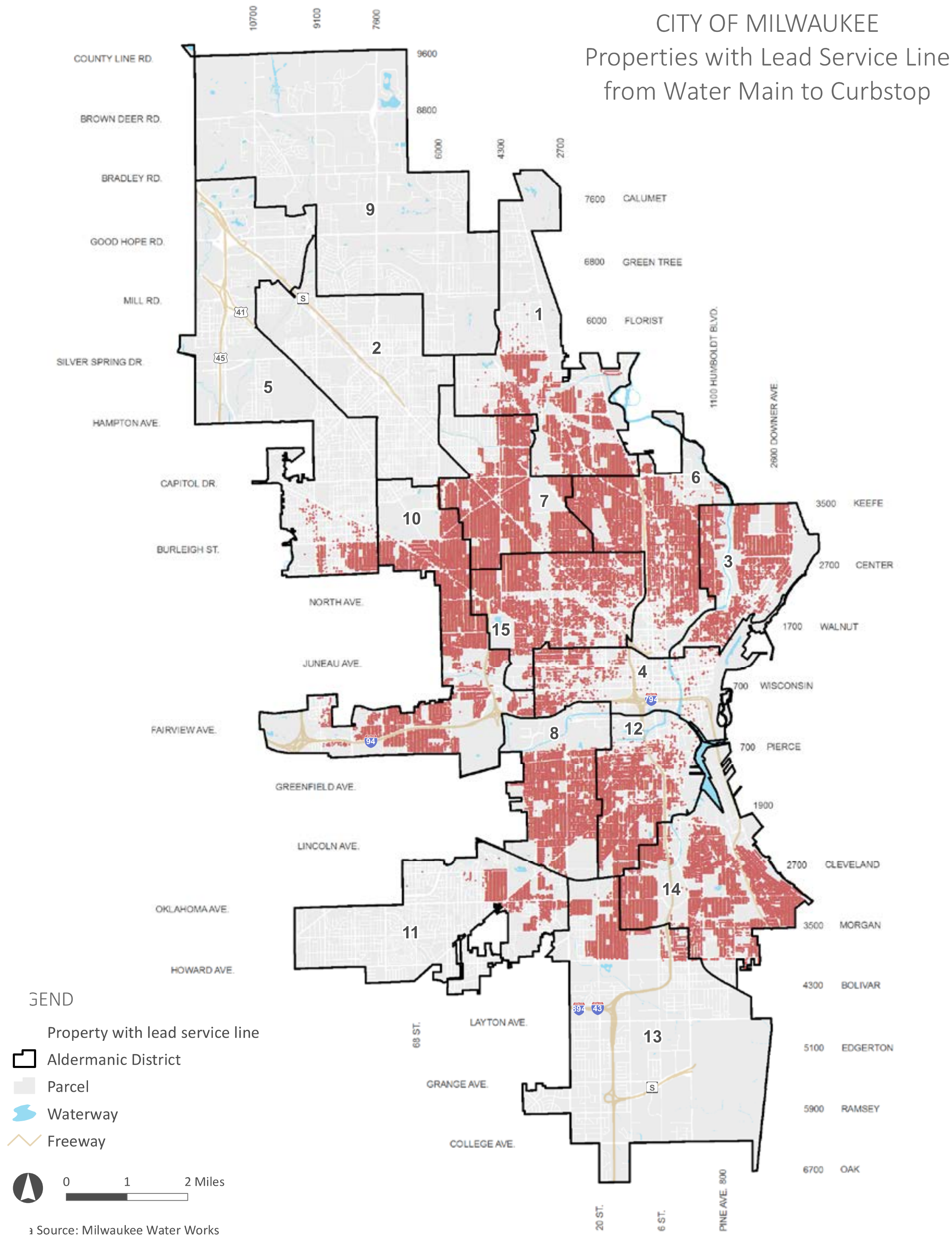

Figure 1.2. Map of Pb service lines owned by the Milwaukee municipal water supply.[117]

While residents using the water distribution infrastructure in Milwaukee are at greatest known risk of Pb contamination, private well owners in Wisconsin are not free of risk. Private wells also use plumbing lines to transport water from the well to the house, and older homes may still be using Pb pipes and solder. A large portion of the Wisconsin population depends on groundwater as its primary water source, particularly rural areas served mostly by private wells. Water monitoring is conducted regularly by municipal water supplies, however, similar monitoring does not occur in private wells. Because there are no federal regulations about private well monitoring, the responsibility of well stewardship falls to the owners. Samples of drinking water from Wisconsin have been found to be contaminated with nitrate, coliform bacteria (a marker of fecal contamination), and heavy metals, and of 4,000 private rural water samples, 11% had elevated levels of several metals including Pb.[119] Furthermore, results from the Survey of the Health of Wisconsin (SHOW), indicate that most private well owners in Wisconsin report not testing their water, and most who treat their water use only a water softener,[25] which may actually increase Pb contamination if Pb pipes or solder are used in the plumbing between the water softener and the taps.

While it is hard to fully characterize water quality, use of a water filter can alter water quality and can eliminate both metals and bacteria depending on the type of filter.[120] After the water system treatment failure in Flint, Michigan in 2015, residents turned to bottled water and household water filters as a temporary solution to reduce Pb exposure.[116] Different types of water filters and treatment systems are effective for treating different contaminants, and the most effective treatment methods for removing Pb are distillation, reverse osmosis, and certified carbon filters.[120] Household water filters and other treatment systems can either be placed at the point of entry into the household, or at the point of use, such as attaching a filter to the kitchen tap.[120] Because household water treatment can reduce levels of xenobiotics, including Pb, as well as microbes, their effects on the human microbiota are likely multi-faceted.

Not only do household treatment options differ fundamentally in function, but, depending on source water (groundwater vs. surface water), including its unique chemical and biological composition, and water source (public vs. private well), including any treatment prior to distribution, and the plumbing used to bring the water into the home, water quality can vary even when using the same household treatment system. Moreover, based on these factors, water quality can differ greatly between individuals even when they live within a relatively small geographical area. Few studies, if any have examined differences in microbiome by water filtration, however, if microbiome is a surrogate or mediating biomarker of exposure to xenobiotics, then it is important to test for associations.[66]

1.2.9. Theory and Conceptual Framework

The purpose of this dissertation is to examine the relationships among Pb exposure, household water treatment, and the gut microbiota, including colonization by ARB. These relationships are hypothesized based upon the conceptual framework illustrated in Figure 3. When humans ingest Pb at levels that are toxic to microbes, some bacteria are killed and some survive. Those that are susceptible are killed, many of which are presumably beneficial commensal bacteria. Reduction of these beneficial bacteria causes dysbiosis, leading to reduced competitive inhibition of pathogens, increased inflammation, and reduced Th1 immune response.[70–73] Those bacteria that survive otherwise toxic levels of Pb exposure are Pb resistant, and likely antibiotic resistant as well.[20,109] This Pb exposure would decrease diversity within the gut microbiota by reducing the abundance, if not eliminating the presence, of some beneficial microbes, and increase the prevalence (relative abundance) of Pb and antibiotic resistant bacteria. It would also select for metal and antibiotic resistance and proliferate these genes to previously susceptible bacteria, which also leads to increased prevalence of resistant bacteria. Pb's ability to reduce Th1 immune response, independent of gut microbial dysbiosis, would also increase the likelihood of colonization and infection by ARB. A directed acyclic graph (DAG) has been constructed (Figure 1.3) to better illustrate the mechanisms and confounding associations of Pb, the gut microbiota, and ARB colonization.

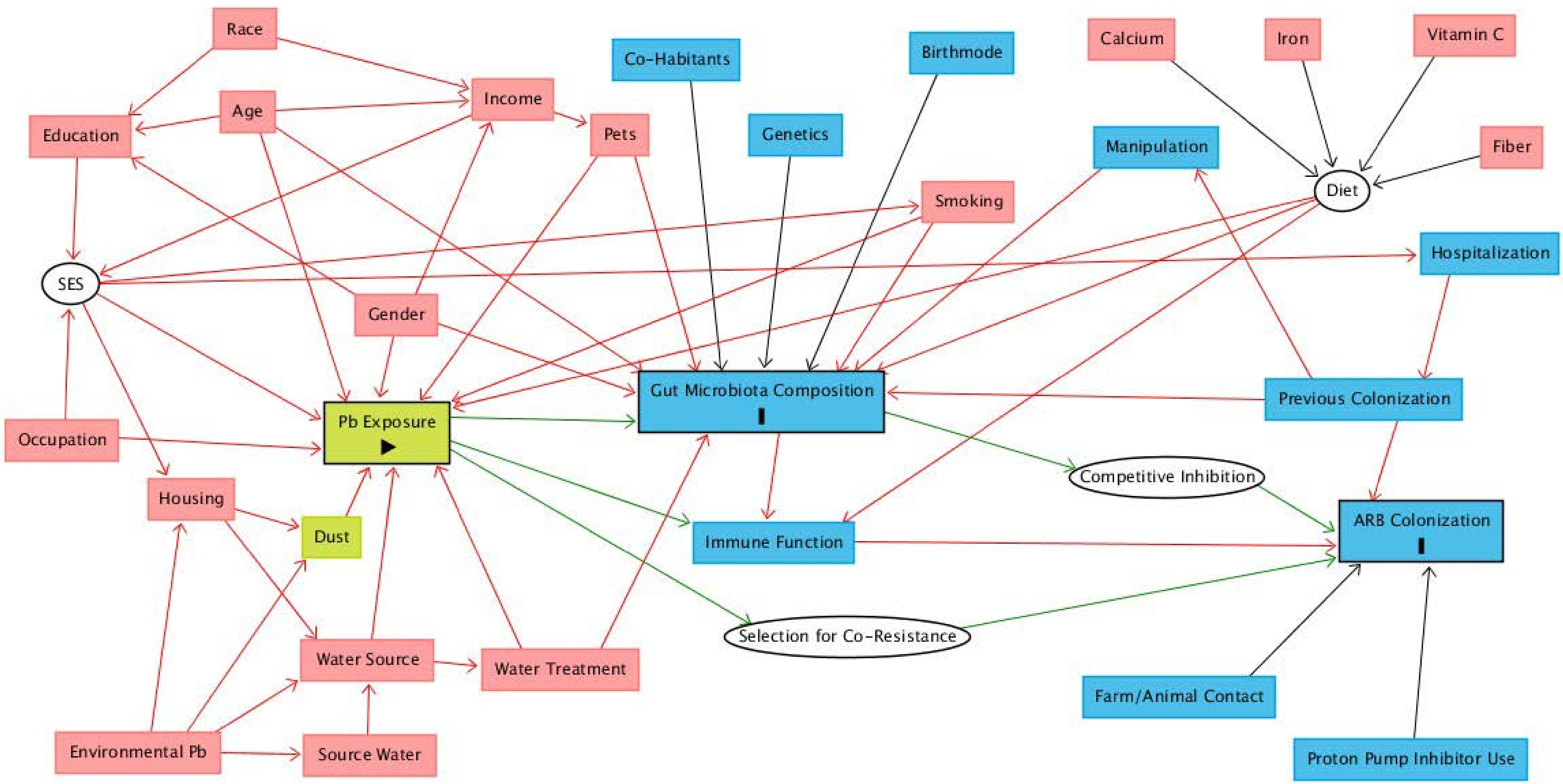

Figure 1.3. Directed acyclic graph of the relationship between Pb, gut microbiota, and ARB colonization.

1.2.10. Research Gap

While studies of the effects of environmental exposures on the gut microbiota are starting to emerge, most use animal models, and the few human epidemiologic studies so far are typically cross sectional and have small sample size. For instance, a study by Bisanz et al that examined the association between gut microbiota and Pb exposure, used a sample of 44 children, with two time points, but appeared to pool the time points for analysis as if they were independent.[9] Moreover, the main purpose of that study was a clinical trial of probiotics to ameliorate the effects of Pb exposure, so the gut microbiota were likely altered by the probiotic exposure. While there have been large scale human microbiome studies, like the HMP which used samples of 15 body sites from 300 subjects,[121] they have not been used to examine associations with environmental factors or specific exposures to chemical pollutants. Studies specifically examining the effects of environmental exposures have examined a wide range of xenobiotics, including pesticides, metals, air pollutants, and polychlorinated biphenols (PCBs), all of which showed compositional changes in gut microbiota, suggesting that this is an important area of further research to determine how the microbiota composition changes, whether the effects are lasting, and to explore downstream health effects in humans.[57]

There is a need for more human studies with larger sample sizes examining a wide range of environmental exposures. Some studies have been published examining associations between geography and the gut microbiota, using samples of between 190 and 400 participants, however, the level of geography examined is typically at the country or continent level, and does not separately account for cultural and ethnic differences that also occur at that level of geography.[37,122,123] While these studies have been helpful in beginning to uncover the role of geography, there are many more granular aspects of geography that may be more modifiable and have impacts on gut microbiology that have yet to be explored. Studies examining many aspects of the built and physical/chemical environment and the gut microbiota in varied populations and settings are critical to our understanding and preventing dysbiosis in the future.

Reviews have highlighted the potential role of the gut microbiota in mediating the toxicity of environmental exposure to xenobiotics, and the recent development of culture-free microbial identification methods makes these more complex investigations possible. The bacteria within the human gut microbiota are capable of metabolizing a wide range of chemical components including xenobiotics. When xenobiotics enter the GI tract, the microbes there may biotransform them into other forms which alter their absorption and metabolism in the body.[4] Although some bacterial Pb resistance mechanisms are known to transform, bind and precipitate Pb, there has not been an examination of the effects of that on human Pb absorption and toxicity. Another review by Wang and Kasper examined the role of the gut microbiota and its effect on the central nervous system, via the gut-brain axis.[124] The microbiota interact with the central nervous system in a number of different ways, and could potentially mediate the neurotoxicity of Pb. Both of these interactions between microbes, xenobiotics, and toxicity could be potential mechanisms of differences in absorption and resulting neurotoxicity between individuals, and are important avenues of future study.

To date, only one epidemiologic study has been conducted (by this research team as preliminary analysis for this dissertation) examining the link between Pb exposure and ARB colonization, although there are several plausible biological mechanisms for this relationship. While studies have been done examining different pieces of these mechanisms, there has been no examination of the potential natural history of Pb and ARB colonization. Animal evidence has found that this relationship is temporal, and chronic Pb exposure can change gut microbial composition over time.[12] Additional epidemiological studies of the effects of Pb on the gut microbiota, Pb and selection for antibiotic resistance, and analysis of the mediating effect of the gut microbiota on Pb and antibiotic resistance to examine potential natural histories are needed. Further, reducing Pb exposure is difficult, water filtration may be a viable policy solution for reducing some low level lead exposures and improving overall water quality. It is beyond the scope of this dissertation to assess Pb in water directly, however, water source, water filtration and association with ARB can be studied with the novel SHOW resources paired with the Wisconsin Microbiome Study,[125] one of the largest microbiome cohorts to be studied to date.

1.2.11. Preliminary Data

Given the paucity of data on the association between Pb and antibiotic resistance in human populations, we examined the relationship using data from NHANES 2001-2004.[92] There is a dose response relationship between the level of blood Pb and the odds of MRSA nasal colonization (Table 1.1.A), which supports

Table 1.1. Results of logistic regression examining the effects of lead (Pb) and cadmium (Cd) exposure with MRSA (A) and MSSA (B) colonization as the outcome. Data from NHANES 2001-2004.[92]

A	Q1 OR	Q2 OR (95% CI)	Q3 OR (95% CI)	Q4 OR (95% CI)
Pb*				
Model 1[a]	1.00	1.44 (0.82, 2.55)	1.59 (0.91, 2.78)	**1.82** (1.01, 3.29)
Model 2[b]	1.00	1.27 (0.71, 2.26)	1.36 (0.65, 2.88)	1.52 (0.66, 3.51)
Model 3[c]	1.00	1.52 (0.83, 2.76)	1.56 (0.75, 3.24)	1.82 (0.81, 4.10)
Cd				
Model 1[a]	1.00	0.63 (0.38, 1.03)	1.21 (0.71, 2.07)	1.26 (0.85, 1.86)
Model 2[d]	1.00	**0.50** (0.29, 0.86)	0.74 (0.45, 1.23)	0.82 (0.50, 1.35)
Model 3[e]	1.00	**0.41** (0.20, 0.83)	0.60 (0.34, 1.08)	0.60 (0.36, 1.03)

B	Q1 OR	Q2 OR (95% CI)	Q3 OR (95% CI)	Q4 OR (95% CI)
Pb**				
Model 1[a]	1.00	1.01 (0.89, 1.14)	0.96 (0.84, 1.11)	**0.79** (0.67, 0.92)
Model 2[b]	1.00	1.05 (0.93, 1.18)	1.05 (0.91, 1.21)	**0.84** (0.69, 1.00)
Model 3[c]	1.00	1.07 (0.95, 1.21)	1.10 (0.94, 1.28)	0.91 (0.76, 1.09)
Cd***				
Model 1[a]	1.00	**0.87** (0.76, 1.00)	**0.74** (0.64, 0.85)	**0.57** (0.50, 0.66)
Model 2[d]	1.00	0.96 (0.82, 1.11)	0.88 (0.75, 1.03)	**0.67** (0.58, 0.78)
Model 3[e]	1.00	1.16 (0.95, 1.42)	0.86 (0.66, 1.20)	**0.77** (0.60, 0.99)

*P for trend ≤0.05. **P for trend ≤0.005. ***P for trend ≤0.0001. a) Unadjusted; b) Adjusted for age, gender, race, and income; c) Adjusted for age, gender, race, income, smoking, iron, calcium, and Vitamin C; d) Adjusted for age, gender, income, and smoking; e) Adjusted for age, gender, income, smoking, and dietary factors. Data from NHANES 2001–2004, n = 18,626. Percentages are adjusted using survey weights to be representative of the United States population. Bold text indicates that the 95% CI does not cross 1.00, and the finding is considered significant.

the hypothesis of a positive association between Pb exposure and antibiotic resistance. Conversely we see that the highest quartile of Pb exposure is associated with lower odds of *methicillin-susceptible Staphylococcus aureus* (MSSA) nasal colonization (Table 1.1.B), which supports the hypothesis of Pb exposure reducing the prevalence of non-resistant bacteria. Both of these results also lend support to the association of Pb exposure to changes in microbial composition, however, more data on other bacteria present are needed to obtain a more accurate understanding of these relationships.

1.3. SPECIFIC AIMS

To further examine the relationships between Pb exposure and the gut microbiota, including colonization by ARB, I used novel survey data and biological samples collected from 466 adults as part of an ongoing population based microbiome study. Using analysis 16S rRNA amplicon sequencing of bacterial DNA extracted from stool samples, the subsequent chapters address the following specific aims:

Aim 1: Determine if individual differences in urine Pb levels are associated with differences in gut microbiota composition among a general population-based sample of adults.

<u>H1a.</u> Increasing urine Pb levels are associated with decreasing microbial α-diversity and richness, because Pb exposure will reduce abundance of Pb-susceptible bacteria.

<u>H1b.</u> Total community structure and composition (β-diversity) differs significantly between levels of urine Pb concentration.

<u>H1c.</u> Increasing urine Pb concentration is associated with differential abundance of specific bacterial taxa.

Aim 2: Determine the association between adult urine Pb concentration and ARB colonization, and the potential mediating effect of gut microbial α-diversity.

H2a. Urine Pb concentration is associated with increased odds of ARB colonization.

H2b. Microbial α-diversity will be associated with decreased odds of ARB colonization.

H2c. Higher levels of urine Pb concentration will be associated with lower levels of gut microbial α-diversity, which will be associated with higher ARB colonization.

H2d. Isolated strains of ARB from the study population will also be resistant to Pb.

Aim 3: Identify difference in gut microbial composition by household water filter use, as a potential intervention to reduce Pb exposure and other water contaminants.

H3a. Differences in household use of water filtering systems are associated with differences in microbial α-diversity and richness, because water filter use will reduce exposure to chemical and biological water contaminants that would effect growth of different bacterial taxa.

H3b. Total community structure and composition (β-diversity) differs significantly by water filter use.

H3c. Differences in household use of water filtering systems are associated with differential abundance of specific bacterial taxa.

Chapter 2. Relationship of Urinary Lead Concentration with Composition of the Adult Gut Microbiota

2.1. ABSTRACT

Background: Lead (Pb) is a ubiquitous environmental contaminant with a wide array of detrimental health effects in children and adults, including neurological and immune dysfunction, and cardiovascular disease. Emerging evidence suggests that Pb exposure by ingestion may alter the composition of the gut microbiota. Imbalance of the gut microbiota has been similarly linked to adverse health outcomes including infection, obesity, allergic disease, and mental health conditions. The purpose of this study is to examine the association between urinary Pb concentration and the composition of the adult gut microbiota in a population-based, sample of adults.

Methods: Data come from the Survey of the Health of Wisconsin (SHOW) and its ancillary microbiome study in 2016. SHOW is a household based examination survey of Wisconsin residents, collecting a variety of survey data on health determinants and outcomes, as well as objective measurements of body habitus, and biological specimens including urine. The ancillary microbiome study collected some additional survey data as well as several biological specimens, including stool, for microbiota analysis, from participants age 18 and over. Pb concentration was analyzed in urine samples using inductively couple plasma mass spectrometry. Gut microbiota composition was assessed using DNA sequencing of the 16S rRNA V4 region, extracted from stool samples. Statistical analysis, performed in mothur, R, and SAS, includes calculation of alpha and beta diversity metrics, frequency tables and χ^2 calculations, multiple linear regressions, permutational analysis of variance, and a quasi-conditional association test, adjusted for relevant confounders.

Results: Of 466 participants, urinary Pb concentration was highest in those age 70+, females, non-Hispanic whites, and former smokers. Increasing urinary Pb concentration was associated with differences in α-diversity for females. Differences in β-diversity were significant with increasing levels of urinary Pb. Presence of bacterial genus *Desulfovibrio* was significantly associated with increased urinary Pb.

Conclusion: These novel results suggest that Pb exposure is associated with differences in the composition of the adult gut microbiota in a community-based human population. Further investigation of this association is warranted.

2.2. INTRODUCTION

Xenobiotics, including lead (Pb) remain persistent public health problems across the globe. The bacteria that cover the body, known as the microbiota, and their collective genomes, the microbiome, may play a role in mediating the relationship between Pb exposure and its downstream health outcomes. The gut is a particularly rich site for bacterial colonization, and the gut microbiota play key functions in our metabolism and health.[1,73] Although many have postulated that the environment and geography may affect the composition of the gut microbiota, research into the mechanisms by which these factors assert their influence is in its infancy.[37,126] Xenobiotics are potentially toxic to the bacteria in our gut, and can cause dysbiosis by altering the composition of the gut microbiota.[4] Likewise, the bacteria in the gut can change the metabolism of xenobiotics and potentially mediate the toxic effect of an internal dose on the human body.[4,65] Many factors affect the composition of the gut microbiota, of which traditional environmental pollutants are just beginning to be investigated.

Like any ecosystem, the gut microbiota maintain an ecological balance. When that system is in a state of imbalance, or dysbiosis, host health can be affected through changes in metabolic functions and production of signaling molecules, as well as infection.[2] Gut dysbiosis has been associated with a range of acute and chronic diseases including infection, digestive conditions, and mental health.[2] One facet of gut microbial balance is diversity, a combined measure of the number of species present and the abundance of individuals within each species.[127] Having a large number of species present, and a relatively even abundance of those species, can be beneficial because those microbes take on a variety of vital immune and metabolic functions and compete with each other for resources and mucosal binding sites within the gut.[9] A diverse gut microbiota can help prevent colonization by invading pathogens by competitive inhibition, as well as reduce the possibility of opportunistic infections by normally beneficial gut microbes.[128] Gut microbes also influence risk of chronic diseases as they impact our ability to harvest energy from the diet, metabolize the xenobiotics that enter our systems, and create signaling molecules that affect processes throughout the body.[63,65,67] Having a diverse gut microbiota also helps prevent an imbalance of particular metabolites and signaling molecules that can lead to chronic disease.

Pb is a pervasive environmental contaminant that plays no known necessary biological function for humans and most bacteria.[5] Pb has been causally linked to myriad detrimental health effects in children and adults including neurological disorders, kidney malfunction, anemia, and reduced immune function, and no “safe” threshold of Pb exposure exists.[6] Health effects are more prominent in children than adults, but immune effects are seen in adults even at low levels of exposure.[6,89,91] Pb's toxicity comes from its ability to substitute for calcium and other essential metal ions. It uses this ability to enter human and bacterial cells.[90] Pb also plays a toxic role for many microbes, which can cause significant shifts in the bacterial community within the gut. [7–9] In animal models, Pb exposure has been found to alter microbial communities and metabolic pathways including oxidative stress, energy and defense mechanisms that can alter Pb toxicity in humans.[12]

Human immune altering effects of Pb can also affect the microbiome. These effects include a shift from Type 2 helper (Th2) cells to Th1 cells, an increase in interleukin 4 secretion, and increased IgE secretion, all of which reduce the immune system's ability to fight infection, and increase its autoimmune tendencies.[91] These immune effects could alter the gut microbiome by reducing the body's ability to keep it in balance, thus allowing greater potential for opportunistic species to become overly abundant.

Several studies have been done in animal models that found that both chronic and acute Pb exposure lead to changes in microbial diversity, composition of bacterial taxa, and metabolic function within the gut.[7,8,12–15] Not only do gut microbial composition and function shift with Pb exposure in mice, but the gut microbiota also alter absorption of Pb into the blood stream, both by acting as a barrier to absorption, and by altering host gene expression of proteins involved in metal metabolism.[65] However, very little has been done to see if these relationships translate to human populations. One randomized trial of probiotics, examined the association between Pb exposure and gut microbes in a small population of children exposed to toxic levels of Pb, and found a significant increase in Succinivibrionaceae and Gammaproteobacteria with increased blood Pb.[9] Given the small sample size, the probiotic treatment, the high exposure level, and restricted age of the children, there is much to still be investigated in the relationship between Pb and the human gut microbiota. No study to date has examined the relationship between Pb exposure and the microbiota of adults with more typical levels of Pb exposure.

The objective of this study is to improve the understanding of the association between Pb exposure in adult humans and composition of the gut microbiome. The goals are for the first time to examine associations between urinary Pb levels and gut microbial diversity in a sample of 466 adults recruited from a population based household examination survey. We hypothesize that increasing urine Pb levels would be associated with a significant decrease in α-diversity, and significant shifts in β-diversity within the gut microbiome, driven by differences in specific bacterial taxa.

2.3. METHODS

2.3.1. Data Source

Data and biological specimens used in this analysis came from the Survey of the Health of Wisconsin (SHOW) and its ancillary microbiome study, both described in previous publication.[125,129] SHOW is a yearly statewide health survey that collects a wide range of health exposure and outcome data addressing all major determinants of health including health care access, social determinants, lifestyle and behavioral factors. Modeled after the National Health and Nutrition Examination Survey, SHOW began in 2008, and uses a three tier clustered randomization scheme to select participants from around the state of Wisconsin. SHOW collects survey, as well as objective measures of body habitus and biological specimens including urine, plasma, and serum. In 2016 the microbiome ancillary study was added to the SHOW protocol, recruiting participants age 18 and older to investigate the relationship between dietary fiber consumption, the gut microbiome, and colonization by multidrug resistant organisms

(MDROs).[125] Participants completed the standard SHOW components plus additional survey components querying further dietary recall and MDRO risk factor exposures, as well as submitting swabs of the skin, nose, and mouth, and samples of saliva and stool. The analysis for this study uses survey data, urine samples, and stool samples collected by SHOW and the microbiome study in 2016. The SHOW protocol and the protocol for this study have both been approved by the University of Wisconsin Institutional Review Board, and all participants completed written consent to participation.

2.3.2. Variables

The main predictor variable is creatinine adjusted urinary Pb concentration (original measurement in µg/L, after creatinine adjustment units are pg/L) as a measure of chronic Pb exposure.[130] Urinary Pb concentration was measured using inductively coupled plasma mass spectrometry. One value was below the limit of detection (LOD), and was replaced with the LOD/$\sqrt{2}$. For regression analyses, to account for variation in Pb concentration by urinary output and kidney function, Pb exposure was adjusted for urinary creatinine by standardizing the units and dividing Pb by creatinine. For descriptive tables, creatinine adjusted urinary Pb was then categorized by quartiles. For regression analyses, creatinine adjusted Pb was log transformed for normality.

The main outcome variables include α-diversity, measured using the Inverse-Simpson index,[131] richness, measured using the abundance-based coverage estimator (ACE)[132], and β-diversity measured using the Bray-Curtis dissimilarity index,[133] as well as taxonomic units from phylum to genus levels. Richness is an estimate of the number of different species, or in this case operational taxonomic units (OTUs), present within the gut of an individual, and α-diversity is a combination of richness and evenness of abundance among the OTUs present. Both measures can be interpreted as higher estimates representing a richer or more diverse gut microbiota. The β-diversity measurement is a distance matrix comparing the similarity (or dissimilarity in the case of Bray-Curtis) of the OTU composition of each sample to each other. Samples that are more distant from each other are more different in composition than samples that are less distant. Sensitivity analysis included the use of alternate measures of α-diversity, richness, and β-diversity, namely Shannon, Chao-1, and Jaccard, respectively.[36,134,135]

Many demographic variables were identified as potential confounders based on *a priori* hypotheses. A direct acyclic graph (DAG) was used to identify both predictors for inclusion in statistical models. Demographic variables include age, gender, income, poverty status, and race-ethnicity. Age was calculated based on self-reported date of birth and date of interview. Gender was self-reported as either male or female. Income was operationalized using self-reported total household income which was then calculated as a percentage of the Federal Poverty Level (FPL) based on the Health and Human Services guidelines for the number of people in the household. Regression models used %FPL as a continuous variable, but to examine distribution across Pb quartiles, %FPL was categorized into three groups: low income (<200%FPL), middle income (200-399%FPL), and high income (≥400%FPL). Education was self-reported as the highest level of primary and secondary education attained, and then categorized into similar sized groups of high school diploma/GED or less, some college, including attainment of an associate's degree, and attainment of a bachelor's degree or higher. Race and ethnicity were self-reported and then collapsed into two categories of non-Hispanic White, and all others due to lack of sample size to represent other race/ethnicity status.

Other potential confounding factors include behavioral variables such as smoking, antibiotic use in the last year, and dietary components. Diet was analyzed using the Diet History Questionnaire (DHQ-II), which asks about usual diet consumption over the last year.[136] Dietary factors that are known to influence Pb metabolism (Iron, Vitamin C, Calcium, and Fiber) and likely also affect the gut microbiota were included in the analysis. Antibiotic use in the last year was self-reported as either yes or no. Although it was unlikely that antibiotic use was associated with Pb exposure, it is an important variable due to its strong effects on microbial composition. The study from which these data came did not exclude participants based on antibiotic use, and only asked about use in the past year. Because the gut microbiome can get back to a normal state in less than a year, and excluding all those who answered yes would have reduced our sample size by approximately 50%, those participants are included, and the variable is included in the model building process.

Lastly, additional physiological and environmental factors were also considered as covariates, including indoor pet ownership, body mass index (BMI), urbanicity, and length of residence in current home. Indoor pet ownership was self-reported as keeping any pet inside the house. Pet ownership was originally included as it has been shown to affect gut microbial diversity,[137] and could be associated with Pb as pets spend time in the dirt and dust in and around the house where Pb is most prevalent, and may transfer Pb to their owners. BMI was calculated based on measured height and weight, using the equation: weight(kg)/height(cm)^2. BMI was modeled as continuous, but displayed in Table 1 by category: underweight (<18.5), normal weight (18.5-24.9), overweight (25-29.5), and obese (≥30). Urbanicity is defined using the 2010 census definition of urban and rural areas.[138] Length of residence in current home was self-reported as 0-1 years, 1-3 years, 3-10 years, and >10 years.

2.3.3. Microbiota Analysis

Genomic DNA was extracted from stool samples as described previously.[125] Briefly, chemical and mechanical disruption were used to lyse the bacterial cells. DNA was purified by phenol-chloroform-isoamyl alcohol extraction, and further purified using NucleoSpin Gel and PCR clean-up kit (Mcherey-Nagel, Germany). Purified DNA was quantified using PicoGreen in a microplate reader. The 16S rRNA V4 region of the extracted DNA was barcoded and amplified using custom PCR primers following the protocol in by Kozich et al.[139] The PCR reaction consisted of 5µL (25ng) sample DNA, 0.5 µL (10µM) of each primer, 12.5 µL of 2X KAPA Hotstart Ready Mix (Kapa Biosystems, Wilmington, MA, United States), and water to 25 µL total volume. Amplification conditions were 95^{o}C for 3 min, 25 cycles of 95^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 30 sec, followed by 72^{o}C for 5 min. After PCR, samples were run through 1.0% low melt agarose gel (National Diagnostics, Atlanta, GA) electrophoresis to further remove unwanted DNA. Bands of the correct length were then extracted using Zymo Gel DNA Recovery Kit (Zymo Research, Irvine, CA, United States). Once the gel was removed, samples were quantified by Qubit® Fluorometer (Invitrogen, San Diego, CA, United States) and pooled to 4 nM. A DNA sequencing control of 10% PhiX was added to the aliquot and sequenced on Illumina's MiSeq using MiSeq v2 Reagent Kit (Illumina, Inc., San Diego, CA) per manufacturer's instructions. The samples for this analysis were sequenced in two runs of equal size. To avoid sequencing batch effects, samples were stratified by urinary Pb concentration, age, and gender and randomized in a 1:1 ratio to each plate.

Raw sequencing data was processed using mothur (v. 1.37[139] using the Standard Operating Procedure for MiSeq data[140]). Briefly, contigs (overlapping sequences) were aligned using the SILVA 16S rRNA gene reference database,[141] and low quality reads were removed. Sequences of the wrong length were removed, and chimeras were detected and removed using UCHIME.[142] Sequences were assigned to operational taxonomic units (OTUs) at the species level (97% similarity) using the GreenGenes database.[143] Coverage was assessed by Good's coverage. OTU counts were normalized to 11,000 per sample. Normalized OTU counts were used for α–diversity and richness calculations performed in mothur. Additional data processing was done using Python, SAS and R.

2.3.4. Statistical Analysis

Statistical analysis was performed in SAS and R. Frequency tables were calculated for all potential confounders by creatinine adjusted Pb quartiles. Univariate analyses including P-values based on χ^2 are shown for categorical variables, and p for trend is shown for continuous variables to test for predictors of Pb exposure. Linear regression of log creatinine adjusted urinary Pb was performed for inverse-Simpson and ACE to determine if increased Pb exposure was associated with α-diversity and richness. The β-diversity distances (Bray-Curtis) were calculated in R using the vegan package.[144] PERMANOVA was then used to estimate associations between log creatinine adjusted urinary Pb and β-diversity, indicating significant differences in distance between samples at increasing levels of Pb exposure. Linear regression and PERMANOVA analysis included four models adjusted for different confounders and interactions. Model 1 is unadjusted. Model 2 adjusts for demographics (age, gender, race, income, education), and behavioral variables (smoking, antibiotic use, diet: iron, vitamin C, calcium, and fiber). Model 3 includes all measures in Model 2 and further adjusts for additional physiological and environmental covariates including BMI, urbanicity, and length of residence in current home. To test for moderating effects of key demographic and dietary factors, Model 4 included all components of model 3, but added interaction terms between Pb and age, gender, income, and diet variables. To examine specific taxa that contributed to differences in β-diversity, the Quasi-Conditional Association Test using General Estimating Equations (QCAT-GEE) in the miLineage R package was used, that includes a Benjamini-Hochberg correction for multiple comparisons (FDR).[145] The QCAT-GEE has three parts: the zero-part which assesses differences in presence/absence of each taxa, the positive-part which assesses differences in abundance of each taxa, and the two-part which combines the zero and positive-parts. This test is adjusted for significant covariates from the previously listed analyses, and outputs the names of taxa that are significantly different by continuous Pb exposure. To determine direction of association, logistic regression was used for taxa significant in the zero-part, and linear regression was used for taxa significant in the positive-part.

Several sensitivity analyses (included in Appendix A) were conducted to examine the robustness of the main results. Models 1-4 examining α-diversity, richness, and β-diversity, were also run with Shannon, Chao-1, and Jaccard as outcomes, respectively. Further sensitivity analysis of α-diversity, richness, and β-diversity were conducted with all missing values imputed using the missForest package in R, which imputes missing values using a Random Forest technique.[146]

2.4. RESULTS

Geometric mean urinary Pb for the total sample was 0.30 μg/L (95% CI 0.28-0.32). Creatinine adjusted urinary Pb concentration, increased with age, was higher in those who identify as women, non-Hispanic White, current or former smoker, and those who do not own an indoor pet (Table 2.1). Dietary calcium intake shows a marginally significant inverse association with Pb quartile (Table 2.1).

Table 2.1. Demographics and covariates by creatinine adjusted urinary Pb quartile.

	1st Quartile		2nd Quartile		3rd Quartile		4th Quartile		
	GM	95% CI	GM	95% CI	GM	95% CI	GM	95% CI	
Urinary Pb	0.16	0.14-0.19	0.25	0.22-0.29	0.38	0.33-0.44	0.52	0.45-0.60	
Demographics	N	%	N	%	N	%	N	%	P-value
Age									<0.001
18-29	27	58.7	17	37.0	1	2.2	1	2.2	
30-49	53	49.1	34	31.5	8	7.4	13	12.0	
50-69	33	15.1	47	21.5	81	37.0	58	26.5	
≥ 70	4	4.4	18	19.8	25	27.5	44	48.4	
Gender									0.026
Female	58	22.2	59	22.6	66	25.3	78	29.9	
Male	59	29.1	57	28.1	49	24.1	38	18.7	
Race/Ethnicity									0.026
Non-Hispanic White	90	22.7	101	25.5	105	26.5	100	25.3	
Other	26	38.8	15	22.4	10	14.9	16	23.9	
Family Income									0.839
Low Income	33	26.0	33	26.0	27	21.3	34	26.8	
Middle Income	36	25.5	36	25.5	33	23.4	36	25.5	
High Income	45	24.9	44	24.3	52	28.7	40	22.1	
Education									0.142
≤ High School	23	18.0	31	24.2	38	29.7	36	28.1	
Some college	37	23.6	42	26.8	37	23.6	41	26.1	
≥ Bachelor's Degree	57	32.0	43	24.2	40	22.5	38	21.4	
Behaviors									
Smoking									0.005
Current	9	15.0	18	30.0	17	28.3	16	26.7	
Former	20	16.1	26	21.0	39	31.5	39	31.5	

Never	84	30.7	72	26.3	57	20.8	61	22.3	
Antibiotic Use									0.530
Yes	41	26.3	42	26.9	33	21.2	40	25.6	
No	69	24.4	71	25.1	78	27.6	65	23.0	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P-value
Iron	16.17	17.38	16.51	28.51	13.79	6.88	13.35	12.97	0.142
Calcium	1410.39	1169.64	1449.74	1517.76	1319.32	867.35	1156.10	826.02	0.056
Fiber	19.35	18.03	20.97	28.66	19.80	9.93	19.24	12.64	0.849
Vitamin C	96.40	71.26	121.34	120.41	106.91	83.38	105.03	104.89	0.771
Other Covariates	N	%	N	%	N	%	N	%	P-value
Indoor Pet									0.007
Yes	78	29.8	71	27.1	58	22.1	55	21.0	
No	38	18.9	45	22.4	57	28.4	61	30.4	
BMI									0.116
Underweight	0	0.0	0	0.0	2	50.0	2	50.0	
Normal Weight	25	22.1	31	27.4	24	21.2	33	29.2	
Overweight	36	24.7	29	19.9	38	26.0	43	29.5	
Obese	56	28.4	56	28.4	49	24.9	36	18.3	
Urbanicity									0.050
Urban	78	27.9	77	27.5	60	21.4	65	23.2	
Rural	39	21.2	39	21.2	55	29.9	51	27.7	
Length of Residence									<0.001
< 1 year	18	40.9	11	25.0	9	20.5	6	13.6	
1-3 years	27	37.0	18	24.7	14	19.2	14	19.2	
3-10 years	26	26.5	41	41.8	15	15.3	16	16.3	
> 10 years	44	18.0	46	18.8	75	30.6	80	32.7	

Sequencing produced Goods coverage of 98.7% or higher for all samples, indicating that less than 2% of sequencing reads in each sample are from OTUs that only appear once. The number of sequences per sample ranged from 10,920-11,067. Firmicutes were the most prevalent bacterial phylum across all four creatinine adjusted urinary Pb quartiles, with Bacteriodetes and Actinobacteria following (Figure 2.1). A heat map of a subsample of 20 individuals from the 1st and 4th quartiles of Pb exposure demonstrates the wide range of composition, and inter-individual variability in OTUs even within the highest and lowest Pb exposure groups (Figure 2.2).

Figure 2.1. Bar graph of relative abundance of each bacterial phylum found in the study samples by creatinine adjusted urinary Pb quartiles (A=Q1, B=Q2, C=Q3, D=Q4).

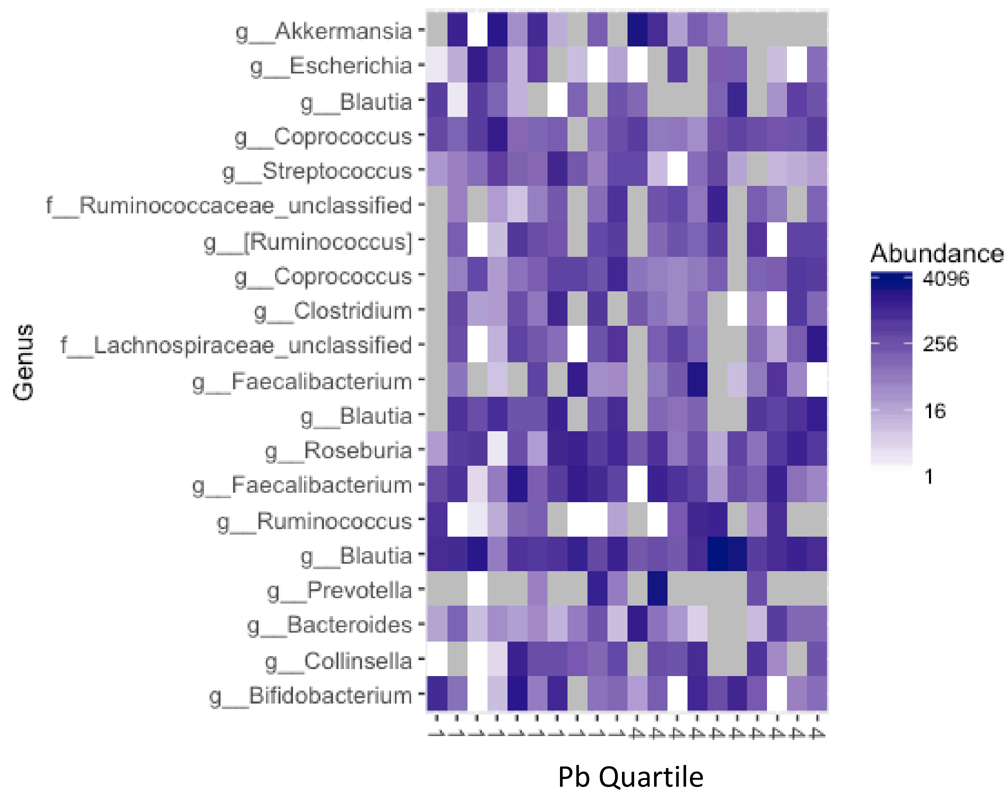

Figure 2.2. A heat map illustrating abundance of the top 20 most abundant OTUs in a subsample of 20 individuals, 10 from each of the 1st and 4th quartiles of creatinine adjusted urinary Pb level.

Inverse-Simpson values ranged from 1.5-45.0, with a mean of 14.3. In models of α-diversity (inverse-Simpson) the main effect of log creatinine adjusted urinary Pb was a slight increase in diversity, although only significant (p=0.032) when adjusting for demographics (model 1) (Table 2.2). In model 4, the interaction between Pb and gender was statistically significant (p=0.043), indicating a decrease in diversity with increase Pb level for females. The interaction with dietary fiber was marginally significant (p=0.082), indicating increased diversity with increasing Pb level and fiber consumption. Other significant predictors of α-diversity were age, education, smoking, dietary iron, and dietary fiber.

Table 2.2. Linear regression estimates of α-diversity (inverse-Simpson)								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	15.397	0.000	9.260	0.000	8.456	0.005	8.524	0.049
Log Pb^{+}	1.010	**0.032**	0.965	0.076	0.818	0.139	1.562	0.535
Demographics								
Age			0.026	0.245	**0.056**	**0.038**	**0.109**	**0.016**
Gender (Female)			-0.980	0.135	-0.828	0.207	**-2.948**	**0.018**
Race (Non-White)			0.929	0.345	0.946	0.347	0.828	0.417
Income			0.120	0.365	0.107	0.423	0.078	0.764
Education - Some College			**2.509**	**0.002**	**2.616**	**0.002**	**2.397**	**0.004**
Education - ≥ Bachelor's degree			**2.385**	**0.007**	**2.403**	**0.008**	**2.519**	**0.005**
Behaviors								
Smoking (Former)			1.275	0.255	1.402	0.217	1.139	0.317
Smoking (Never)			**2.144**	**0.042**	**2.246**	**0.036**	2.074	0.055
Antibiotic Use (Yes)			-1.002	0.124	-1.055	0.106	-1.153	0.077
Dietary Iron			**-0.108**	**0.049**	-0.107	0.052	**-0.225**	**0.023**
Dietary Vitamin C			0.000	0.992	0.001	0.907	-0.002	0.840
Dietary Fiber			0.099	0.055	0.100	0.054	**0.222**	**0.017**
Dietary Calcium			0.000	0.778	0.000	0.862	0.000	1.000
Other Covariates								
Indoor Pet					-0.832	0.234	-0.959	0.176
BMI					-0.028	0.517	-0.021	0.637
Urbanicity (Rural)					0.717	0.281	0.626	0.348
Length of Residence (1-3 years)					1.654	0.207	1.608	0.221
Length of Residence (3-10 years)					-0.427	0.736	-0.141	0.912
Length of Residence (>10 years)					-0.989	0.420	-0.909	0.461
Interaction Terms								
Log Pb^{+}*Age							0.041	0.186

Log Pb^{+}*Gender (Female)	**-2.012**	**0.043**
Log Pb^{+}*Income	-0.033	0.873
Log Pb^{+}*Dietary Iron	-0.129	0.129
Log Pb^{+}*Dietary Vitamin C	-0.003	0.674
Log Pb^{+}*Dietary Fiber	0.140	0.082
Log Pb^{+}*Dietary Calcium	0.000	0.863

$^{+}$ Creatinine adjusted. **Bold** values are significant at the <0.05 level.

ACE richness estimates ranged from 67.9-810.4, with a mean of 286.2. Models of richness (ACE) showed a positive effect of increased Pb level of varying sizes, which was only significant ($p=0.009$) in model 1 (Table 2.3). There were no significant interactions with Pb level in model 4. Other variables significantly associated with richness were BMI (model 3) and dietary fiber (model 4).

Table 2.3. Linear regression estimates of Richness (ACE).

	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	309.7	0.000	201.5	0.000	208.2	0.000	191.6	0.021
Log Pb^{+}	**22.8**	**0.009**	15.2	0.140	11.4	0.279	10.4	0.828
Demographics								
Age			0.6	0.126	0.7	0.164	1.6	0.062
Gender (Female)			-0.8	0.947	1.6	0.895	-27.8	0.243
Race (Non-White)			23.3	0.214	28.6	0.134	26.6	0.173
Income			0.6	0.815	0.7	0.771	1.8	0.715
Education - Some College			17.1	0.273	17.5	0.263	14.6	0.357
Education - ≥ Bachelor's degree			19.4	0.251	15.3	0.373	17.5	0.311
Behaviors								
Smoking (Former)			12.2	0.567	15.2	0.480	10.9	0.615
Smoking (Never)			15.9	0.428	13.8	0.497	9.5	0.643
Antibiotic Use (Yes)			-18.6	0.133	-17.3	0.163	-19.0	0.128
Dietary Iron			-1.3	0.208	-1.4	0.174	-2.7	0.149
Dietary Vitamin C			0.0	0.604	0.0	0.603	-0.1	0.487
Dietary Fiber			1.8	0.062	1.9	0.051	**3.9**	**0.027**
Dietary Calcium			0.0	0.504	0.0	0.449	0.0	0.472
Other Covariates								
Indoor Pet					4.1	0.757	2.5	0.854
BMI					**-1.7**	**0.036**	-1.6	0.053
Urbanicity (Rural)					18.6	0.142	17.9	0.161
Length of Residence (1-3 years)					23.5	0.345	23.9	0.340
Length of Residence (3-10 years)					4.8	0.842	11.0	0.650
Length of Residence (>10 years)					1.6	0.945	4.4	0.851
Interaction Terms								
Log Pb^{+}*Age							0.7	0.232

Log Pb[+]*Gender (Female)	-28.7	0.131
Log Pb[+]*Income	0.9	0.823
Log Pb[+]*Dietary Iron	-1.5	0.348
Log Pb[+]*Dietary Vitamin C	-0.1	0.605
Log Pb[+]*Dietary Fiber	2.2	0.149
Log Pb[+]*Dietary Calcium	0.0	0.636

[+] Creatinine adjusted. **Bold** values are significant at the <0.05 level.

The PERMANOVA analysis indicated highly significant differences in β-diversity by level of log creatinine adjusted urine Pb level (Table 2.4), and the effect was robust to confounders and interactions across all four models (model 1 $p=0.007$, model 2 $p=0.002$, model 3 $p=0.002$, model 4 $p=0.001$). The interaction between Pb and gender in model 4 was marginally significant ($p=0.052$), indicating that the effect of Pb on β-diversity was amplified for females. The β-diversity distance (Bray-Curtis) was calculated and displayed by quartiles of creatinine adjusted urine Pb level in a non-metric multidimensional scaling (NMDS) plot in Figure 2.3.

Table 2.4. PERMANOVA p-values of Bray-Curtis dissimilarity distances (β-diversity).

Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Log Pb⁺	**0.007**	**0.002**	**0.002**	**0.001**
Demographics				
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.002**	**0.001**	**0.002**
Race (Non-White)		**0.013**	**0.009**	**0.007**
Income		0.084	0.101	0.110
Education - Some College		0.192	0.206	0.195
Education - ≥ Bachelor's degree		0.061	0.072	0.058
Behavior				
Smoking (Former)		0.077	0.069	0.067
Smoking (Never)		0.089	0.111	0.087
Antibiotic Use (No)		**0.001**	**0.002**	**0.002**
Dietary Iron		0.477	0.502	0.488
Dietary Vitamin C		0.465	0.489	0.477
Dietary Fiber		**0.001**	**0.001**	**0.001**
Dietary Calcium		0.057	0.056	**0.060**
Other Covariates				
Indoor Pet			0.188	0.163
BMI			**0.016**	**0.011**
Urbanicity (Rural)			0.348	0.352
Length of Residence			**0.024**	**0.022**
Interaction Terms				
Log Pb⁺*Age				0.217
Log Pb⁺*Gender (Female)				0.057
Log Pb⁺*Income				0.533

Log Pb⁺*Dietary Iron	0.225
Log Pb⁺*Dietary Vitamin C	0.534
Log Pb⁺*Dietary Fiber	0.794
Log Pb⁺*Dietary Calcium	0.214
⁺ Creatinine adjusted. **Bold** values are significant at the <0.05 level.	

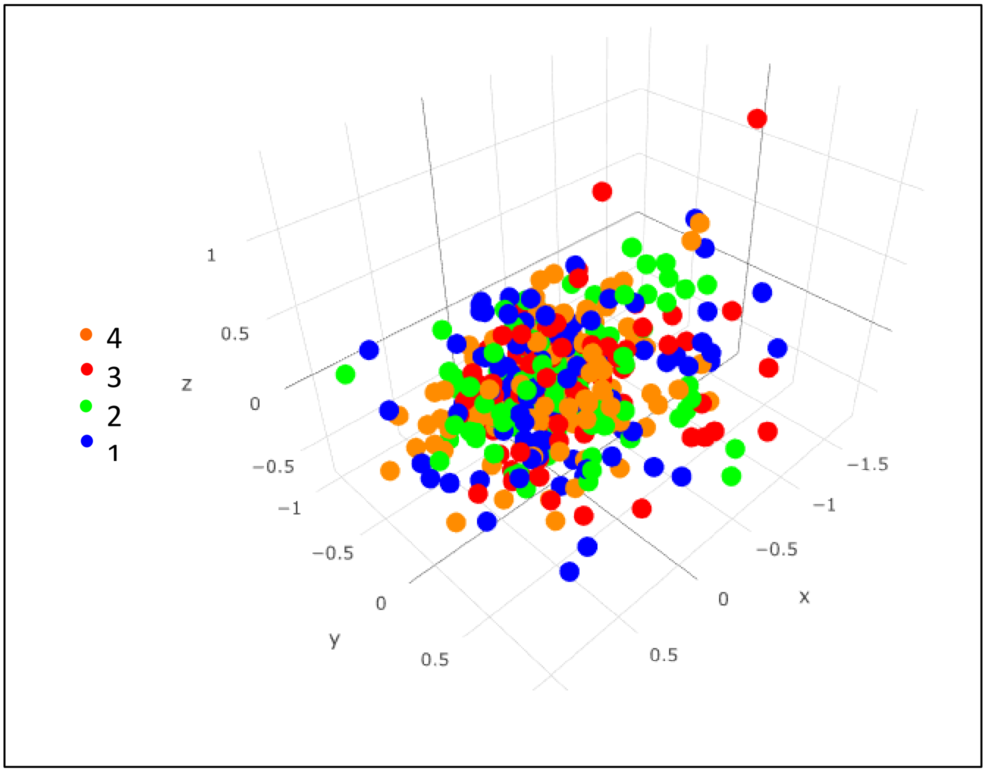

Figure 2.3. A 3 dimensional NMDS plot of Bray-Curtis dissimilarity distances, colored by quartile of creatinine adjusted urinary Pb level. Distance between dots represents the difference in OTU composition between samples.

Several genera, families, orders, classes, and phyla that were significantly different by level of log creatinine adjusted urine Pb before correction for multiple comparisons using the QCAT_GEE Test (Table 2.5). After FDR correction, only genus *Desulfovibrio* remained significant at the <0.05 level. The presence of *Desulfovibrio* increased with increasing Pb quartile, with 34% of the 4th quartile and 20% of the 1st quartile colonized. Odds of *Desulfovibrio* colonization with increasing log creatinine adjusted Pb were 1.47 (95% CI = 1.05-2.06).

Table 2.5. Output from QCAT_GEE analysis displaying bacterial taxa that differ by log creatinine adjusted urinary Pb level. Results shown are p-values before correction for multiple comparisons.

	Raw P-value		
Phylum	Two-part	Zero-Part	Positive-Part
Proteobacteria	0.0228	0.0455	0.0881
Tenericutes	0.8911	0.0198	0.9604
Class			
Deltaproteobacteria	0.0228	0.0228	1.0000
Betaproteobacteria	0.8317	0.0396	0.8317
Mollicutes	0.4950	0.0198	0.5050
Order			
Burkholderiales	0.1089	0.0297	0.9802
Desulfovibrionales	0.0842	0.0426	0.5545
Family			
Oxalobacteraceae	0.0347	0.0347	1.0000
Barnesiellaceae	0.3960	0.0297	0.3960
Enterobacteriaceae	0.1980	0.8119	0.0198
Veillonellaceae	0.0960	0.3822	0.0436
Genus			
Coprococcus	0.0267	0.0218	0.2327
Desulfovibrio	**0.0003**	**0.0003**	1.0000
Streptococcus	0.1188	0.6733	0.0396

Bold values were significant at the <0.05 level after FDR correction. Analysis was adjusted for age, gender, education, education, race, smoking, antibiotic use, indoor pets, dietary fiber and calcium.

Sensitivity analysis using the Shannon index as a measure of α-diversity showed effects in the same direction of the Inverse-Simpson models, however the interaction between gender and Pb in model 4 was not significant (Appendix A: ST1). Analysis using Chao-1 instead of ACE for richness showed very similar estimates, with significance on the same variables in the same models (ST2). Analysis with Jaccard distance instead of Bray-Curtis as a measure of β-diversity also showed very similar p-values for all variables in all models (ST3.) Inverse-Simpson models run with imputed data showed very similar effect sizes, and the only change in significance was on the interaction between Pb and dietary fiber, indicating increased diversity with increased Pb and increased fiber (ST4). ACE (ST5) and Bray-Curtis (ST6) models run with imputed data showed similar effects with no changes in significance.

2.5. DISCUSSION

In this analysis of adult Pb exposure and the gut microbiome, urinary Pb, as a surrogate for chronic Pb exposure, was associated with significant differences in α-diversity for females but not richness. This suggests that the Pb exposure is associated with reduced evenness of abundance but not the number of species present within an individual's gut. Dietary fiber had significant effects on α-diversity, and a marginally significant interaction with Pb, indicating that increasing dietary fiber intake may reduce the negative impacts of Pb on microbial diversity. Pb exposure was also associated with significant differences in β-diversity, meaning that composition of the gut microbiota was increasingly different with increased Pb exposure. Pb level was associated with significantly increased colonization by *Desulfovibrio*.

While this is among the first human studies to look at Pb exposure and altered gut microbiota, the findings are consistent with recent studies of oral Pb exposure and gut microbiota in mice. A recent study by Gao, et. al, in mice found significant differences in abundance of several bacterial taxa upon Pb exposure, including genus *Coprococcus*, which we found to be significantly altered before correction for multiple comparisons.[12] That study also found significant changes in trajectories of α and β-diversity, and metabolic function with Pb exposure that became stronger over time. This finding underscores the importance of our examination of chronic Pb exposure, rather than acute, as changes in composition of the microbiota over time lead to changes in metabolic function that persist over time, and can have stronger effects on our health.

Our findings are dissimilar to those of Bisanz, et al, the only other published human study of Pb and the gut microbiota, who found increased prevalence of Succinivibrionaceae and Gammaproteobacteria in children with highly elevated blood Pb levels who underwent probiotic or placebo treatment.[9] The differing results found between these studies are not surprising given the vastly different population composition, sample size, exposure level, exposure measurement type, and primary study purpose and design. Several investigations of different animal models also found the abundance of varying taxa to be associated with increased Pb exposure.[7,12–15] When our findings are added to the existing body of literature they suggest that Pb exposure affects gut microbial composition, even in adult human populations with relatively low levels of exposure.

The finding that α-diversity was associated with higher urinary Pb for some subsets of the population was not consistent with our hypothesis that α-diversity would decrease upon Pb exposure. There are multiple possible explanations for the unanticipated direction of effect. Pb could be reducing the abundance of highly abundant taxa in a way that increases the evenness of abundance across all taxa. Alternatively, Pb exposure may be introducing different taxa or increasing the survival ability of taxa that would not otherwise thrive in that environment. In either case, this finding does support the assertion that Pb exposure is associated with changes to the composition of the gut microbiome.

The odds of presence of *Desulfovibrio* in this sample increased with Pb level. *Desulfovibrio* is a bacterial genus commonly found in the human gut. They are sulfur-reducing bacteria (SRB) that use sulfur as a terminal electron acceptor in cellular metabolism, producing hydrogen sulfide (H_2S).[147] SRB have been implicated in the pathogenesis of multiple adverse health outcomes including inflammation, colitis and autism.[148–150] However, the presence of H_2S within a microbial ecosystem helps maintain redox homeostasis, which is vital to cellular survival.[151] Through this mechanism, the presence of H_2S can also protect bacteria against the toxic effects of both heavy metals and antibiotics, by reducing oxidative stress, a primary mechanism of toxicity for heavy metals and antibiotics, thus increasing antibiotic resistance. These potential adverse health effects of *Desulfovibrio* may mediate the health risks of Pb exposure in adults.

The sample used in this study may not be comparable to the general US population in a couple of key ways. Geometric mean urinary Pb concentration was much lower in this study population than in NHANES's nationally representative sample (0.45 μg/L).[152] While this is good news for these Wisconsin residents, it means that there is more work to be done in examine dose response relationships between Pb and gut microbial composition. The racial/ethnic composition of this study sample is also more predominantly non-Hispanic white than the country as a whole. Another point to note is that approximately 75% of this study population is overweight or obese based on objectively measured height and weight. This is consistent with previous estimates using a representative sample of the Wisconsin population.[153] This skew towards a high body mass index is likely the reason that the Firmicute to Bacteriodetes ratio is so much higher in this study population than in previous studies of healthy adults.[29] However, the high level of variability in community structure between individuals is consistent with previous findings.[29]

While pet ownership was initially included because pets may be a mechanism of increased Pb exposure, in this study sample, urinary Pb level was actually higher in those who do not own pets. A likely explanation for this finding is that pet ownership is associated with both socioeconomic status (SES),[154] and in this sample, the pet ownership variable may be capturing residual confounding that our measurements of SES are missing.

Eating a healthy diet has been previously suggested as an intervention to mitigate the toxicity of Pb exposure.[155] Mechanisms include competition by essential metals, reduction of oxidative stress, a key mechanisms of heavy metal toxicity, and improved immune function, counteracting the negative effects of heavy metals.[155] In our analysis, dietary components, particularly fiber, were significantly associated with gut microbial composition, and in some cases, adding dietary components to the models reduced the effects of Pb. Moreover the interaction between Pb and fiber moderately increased α-diversity. These findings suggest that dietary fiber consumption may mediate or modify the effects of Pb on gut microbial composition. This may be a previously unconsidered pathway by which healthy diets ameliorate the toxic effects of Pb. This area is ripe for further investigation including observational and clinical studies.

Although this analysis contributes novel insights to what is known about Pb and the gut microbiome, it has limitations. Fecal samples are a useful matrix for examining the contents of the gut microbiota, however, some aspects of sample collection could have been improved upon in this study. For example, the Wisconsin Microbiome study collects only one stool sample per participant, which gives a cross-sectional snapshot of the microbiota, but may not accurately represent the usual composition. Moreover, the cross-sectional nature of this study lacks the temporal precedence required to assert causality, therefore these findings are limited to associations. A recently funded expansion of the Wisconsin Microbiome study starts in 2018 and will collect another fecal sample (as well as several environmental samples) from the participants in this study. Future analysis will be able to examine gut microbiota composition longitudinally as well as urinary Pb level over time, and may be able to gain more clear insights into the relationship between the two.

Extracting DNA from fecal samples that have not been frozen is ideal for getting the most accurate sequencing results, as some bacterial DNA is damaged with each freeze-thaw cycle. Fecal samples from the Wisconsin Microbiome study go through at least one freeze-thaw cycle before sequencing, which could prevent some bacteria from being detected. The gut microbiota analysis is further limited by the use of 16S rRNA amplicon sequencing as opposed to shotgun metagenomic sequencing. Sequencing entire genomes would allow for more in depth analysis of potential metabolic function within the gut microbiome. This type of analysis is useful given the interpersonal variable in taxonomic composition of the gut microbiota. Using metagenomic data would allow for the examination of how the functional capacity of the microbiome is altered upon exposure to Pb.

The use of urine samples for the measurement of Pb exposure, while useful in measuring chronic exposure, is also not ideal. Because this study is observational, determination of Pb exposure is limited to post-exposure measurement. This is problematic for this particular research question as the gut microbiota play a role in the metabolism of Pb within the gut, which affects the level of Pb that is then absorbed into the bloodstream and later exits the body through the urine.

The most natural extensions of these findings would be to use metagenomic sequencing to further study this population, to examine the strength of these association in other study populations, and to determine the role that these gut microbial changes play in affecting downstream health outcomes. Further investigation using different measures of Pb exposures, and of important sources of Pb exposure on the gut microbiome would also be helpful in designing useful prevention and intervention strategies.

2.6. CONCLUSION

This study found that community-based population levels of adult urinary Pb concentration are associated with significant differences in gut microbial composition, including changes in α and β-diversity, as well as significantly increased prevalence of *Desulfovibrio*. This study sets a basis for comparison of future studies of Pb exposure and the human microbiota. Further examination of this association and its downstream health effects is warranted.

Chapter 3. Urinary Lead Level and Gut Colonization by Antibiotic Resistant Bacteria: Evidence from a Population-Based Study.

3.1. ABSTRACT

Background: Infection by antibiotic resistant bacteria is a global health crisis, and asymptomatic colonization increases risk of infection. Non-human studies have linked heavy metal exposure to selection of antibiotic resistant bacteria (ARB), however, few epidemiologic studies have examined relationships. This study analyzes the association between urinary lead level and colonization by ARB in a non-clinical human population.

Methods: Data came from the Survey of the Health of Wisconsin (SHOW) and its ancillary microbiome study. SHOW is a population-based health survey collecting data on many health determinants and outcomes, and biological specimens. Participants for this study are Wisconsin residents, age 18 and older, who participated in SHOW in 2016 and submitted urine and stool specimens. ARB included in this analysis were methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and fluoroquinolone resistant Gram-negative bacilli (RGNB). Statistical analyses were performed in SAS version 9.4.

Results: Among 466 participants, 152 (32%) tested positive for ARB. ARB colonization was highest in the 4th quartile of Pb exposure, those age 70 and over, females, non-Whites, those with the highest education, those in the middle income group, and never smokers. Geometric mean urinary Pb was 0.286 µg/L (95% CI 0.259-0.317) for negative participants and 0.326 µg/L (95% CI 0.282-0.378) for positive participants. Carefully adjusted models showed significantly elevated odds of positive colonization with increasing Pb level only for those in the highest income level. Resistant Gram-negative bacilli were most resistant to Pb.

Conclusion: These novel results suggest that Pb exposure is associated with colonization by ARB, particularly RGNB, for those with high income in a community-based human population. Reduction of Pb exposure may be a useful strategy for prevention of ARB colonization in those at high risk.

3.2. INTRODUCTION

Infection by antibiotic resistant bacteria (ARB) is a worldwide public health crisis. As bacterial resistance to antibiotics continues to evolve and spread, treatment options become increasingly sparse, therefore preventing colonization by ARB is increasingly important. Some pathogens of particular concern are methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), and antibiotic resistant Gram-negative bacilli (RGNB). Infection by these bacteria lead to increased medical care usage and can result in severe morbidity and mortality. In the United States infections by ARB cause more than 23,000 deaths annually.[19] Exposure to these bacteria is common in health care settings, often affecting outcomes of other medical treatment,[94] but community acquisition is also on the rise.[95–97]

Over prescription and overuse of antibiotics as well as leaching of antibiotics into the environment through medical and agricultural waste and runoff are well known causes of selection for antibiotic resistance,[156] however, environmental heavy metals can also select for antibiotic resistance.[157] Lead (Pb) is one such heavy metal. A ubiquitous environmental pollutant, Pb plays no necessary biological function for humans, and causes serious detrimental health outcomes throughout the life-course including immune dysfunction, neurological defects, and cardiovascular disease.[6,91] Likewise, many bacteria are also susceptible to the toxic effects of Pb, which enters cells through essential metal ion transports and alters nucleic acids and proteins, hinders enzyme activity, inhibits membrane functions, and changes osmotic balance.[5,108] However, much like antibiotic resistance, many bacteria are also Pb resistant.[5]

Typical mechanisms for bacterial resistance to heavy metals, including Pb, are binding it to intra- or extra-cellular compounds or precipitating into salt so that it cannot interfere with cellular mechanisms, transforming it into non-volatile forms, or effluxing it from the cell.[5,108] The genes that code for heavy metal resistance are often physically or transcriptionally linked to antibiotic resistance genes, or the same set of genes can encode proteins that confer resistance to both heavy metals and antibiotics.[20] Often these genes reside on mobile genetic units called plasmids that can be vertically transferred to offspring or horizontally transferred to other bacteria.[158] Bacterial plasmids need selective pressure in order to be maintained; otherwise they are easy targets to cut in replication when energy and resources are limited. When antibiotic and metal resistance genes are encoded on these plasmids and either xenobiotic enters the environment, the selective advantage of maintaining rather than eliminating the plasmid is greatly increased and proliferation of these genes is amplified.

Pb exposure may not only select for heavy metal and antibiotic resistance, but in altering essential cellular mechanisms within susceptible bacteria, also has the ability to cause major shifts in bacterial communities.[7–9] Bacterial ecosystems, known as microbiota, and the genes that they encode, the microbiome, cover nearly every surface on earth, including humans. The human microbiome, particularly the gut microbiome has been shown to play a significant role in our health. Microbiome imbalance, or dysbiosis, has been linked to many health outcomes including obesity, diabetes, Alzheimer’s disease, and infection.[3,159,160] Having a balanced gut microbiome can help defend against pathogenic bacteria in multiple ways. Commensal (healthy, beneficial) bacteria that colonize the gut often compete with pathogens for vital resources and mucosal binding sites. The more prevalent and varied commensal bacteria in the gut, the more competitive inhibition of pathogenic bacteria, and the more difficult it is for them to colonize and cause infection. Moreover, the immune system and the microbiome develop concurrently, in interaction with each other, and must maintain a homeostasis throughout our lives.[70,72] According to the hygiene hypothesis, higher levels of bacterial exposure early in life allow the immune system to become more adept at defending against bacterial pathogens, and less inclined to cause autoimmune disease and inflammation through the life-course.[161]

Pb accumulates in bones and as the body ages, can be excreted in to the blood stream, especially in women, causing adverse health effects throughout the lifecourse. An important and detrimental health effect of Pb in adults is changes to immune function.[91] Pb has been shown to affect almost every aspect of immune function, even at low levels of Pb exposure.[91] One of the most prominent immune effects of Pb is a shift toward Type 2 T helper Cell (Th2) response and increased Interleukin-4 (Il-4) secretion, reducing the Th1 response, an important mechanism for fighting bacteria and viruses. This change in immune function reduces the body's ability to fight infection, and an epidemiologic study using data from the National Health and Nutrition Examination Survey (NHANES) shows a significant association between Pb exposure and several chronic infections including *Helicobacter pylori, Toxoplasma gondii,* and Hepatitis B virus (HBV).[93]

Taken together, Pb's potential to select for antibiotic resistance, reduce competitive inhibition by altering the gut microbiota, and impede immune function, make it highly plausible that human Pb exposure would be associated with colonization by ARB. Although this link has been established in environmental and animal studies,[22,23] epidemiologic evidence is in its infancy, with the first study published by our group, examining the association between Pb and

MRSA in NHANES.[92] The aim of this study is to identify the association between urinary Pb concentration and colonization by ARB in a non-clinical, population-based sample of 466 adults, and to assess the potential mediating effects of gut microbial diversity. As conformational analysis, we tested isolated ARB for resistance to Pb.

3.3. METHODS

3.3.1. Data Source

Participants were identified as part of the Survey of the Health of Wisconsin (SHOW) and it's ancillary microbiome study. Details on both have been previously described.[125,129] In brief, SHOW is an ongoing, statewide, population health survey, modeled after NHANES. SHOW was initiated in 2008, and has been conducted annually using a representative cross-sectional sample from different counties within Wisconsin. Data for this analysis come from the 2016 cohort. SHOW collects data on health history, behaviors, diet, neighborhood and housing characteristics, current health status, and biological measurements and samples. A 2-stage cluster sampling method is used to sample households, and in 2016 all residents of selected households are invited to participate. The Wisconsin Microbiome Study, also known as the Winning the War Against Antibiotic Resistance (WARRIOR) project, is an ancillary study building off of SHOW's established infrastructure during survey waves 2016-2017.[125] The main purpose of the Wisconsin Microbiome Study is to assess the relationship between dietary fiber intake, gut microbial composition, and multi-drug resistant organism (MDRO) colonization. In addition to the full SHOW survey and examination, the Wisconsin Microbiome Study has added extensive dietary data collection methods, survey questions on factors known to influence the gut microbiota and risk of MDRO colonization, and collection of oral, nasal and skin swabs, and saliva and fecal samples. Both SHOW and WARRIOR study protocols have been reviewed and approved by the University of Wisconsin Institutional Review Board, and all participants consented to study participation.

The sample for this study includes adults age 18 years or older who provided urine and fecal samples and consented to long-term storage for additional analysis. Participants were randomly sampled from four counties (Waushara, Milwaukee, Eau Claire and Brown), and include both rural and urban residents. This sample comes from one year of a three-year representative sample, therefore, when used on its own, this sample is not representative of the state, however, for the purposes of this study, representativeness is not necessary to evaluate biological associations. The sample size is 466. A conceptual model illustrating the pathways between Pb exposure and ARB colonization is shown in Figure 3.1.

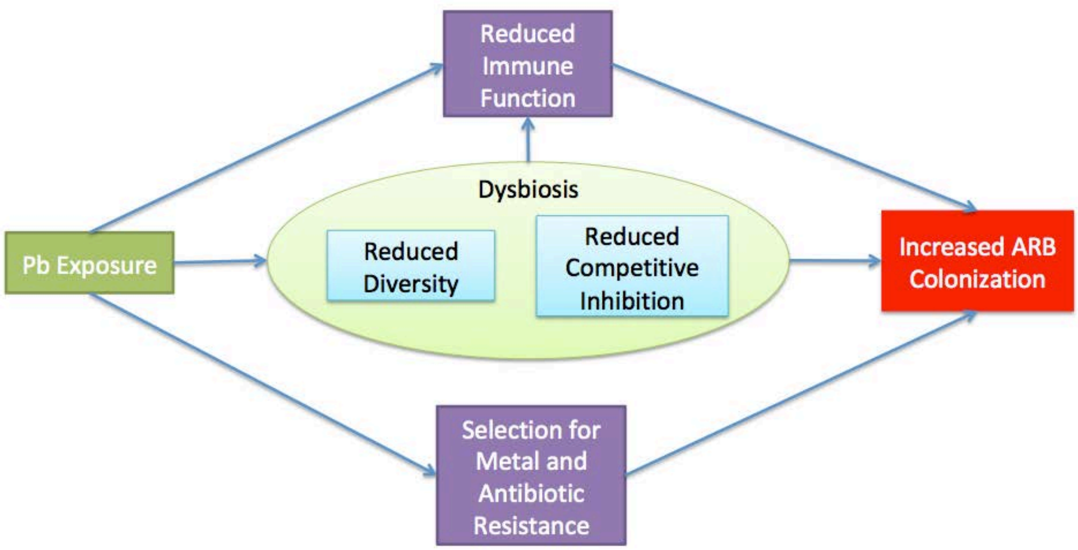

Figure 3.1. Conceptual model of the pathways between Pb exposure and ARB colonization.

3.3.2. Variables

The main predictor variable for this analysis is urinary Pb concentration, as an estimate of chronic Pb exposure, adjusted for urinary creatinine concentration to account for variable hydration and kidney function between participants. Urinary lead was measured using inductively coupled plasma mass spectrometry. One participant had a result below the limit of detection (LOD), which was replaced with the LOD/√2. Pb concentration was adjusted for creatinine concentration by converting both measurements to the same units and dividing Pb concentration by creatinine concentration. The resulting distribution was left skewed, thus log adjustment was performed to normalize the data for statistical analysis.

The primary outcome was colonization by ARB. MRSA, VRE, and RGNB were isolated from stool and saliva samples, and nasal, oral, and skin swabs as previously described.[125] MRSA and RGNB were tested for resistance to cefoxitin, and ciprofloxacin, respectively, using Kirby-Bauer disc diffusion methods and cut points established by the Clinical Laboratory Standards Institute.[162,163] Vancomycin resistance in VRE was determined using the E-test (Bio-Merieux, Marcy l'Etoile, France) of minimum inhibitory concentration (MIC). Results from each biological sample for MRSA, VRE, and RGNB were pooled into a single variable or at least one positive or intermediate resistance result (positive), or no positive or intermediate results (negative). In sub analyses, RGNB positive/negative was considered individually.

Several additional variables were used to control for potential confounding. Demographic variables included age, gender, race/ethnicity, education, income, smoking status, and urbanicity. Age was calculated on the day of the interview based on self-reported date of birth. Gender was self-reported as either male or female. Ethnicity was queried by asking participants if they identify as Hispanic or Latino, and race was determined by asking participants if they identify as White, Black/African American, Asian, Native Hawaiian or Pacific Islander, American Indian, or other, with the option to select multiple categories. These data were collapsed into a dichotomous race/ethnicity variable or Non-Hispanic White and Other due to limited sample size in all other racial/ethnic categories. Income was operationalized as total self-reported household income, divided by the Federal Poverty Level (FPL) from Health and Human Services guidelines for the total number of people in the household. That ratio was then multiplied by 100 to calculate % FPL, and then categorized into low-income (<200% FPL), middle-income (200-399% FPL), and high-income (≥400% FPL) categories. Participants reported highest level of education completed by year of primary education, high school diploma, GED, some college with no degree, and degree options from associates to doctoral. These data were categorized as high school diploma or lower, some college or associates degree, and bachelor's degree or higher. Participants who reported having smoked 100 cigarettes in their lifetime and currently smoke cigarettes were considered current smokers. Former smokers have smoked 100 cigarettes in their lifetime but do not currently smoke cigarettes. Participants were considered never smokers if they had not smoked 100 cigarettes in their lifetime. Urbanicity was defined using geo-coded participant addresses and cross referencing them with the 2010 US Census classifications of urban and rural areas.

Additional potential confounding variables considered were antibiotic use in the last year, household history of antibiotic resistant bacterial infection, use of proton pump inhibitors, farm exposure, ownership of an indoor pet, and dietary nutrients. Antibiotic use in the last year was self-reported as yes or no. Household history of antibiotic resistant bacterial infection was self-reported response to the question "Has anyone in your household had an infection with a drug-resistant germ?" Use of proton pump inhibitors was self-reported use in the past 12 months. Farm exposure was self-reported as living on a farm or hobby farm, or working on a farm.

Indoor pet ownership was self-reported as yes or no to keeping any pets inside the house. Dietary nutrients included were Iron (mg), Vitamin C (mg), Calcium (mg), and Fiber (g). Values were calculated based on self-reported usual diet over the last year as queried by the National Cancer Institute's DHQ-II.[136]

Measures of gut microbial composition were considered as potential mediators of the association between urinary Pb concentration and colonization by ARB, due to the association between Pb and the gut microbiota (Chapter 2), and gut microbiota and ARB infection,[3] and the pathway illustrated in Figure 1. Richness is a measurement of the number of different species present within an ecosystem, and for this analysis the abundance-based coverage estimator (ACE) was used to measure gut microbial richness within each individual participant. Inverse-Simpson is the measurement of α-diversity, the richness and evenness of abundance between species, used for each participant in this analysis. Both measures are interpreted as higher numbers representing a more rich or diverse gut microbiota. The diversity measures were calculated in mothur[164] based on 16S rRNA amplicon sequencing, as described in previous publication,[125] and Chapter 2.

3.3.3. Lead Resistance

Confirmatory *in vitro* analysis was conducted on ARB isolates from stool samples, to determine lead resistance by MIC analysis, using Pb (II) Acetate solution in a microtiter plate. The testing method is based on the method by Kafilzadeh, et al,[165] and the full protocol is included in Appendix B. Data are reported as MIC for MRSA, VRE, RGNB, by participant.

3.3.4. Statistical Analysis

Frequency tables were used to evaluate distribution of demographics and confounding variables by increasing quartile of creatinine adjusted urinary Pb. P-values were calculated by χ^2 for categorical variables, and a p for trend was calculated for continuous variables to test for significant differences by estimated exposure. Primary analyses included use of carefully adjusted, multiple logistic regression models to estimate odds of ARB positive v. negative associated with log creatinine adjusted urinary Pb. Secondary analysis also examined RGNB positive v. negative (not pooled with VRE and MRSA). Four models were run with varying levels of confounding and interaction terms determined *a priori* with the use of a directed acyclic graph (DAG). Model 1 was unadjusted. Model 2 adjusted for demographics including age, gender, race, education, and income, and some behavioral variables including smoking, antibiotic use, and diet (Iron, Vitamin C, Fiber, and Calcium). Model 3 added to model 2 by additionally adjusting for moderate risk factors including indoor pets, urbanicity, and testing potential mediation effects by gut microbial α-diversity and richness. Model 4 built on model 3 by adding additional ARB risk factors including farm exposure, history of ARB infection, and proton inhibitor use, as well as interaction terms between log Pb and demographic and diet variables. Analysis of Pb resistance was done using linear regression of MIC by isolate type, and of MIC by urinary Pb, stratified by isolate type. Statistical analysis was performed in SAS v. 9.4 (Carry, North Carolina, USA).

3.4. RESULTS

Among 466 participants, 152 (32%) tested positive for ARB: 12 (2.6%) tested positive for MRSA, 91 (19.5%) tested positive for RGNB, and 68 (14.6%) tested positive for VRE. Geometric mean urinary lead was 0.286 µg/L (95% CI 0.259-0.317) for negative participants and

0.326 μg/L (95% CI 0.282-0.378) for positive participants. No variables were significantly associated with ARB colonization in univariate analysis (Table 3.1), however ARB colonization was most prevalent in the 4th quartile of Pb exposure, those age 70 and over, females, non-Whites, those with the highest education, those in the middle income group, and never smokers. As previously reported, urinary Pb concentration (adjusted for creatinine) was highest in those age 70 and above, women, non-Hispanic whites, those in the low income group, former smokers, those who do not own an indoor pet, and those who live in rural areas (Chapter 2, Table 2.1). ARB risk factors not previously examined by Pb quartile are shown in Table 2. None were significantly associated with Pb quartile or ARB colonization (Table 3.1) in this sample.

Table 3.1. Demographics and potential confounding variables by ARB colonization.

	Positive		Negative		
	N	%	N	%	P-value
Total	152	32.8	311	67.2	
Demographics	GM (μg/L)	SD	GM (μg/L)	SD	
Pb	0. 286	2.49	0. 326	2.45	
Pb+ Quartiles	N	%	N	%	0.589
1	34	29.1	83	70.9	
2	41	35.3	75	64.7	
3	35	30.7	79	69.3	
4	42	36.2	74	63.8	
Age					0.301
18-29	13	27.7	34	72.3	
30-49	32	29.6	76	70.4	
50-69	70	32.1	148	67.9	
70+	37	40.7	54	59.3	
Gender					0.765
Male	65	32.0	138	68.0	
Female	87	33.3	174	66.7	
Race/Ethnicity					0.573
Non- Hispanic White	128	32.3	268	67.7	
Other	24	35.8	43	64.2	
Education					0.479
≤ High School	39	30.7	88	69.3	
Some college	48	30.4	110	69.6	

≥ Bachelor's Degree	64	36.0	114	64.0	
Income					0.265
Low	41	32.3	86	67.7	
Middle	52	37.1	88	62.9	
High	52	28.6	130	71.4	
Smoking					0.890
Current	19	32.2	40	67.8	
Former	38	30.7	86	69.4	
Never	91	33.1	184	66.9	
Diet	Mean	SD	Mean	SD	P-value
Vitamin C	116.9	120.7	102.9	82.9	0.154
Fiber	20.0	25.1	19.8	14.7	0.928
Iron	15.6	25.2	14.7	13.8	0.640
Calcium	1367.6	1321.7	1329.2	1026.8	0.732
Covariates & Risk Factors	N	%	N	%	P-value
Antibiotics					0.391
Yes	45	28.7	112	71.3	
No	92	32.6	190	67.4	
Indoor Pet					0.998
Yes	86	32.8	176	67.2	
No	66	32.8	135	67.2	
Urbanicity					0.956
Urban	92	32.9	188	67.1	
Rural	60	32.6	124	67.4	
History of ARB infection					0.869
Yes	3	30.0	7	70.0	
No	138	32.5	287	67.5	
Proton Pump inhibitor use					0.867
Yes	22	31.4	48	68.6	
No	122	32.5	254	67.6	
Live or work on a farm					0.574
Yes	14	36.8	24	63.2	
No	135	32.4	282	67.6	
Gut microbiota	Mean	SD	Mean	SD	P-value
Diversity (Inverse-Simpson)	14.8	6.6	14.1	6.2	0.274
Richness (ACE)	292.6	124.2	281.6	113.9	0.342
+ Adjusted for creatinine.					

Table 3.2. ARB risk factors (not previously shown/published) by urinary Pb quartile, adjusted for creatinine.

	Q1		Q2		Q3		Q4		
ARB Risk Factors	N	%	N	%	N	%	N	%	P-value
History of ARB infection									0.558
Yes	1	10.0	2	20.0	4	40.0	3	30.0	
No	106	25.0	108	25.5	103	24.3	107	25.2	
Proton Pump inhibitor use									0.950
Yes	19	27.1	18	25.7	16	22.9	17	24.3	
No	92	24.5	93	24.8	95	25.3	95	25.3	
Live or work on a farm									0.301
Yes	8	21.1	9	23.7	14	36.8	7	18.4	
No	106	25.5	106	25.5	97	23.3	107	25.7	

Results of the logistic regression analysis (Table 3.3) showed a non-significant increase in odds of ARB colonization with increasing Pb level in both the unadjusted and interaction model (1 and 4), no association was found in models adjusting for demographics and potential mediation (2 and 3). In the interaction model (4), the interaction term between Pb exposure and the highest income level was associated with a significant increase in log odds of ARB colonization (p=0.026). Other variables that were significantly associated with ARB colonization were education level and dietary Vitamin C. Potential mediation effects of gut microbial α-diversity and richness were null. These findings did not justify the need for more formal mediation analysis with the measures used from this data.

Table 3.3. Logistic regression estimates and relevant odds ratios for ARB colonization.

	Model 1			Model 2			Model 3			Model 4	
Variable	Estimate	OR	95% CI	Estimate	OR	95% CI	Estimate	OR	95% CI	Estimate	P-value
(Intercept)	-0.67			-2.17			-2.53			-0.82	0.747
Log Pb+	0.10	1.10	0.83 - 1.46	-0.02	0.98	0.68 - 1.41	-0.04	0.96	0.67 - 1.39	0.61	0.535
Age				**0.02**	1.02	1.00 - 1.03	0.02	1.02	1.00 - 1.04	0.00	0.987
Gender (Female)				0.10	1.11	0.70 - 1.75	0.11	1.12	0.71 - 1.78	0.19	0.700
Race (Non-White)				0.13	1.30	0.68 - 2.50	0.14	1.32	0.68 - 2.56	-0.02	0.898
Education (Some College)				-0.01	1.39	0.77 - 2.49	-0.01	1.39	0.77 - 2.53	-0.06	0.844
Education (≥Bachelor's degree)				**0.34**	1.96	1.05 - 3.65	**0.36**	2.02	1.07 - 3.80	0.15	0.674
Income - Middle				0.27	1.26	0.70 - 2.27	0.28	1.24	0.69 - 2.24	-0.20	0.541
Income - High				-0.31	0.70	0.38 - 1.31	-0.33	0.68	0.36 - 1.27	0.30	0.341
Smoking (Former)				-0.12	1.00	0.44 - 2.25	-0.11	1.03	0.45 - 2.34	-0.17	0.426
Smoking (Never)				0.23	1.40	0.65 - 3.01	0.24	1.46	0.67 - 3.17	0.30	0.122
Antibiotic Use (Yes)				-0.10	0.82	0.52 - 1.30	-0.10	0.82	0.52 - 1.30	-0.09	0.497
Dietary Vitamin C				**0.00**	1.00	1.00 - 1.01	**0.00**	1.00	1.00 - 1.01	**0.01**	**0.011**
Dietary Fiber				-0.03	0.98	0.94 - 1.01	-0.03	0.97	0.94 - 1.01	-0.03	0.415
Dietary Iron				0.01	1.01	0.98 - 1.05	0.02	1.02	0.98 - 1.06	-0.02	0.584
Dietary Calcium				0.00	1.00	1.00 - 1.00	0.00	1.00	1.00 - 1.00	0.00	0.684
Indoor Pet							0.14	1.31	0.81 - 2.12	0.09	0.513
Urbanicity (Rural)							-0.05	0.95	0.60 - 1.50	-0.17	0.511
Inverse Simpson							0.00	1.00	0.96 - 1.04	0.01	0.572
ACE							0.00	1.00	1.00 - 1.00	0.00	0.399
Infection with drug-resistant germ										-0.08	0.927
Proton pump inhibitor use										0.11	0.757
Live or work on a farm										-0.55	0.186
Log Pb+*Age										-0.01	0.236

Log Pb^{+}*Gender (Female)		0.06	0.885
Log Pb^{+}*Education (some College)		-0.01	0.967
Log Pb^{+}*Education (≥Bachelor's degree)		-0.20	0.501
Log Pb+*Income - Middle		-0.51	0.058
Log Pb+*Income - High		**0.65**	**0.024**
Log Pb^{+}*Dietary Fiber		0.01	0.725
Log Pb^{+}*Dietary Calcium		0.00	0.983
Log Pb^{+}*Dietary Iron		-0.05	0.246
Log Pb^{+}*Dietary Vitamin C		0.00	0.145

Stratified analysis by income level (Table 3.4) showed that for those in the low-income group, increasing Pb exposure was associated with a non-significant decrease in odds of ARB positive colonization (AOR = 0.55, 95% CI = 0.24-1.26). Those in the middle-income group showed a similar effect of Pb exposure (AOR = 0.57, 95% CI = 0.28-1.14). However, for those in the high-income group there was an increase in odds of ARB positive colonization with increased Pb exposure (AOR = 1.69, 95% CI = 0.90-3.18).

Table 3.4. Stratified analysis of ARB colonization by income level.

	Low Income			Middle Income			High Income		
Effect	Estimate	OR	95% CI	Estimate	OR	95% CI	Estimate	OR	95% CI
(Intercept)	-3.69			-2.31			-2.40		
Log Pb⁺	-0.60	0.55	0.24 - 1.26	-0.57	0.57	0.28 - 1.14	0.53	1.69	0.90 - 3.18
Age	0.03	1.04	1.00 - 1.07	0.01	1.01	0.98 - 1.04	0.02	1.02	0.99 - 1.05
Gender (Female)	-0.22	0.80	0.32 - 2.00	0.31	1.37	0.56 - 3.31	0.25	1.28	0.57 - 2.86
Race (Non-White)	-0.34	0.51	0.16 - 1.62	0.38	2.14	0.64 - 7.20	0.34	1.97	0.39 - 10.01
Some College	-0.05	0.96	0.35 - 2.69	0.05	1.94	0.68 - 5.60	-0.03	1.30	0.37 - 4.66
≥Bachelor's degree	0.06	1.07	0.29 - 3.92	**0.56**	3.21	1.08 - 9.59	0.33	1.87	0.57 - 6.16
Smoking (Former)	-0.23	0.74	0.20 - 2.71	-0.37	0.64	0.12 - 3.30	0.58	5.33	0.51 - 55.53
Smoking (Never)	0.15	1.08	0.33 - 3.61	0.30	1.25	0.27 - 5.80	0.51	4.94	0.52 - 47.40
Antibiotic Use (Yes)	-0.05	0.90	0.33 - 2.42	-0.03	0.95	0.42 - 2.15	-0.18	0.69	0.32 - 1.50
Dietary Vitamin C	**0.01**	1.01	1.00 - 1.01	0.01	1.01	1.00 - 1.02	0.00	1.00	1.00 - 1.01
Dietary Fiber	-0.01	0.99	0.92 - 1.05	0.02	1.02	0.94 - 1.12	-0.06	0.94	0.89 - 1.00
Dietary Iron	0.00	1.00	0.93 - 1.07	-0.06	0.95	0.85 - 1.05	0.06	1.06	0.96 - 1.17
Dietary Calcium	0.00	1.00	1.00 - 1.00	0.00	1.00	1.00 - 1.00	0.00	1.00	1.00 - 1.00

Linear regression of Pb MIC by isolate type (MRSA, VRE, RGNB) showed that RGNB had significantly (p<0.0001) higher average value of MIC than the other two isolate types (Figure 3.2). However, regression of Pb MIC of the ARB, by Pb exposure in the study subject, stratified by isolate type, showed null effects for RGNB. Moreover, secondary analysis of RGNB colonization by participant Pb exposure showed no significant association (Appendix B, ST7).

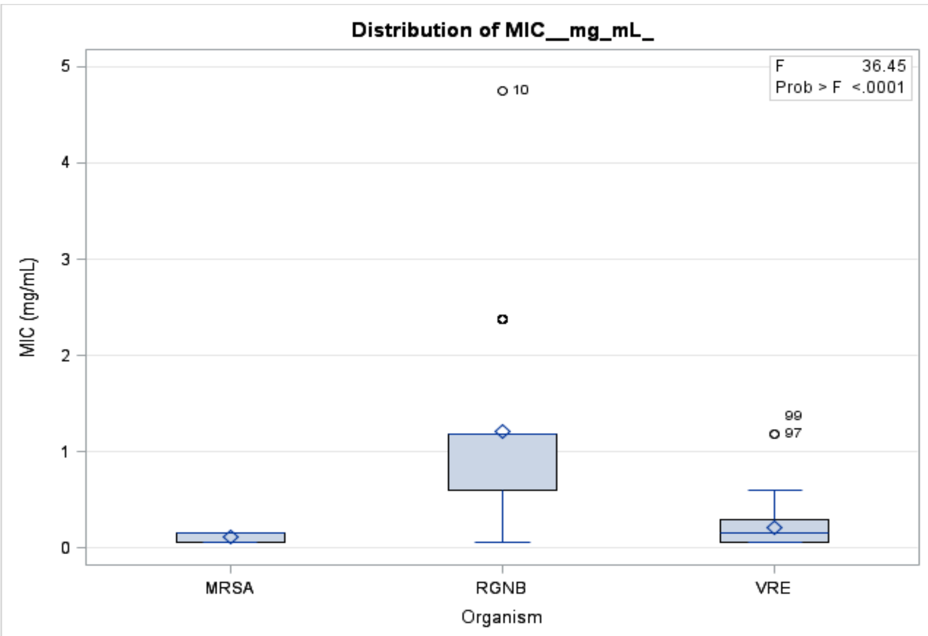

Figure 3.2. Box plot of Pb MIC by ARB isolate type.

3.5. DISCUSSION

This novel epidemiologic investigation provides new insights into the relationship between heavy metal exposure and its association with antibiotic resistant bacteria in a human population. We found that Pb exposure only significantly affects odds of ARB positive colonization among those in the highest income level, and α-diversity and richness within the gut microbiota do not seem to mediate this relationship. *In vitro* testing of Pb resistance in ARB positive isolates from stool, shows that on average RGNB positive isolates have higher tolerance to the presence of Pb, but increasing Pb exposure in humans was not associated with increased Pb tolerance in their colonizing RGNB.

Some of the most medically important RGNB include *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Helicobacter pylori*, *Pseudomonas aeruginosa,* and *Vibrio cholera*. While infections by MRSA have decreased in recent years, there has been increased incidence of RGNB infections.[166] The Center for Disease Control and Prevention (CDC) issued a report in 2013, which classified several RGNB as urgent threats to public health.[19] RGNB often have intrinsic antibiotic resistance due to their outer membrane, which serves as a permeability barrier that is not present in Gram-positive bacteria. However, RGNB can also acquire resistance through horizontal gene transfer that can occur between members of the same species as well as members from other species or genera.[167] It is not clear from this analysis whether the RGNB resistance to Pb and/or ciprofloxacin in this population is plasmid derived or intrinsic. Resistance genes, in RGNB in particular, are carried on plasmids with large concentrated multi-resistance regions that confer resistance to multiple classes of antibiotics.[168] Death attributable to bacterial infections is twice as likely for patients infected with ARB than antibiotic susceptible bacteria.[94] Although most deaths related to antibiotic resistance happen in healthcare settings, most antibiotic resistant infections occur in the general public.[19] Natural reservoirs for RGNB include water, soil, sewage, dairy and meat products, plants, and the gut of animals, including humans.[167] Environmental heavy metal contamination may play a role in selection for antibiotic resistance within all of these reservoirs.

The finding that increased Pb exposure in subjects is not associated with increased Pb tolerance in colonizing bacteria may be explained in several ways. This suggests that the relationship between Pb exposure and ARB colonization seen in subsets of this population is not due to *in vivo* selection for antibiotic resistance, but rather due to the immune effects of Pb, or shifts in gut microbial composition not captured by α-diversity and richness measurements. The geometric mean urinary Pb in this population was lower than the national average,[152] thus Pb exposure in this population may not have been high enough for antibiotic resistance selection, but would likely be high enough to affect immune function.[91] The use of a pooled ARB indicator variable across multiple body sites also captures more systemic effects of circulating Pb than selection for resistance via direct exposure. It may also be that increased exposure to Pb is associated with increased abundance of Pb resistant (and likely antibiotic resistant) bacteria, which was not assessed in this study. Such were the findings of Nisanian et. al, who fed increasing levels of Pb to leghorn chickens, and saw a dose response effect between the amount of Pb consumed, and the number ARB present.[23] If this were the case in our study population, the null relationship between Pb exposure and Pb MIC would not be inconsistent.

In this population, ARB colonization is similar across income groups, when not adjusting for other factors. The significant effect of Pb only in the highest income group is somewhat surprising. There is evidence that suggests there is not only variability in exposure to environmental toxicants by level of SES,[169] but that susceptibility to the negative impacts of those toxicants also varies by SES.[170] It may be that those in lower levels of SES are more exposed to other risk factors for ARB colonization; therefore the effects of Pb are strongest in the high income group. In the case of Pb exposure and ARB colonization however, it is not clear whether this explanation applies. Using cross-sectional survey data such as these, it is impossible to determine the natural history of Pb exposure and colonization by ARB, and where along that timeline the antibiotic resistance develops.[92] This finding might suggest that the antibiotic resistance selection is not happening *in vivo* in this study population. Rather, differences in exposure to Pb and ARB by level of SES may be driving this result. Alternatively, if the mechanism for increased ARB colonization is via the immune effects of Pb, or through changes to specific components of the gut microbiome that were not captured by the diversity metrics used, this explanation is more plausible. The high income group in this population tended to be older, and with age often comes weakened immune function and increased health care exposure over time. In this case, the immune effects of Pb may be even stronger in this group than other income groups, and exposure to ARB in health care settings may be higher. Moreover, in this generally healthy population, there may be other protective factors reducing associations seen in the other income groups. More investigation in to this high risk sub-population is warranted.

The results of the potential mediation analysis by α-diversity was not consistent with our hypothesis, however, these measures of diversity are not always ideal for capturing important ecological relationships. These calculations, based on presence or absence and relative abundance of operational taxonomic units (OTUs), are a very general reflection of the microbiota, and do not have the granularity to examine specific bacterial species or metabolic pathways that may modify this relationship. Moreover, cross sectional analysis may not be able to reveal the effects that other bacteria (not ARB) within the microbiome could have on Pb and the selection of antibiotic resistance over time. Given the strong animal evidence emerging to suggest that chronic lead exposure can alter microbial composition over time,[12] further research should aim to examine these associations in humans using metabolomics and metagenomic approaches to see if other related immune functions could be affected with lead exposures. Heavy metal exposure and toxicological relationships are complex; testing of other heavy metals and their mixtures are another important possible future direction.

The *in vitro* testing of Pb MIC indicates a low level of Pb resistance in MRSA isolates which is consistent with null associations found in this study. Previous findings by our group using NHANES data, found increased odds of MRSA nasal colonization and decreased odds of methicillin-susceptible *Staphylococcus aureus* (MSSA) with increased blood Pb level.[92] While the findings between these two studies may seem inconsistent, there are several possible explanations for the differences, including the small sample size available for this analysis, as well as the difference in Pb exposure biomarker used (urine vs. blood). There are many strains of MRSA and many possible Pb resistance mechanisms. The few isolates tested in this analysis may not be representative of the isolates found in the NHANES population, which could be more Pb resistant. Alternatively, the effects of Pb on MRSA colonization seen in NHANES analysis may not have been through direct exposure of the bacteria to Pb, but through Pb's immune effects on the study participants.

This study offers important new epidemiologic insights, however, it is limited by certain factors. The cross-sectional nature of data collection limits the findings to associations, as causality cannot be established. The use of urine Pb level is perhaps not the best measure of Pb exposure to the gut microbes, because only 10% of ingested Pb is absorbed into the blood stream, with even less filtered into urine.[130] Thus the level of Pb that gut microbes are exposed to is likely much higher. Moreover, some of the circulating Pb stored in bones and blood cells can end up in urine without interacting with gut bacteria. Because the gut microbiota likely play a role in metabolizing the Pb that enters the body, any association found between urine Pb level and presence of ARB may be due to reverse causality. For instance, if Pb resistant (and antibiotic resistant) bacteria efflux Pb out of their cells as a resistance mechanism, it is more likely to be absorbed into the blood stream and ultimately leave the body in urine. This would result in higher Pb measurement in the urine because of the presence of Pb and antibiotic resistant bacteria. The use of multiple models allows for interpretation with all covariates determined *a priori*, however, fully adjusted models 3 and 4 should be interpreted with caution given the possible co-linearity of variables and sample size.

The use of ARB colonization as an endpoint instead of infection is also somewhat problematic. Not all bacteria that are resistant to antibiotics are also pathogenic, meaning that not every colonizing organism will lead to an infection. Antibiotic resistant isolates identified in this study are not tested for the presence of pathogenicity genes. Colonization by an ARB is, however, a strong risk factor for subsequent infection, because antibiotic resistance genes and pathogenicity islands can be easily shared via horizontal gene transfer. Although ARB colonization is an imperfect surrogate measure of infection, it is useful in this population-based sample where prevalence of infection by ARB is likely to be low. Another limitation is the use of culture methods to identify antibiotic resistance, and 16S rRNA amplicon sequencing to determine gut microbial diversity. If shotgun metagenomic sequencing were used instead, many more antibiotic resistant bacteria and their pathogenicity genes could be identified, and functional capacity could have been considered in the mediating role of the gut microbiome.

This study establishes a foundation for many different studies in the future, including the metagenomic analysis mentioned. More studies in populations with different Pb exposure levels would help verify the associations found here, and establish a dose response relationship. Such findings may help determine risks of Pb exposure at levels currently thought to be less dangerous. Analysis of ARB colonization in urine samples may also allow for more insight using urinary Pb measurement, as the exposure to outcome pathway is more direct. Examining combined exposure levels to multiple heavy metals would also add to our understanding of the link between heavy metals and ARB colonization in humans. If other findings are consistent, reducing exposure to Pb and other heavy metals may be a useful prevention strategy for ARB colonization, thus trials of prevention and intervention strategies would be a logical next step.

Another avenue of future study is to examine the effects of Pb on ARB colonization across the life-course, starting with children. Because Pb exposure is absorbed differently and leads to different health effects in children than adults, the relationship seen in this study population may not be the same in children. Future analysis of the role of the microbiota on Pb absorption rates, and neurotoxicity in children would also add greatly to the literature. Starting in 2018, a newly funded extension of the current Wisconsin Microbiome Study will collect household environmental samples of dust, soil, and water.

3.6. CONCLUSION

These novel results suggest that Pb exposure is associated with colonization by ARB, particularly RGNB, for those with high levels of SES in a community-based human population. Diversity of the gut microbiota did not modify this association. Reduction of Pb exposure may be a useful strategy for prevention of ARB colonization in those at high risk. Results show translation of animal to human findings is a challenge, but important in identifying at risk populations and reducing global burden of ARB. This study provides a good model to be replicated in other populations with potentially higher Pb exposure and in areas with endemic ARB presence.

Chapter 4. Household Water Treatment and its Association with Composition of the Adult Gut Microbiota

4.1. ABSTRACT

Background: The adult microbiome has been implicated in many health outcomes, but environmental predictors of gut microbiome composition, including drinking water, are poorly understood. The aim of this study is to examine the use of at home water treatments and their association with gut microbial composition in a randomly selected population-based sample of adults.

Methods: Participants included adults over the age of 18 from the 2016 Survey of the Health of Wisconsin (SHOW) and its ancillary microbiome study. SHOW surveys residents of Wisconsin, collecting a wide range of health determinants including drinking water sources and filtration, as well as objective measurements of body habitus, and biological specimens. Data for this analysis came from participants who also submitted a stool sample and microbiome survey questions.

Results: Of 466 participants, 138 reported using a household water treatment system or filter for their drinking water. Water filter use was associated with increased education level, residence in a rural area, and higher income level. Gut microbial α-diversity and richness were associated with water filter use for those sampled from Waushara County, and those with high income, and older age. Similarly, β-diversity was associated with water filter use for those with increasing income level. Phylum Tenericutes, class Clostridia, order Clostridiales, family Lachnospiraceae, Lactobacillaceae, Rikenellaceae, Ruminococcaceae, geneus *Collinsella*, *Coprobacillus*, *Coprococcus*, and *Lactobacillus* were all significantly different by water filter use.

Conclusion: These novel results suggest that use of multiple types of water filtration may alter gut microbial composition. Additional research is needed to fully understand the health impacts of these shifts.

4.2. INTRODUCTION

John Snow's landmark epidemiologic investigation of the London cholera outbreak in 1852 lead back to a specific source of drinking water, and since then drinking water has been a commonly examined predictor of health in epidemiologic studies. Regulatory agencies such as the Environmental Protection Agency (EPA) have set standards for drinking water quality for both chemical and biological agents.[171] Drinking water plays a large role in human health due to its ability to introduce infectious agents, as well as many other contaminants that influence our chronic and infectious disease risk. Drinking water source and treatment type are well documents sources of water quality contributing to human variability in exposure to xenobiotics.[172,173] Water source and treatment often dictate exposure patterns to xenobiotics, including Pb, disinfectants such as chlorine, and other naturally occurring water elements like calcium and arsenic. These contaminants affect our health in myriad ways, along a variety of biological pathways, and the gut microbiota may be a key mediator in the relationships between drinking water quality and health.

Humans are covered in trillions of microbes that play a crucial role in our health, particularly, the bacteria living in the intestinal tract, or the gut microbiota.[1,73] Many studies have linked imbalance, or dysbiosis, of the gut microbiota with adverse health outcomes including autoimmune disease, obesity, infection, and mental health outcomes.[128,174–176] The composition of the gut microbiota is influenced by many factors including age, diet, antibiotic use, and environmental exposures,[37,48,50,57] although investigations into the influence of classic environmental exposures, and mixtures of exposures on the gut microbiota are in their infancy. Moreover, the relationships between the built environment, the human microbiome, and human health is just beginning to be explored.[58,177–179] Recognizing the importance of this line of investigation, the National Academies of Sciences, Engineering, and Medicine (NAS), published a report in 2017, calls for more work to be done characterizing the interrelationships between the built environment, microbes, and health.[180] The places we live determine where our water comes from, how it is treated, and what infrastructure is used to deliver it from the source or treatment facility to our taps. In developed countries, drinking water sources are carefully regulated providing important public health protection for a vast majority of residents. However, much of the infrastructure in the United States is changing and, as in the case of Flint, Michigan, source water quality and maintenance can quickly alter levels of both naturally occurring and man-made contaminants found in household drinking water. Even when the water that leaves the treatment facility meets water-quality safety standards, water quality can change in the distribution system based on characteristics of water source, treatment practices and infrastructure. For example, in the case of Flint, changes in source water from Lake Huron and the Detroit River to the Flint River altered source water quality such that the traditional chlorination and lack of anti-corrosives lead to leaching of iron and Pb from pipes in the distribution systems and homes of residents.[27] While the Flint water crisis is most well-known for the resulting Pb contamination,[116] another major public health challenge was the reduced effectiveness of chlorine treatment against microbial pathogens due to the interactions with iron in the water.[27] Interactions between water treatments, chemical contaminants, and biological contaminants are complex and pose a multifaceted threat to public health, when not properly controlled and maintained.

The water quality of private wells is much less regulated than public water sources, with no federal laws to ensure safety monitoring. While local and state regulations regarding private well monitoring vary, private well owners bear the primary responsibility for stewardship, including quality monitoring, treatment, and maintenance. In Wisconsin, 47% of private wells tested between 2007-2010 exceeded safety standards for at least one health related water contaminant,[119] yet only half of Wisconsin well-users report having their water tested in the last 10 years.[25] Maintaining high quality drinking water is, therefore, an important ongoing public health challenge, regardless of the source.

One mechanism to avoid consumption of potentially harmful contaminants found in drinking water sources is by use of household water treatments and filters. There are many different water filter types and points of filtration. Each filters different types of contaminants, including chemicals and microbes. Filtering either of these may have drastic impacts on the composition of the gut microbiome, and its downstream health effects. Many studies have shown that drinking water properties including chemical contaminants and pH can alter the gut microbiota in animal models.[12,83,181] However, little has been done to examine drinking water and the gut microbiota in humans.

The goal of this study is to examine the association between use of an in home water treatment or filtration system, and composition of the gut microbiota. To achieve this goal we used data collected from a population-based sample of 466 adult Wisconsin residents. Wisconsin is a unique state to study water quality because of the diversity of drinking water sources. Approximately 1/3 of the state receives drinking water from the service water sources, mainly the Great Lakes, and the remaining 2/3 from deep groundwater sources.[182] Among the 2/3 that receive drinking water from groundwater, approximately 1/3 of residents are served by private wells as their primary drinking water source.[119] Municipal water supplies are monitored and have treatment at the source for microbial and other contamination, while, private well owners are dependent on their own testing and treatment. Using a randomly selected sample of households from four different counties, this study aims to determine if differences in water source and water filter use are associated with differences in gut microbiota, hypothesizing that use of a household water treatment system would be associated with significant differences in α-diversity, and significant shifts in β-diversity within the gut microbiome, driven by differences in specific bacterial taxa.

4.3. METHODS

4.3.1. Data Source

Data for this analysis come from the Survey of the Health of Wisconsin (SHOW) and its ancillary microbiome study, for which protocols have been previously published.[125,129] The SHOW is a statewide representative health-examination survey of Wisconsin residents. Beginning in 2008 and conducting yearly survey waves since, the SHOW is modeled after the National Health and Nutrition Examination Survey (NHANES). It collects self-reported data on demographics, health history, health behaviors, health care access, diet, housing, neighborhoods and many other subjects. The SHOW also includes objective measurements of body habitus and collection of biological specimens including urine and plasma. More information about the SHOW, including questionnaires and data, is available at https://www.med.wisc.edu/show/.

The SHOW's ancillary microbiome study, also known as the Winning the War on Antibiotic Resistance (WARRIOR) Project, built off of the SHOW's pre-existing infrastructure in survey years 2016 and 2017. Participants underwent the full SHOW protocol, plus additional questions about diet and exposure to ARB risk factors. Participants also submitted additional specimens for microbiome analysis, including stool samples, which were used in this analysis. The microbiome study sample consists of 730 participants, age 18 and over. Data processing from the 2017 sample is ongoing. This analysis uses survey, health examination, and microbiome data from the SHOW and the ancillary microbiome study, collected in 2016, with a completed stool sample for gut microbial analysis. The University of Wisconsin Institutional Review Board has approved the protocols for this study and its data and specimen sources. All participants completed written informed consent.

4.3.2. Variables

The main predictor variables for this study were water source, self-reported as either private well or community water supply, and water filter use, defined as self-reported use of a home water filter or treatment system for drinking water. The main outcome variables in this analysis are measures of gut microbiota composition including richness, the number of species, or operational taxonomic units (OTUs), within an individual's gut, α-diversity, the richness and evenness of abundance between OTUs within one individual's gut, and β-diversity, differences in the number and abundance of OTUs between groups of individuals. Gut microbial α-diversity was measured using the inverse of Simpson's diversity index.[131] Richness was measured using the abundance-based coverage estimator (ACE) index.[132] B-diversity distance was measured using the Bray-Curtis dissimilarity index.[133] Alternative measurement indices were used as outcomes in sensitivity analysis (Shannon,[134] Chao-1,[35] Jaccard[135]). Specific bacterial taxa were also used as outcomes for further analysis.

The primary covariates considered for analysis based on known associations with altered microbiota included demographics such as age, gender, education, income, race/ethnicity, and urbanicity, as well as antibiotic use. Age was determined based on self-reported date of birth, and calculated for the date of the interview. Gender was self-reported as either male or female. Education was defined as the self-reported highest level of education attained, which was then categorized as high school diploma/GED or below, some college or an associate's degree, and bachelor's degree and above. Income was defined as self-reported total household income, which was then divided by the Federal Poverty Limit (FPL), as set by the guidelines from the department of Health and Human Services, for the number of people supported by that income in the household. That ratio was used as a continuous variable in regression analysis, but was multiplied by 100 to calculate %FPL, and then categorized to examine distribution across the predictor variable. Income categories were defined as low income (<200%FPL), middle income (200-399%FPL), and high income (≥400%FPL). Race and ethnicity were self-reported and then, due to limited sample size of all non-white groups, collapsed into two categories, non-Hispanic white, and all others. Urbanicity was based on geocoded addresses, and defined using the 2010 census classifications of urbanized areas, urban clusters and rural areas.[138] Urbanized areas and urban cluster were combined to create a dichotomous urban versus rural variable. Antibiotic use over the last year was self-reported as yes or no.

Additional covariates included amount of household drinking water consumed on average, body mass index (BMI), age of housing, length of residence in current home, and county of residence. Household tap water consumption was self-reported as average servings of tap water consumed at home in a day. BMI was calculated as weight(kg)/height(cm)^2, based on measurements of height and weight. Continuous BMI was used in regression models, but was categorized to display distribution by water filter use in table 1: underweight (<18.5), normal weight (18.5-24.9), overweight (25-29.5), and obese (≥30). Length of residence at current address was self-reported as 0-1 years, 1-3 years, 3-10 years, and >10 years. Counties of residence included Brown, Eau Claire, Milwaukee, and Waushara. Figure 4.1 shows the spatial distribution of households within each county. Among these, Waushara is the most rural (100% of participants), then Eau Claire (31%), with the most Urban being Milwaukee (100%) and Brown (88%). Source water for Waushara and Eau Claire is primarily groundwater and Brown and Milwaukee draw from Lake Michigan on the Eastern shore.

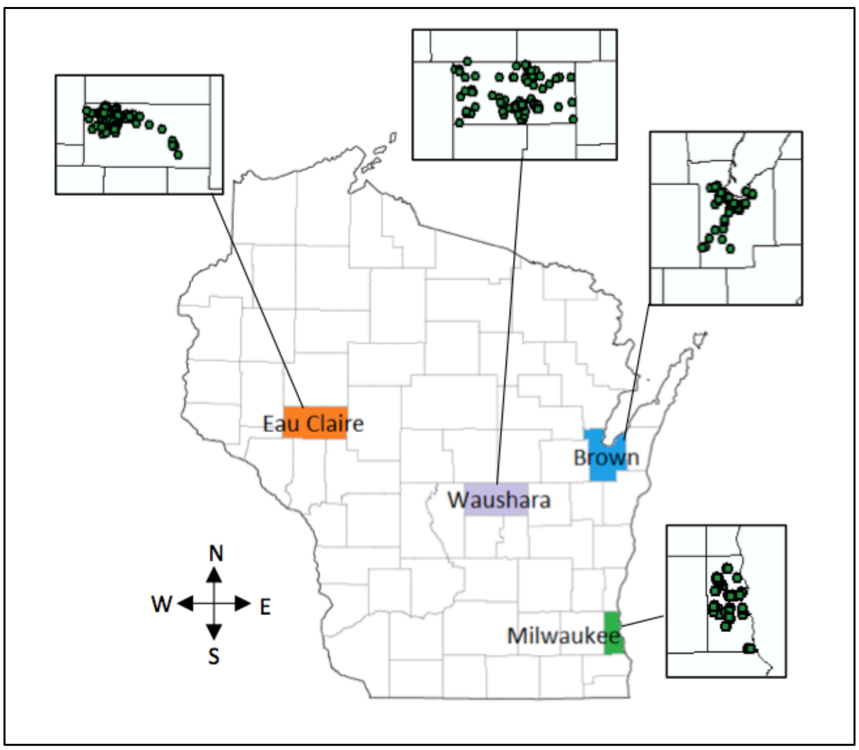

Figure 4.1. A map of Wisconsin with the four sampled counties highlighted. Enlarged portions display the spatial distribution of study households within each county.

4.3.3. Microbiota Analysis

The methods for analysis of the microbiota in this population have been explained in previous publication,[125] and in Chapter 2 of this dissertation. Briefly, DNA was extracted from stool samples, using chemical and mechanical lysis, and purified using phenol-chloroform-isoamyl alcohol and the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Germany). Purified DNA was quantified and the 16S rRNA V4 region was amplified and barcoded using custom PCR primers.[139] The PCR mixture included 5µL (25ng) template DNA, 0.5 µL (10µM) of each primer, 12.5 µL of 2X KAPA Hotstart Ready Mix (Kapa Biosystems, Wilmington, MA, United States), and 6.5 µL water. The amplification protocol was 95ºC for 3 min, 25 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, followed by 72ºC for 5 min. Amplicons were cleaned using 1.0% low melt agarose gel (National Diagnostics, Atlanta, GA) electrophoresis, extracted from the gel using Zymo Gel DNA Recovery Kit (Zymo Research, Irvine, CA, United States), quantified, and then pooled to 4 nM. A DNA sequencing control of 10% PhiX was added to the aliquot and sequenced on Illumina's MiSeq using the MiSeq v2 Reagent Kit (Illumina, Inc., San Diego, CA) according to the manufacturer's protocol.

Sequences were processed using mother (v. 1.37[139] using the Standard Operating Procedure for MiSeq data[140]). Sequences were aligned using SILVA 16S rRNA gene reference database.[141] Reads of the wrong length were and chimeras were removed using UCHIME.[142]OTUs were binned at the 97% similarity level and assigned using the GreenGenes database.[143] OTU counts were normalized to 11,000 per sample, and Good's coverage was calculated for each sample. The Simpson, Shannon, ACE, and Chao-1 indices were calculated based on normalized OTUs in mothur.

4.3.4. Statistical Analysis

Statistical analyses were performed in SAS and R. Frequency tables with p-values based on χ^2 analysis were calculated to examine the distribution of potential confounder variables by water filter use. Multiple linear regression was performed with the inverse-Simpson α-diversity index, and the ACE richness index as outcomes, and water filter use as the main predictor. Multiple PERMANOVA was used to examine Bray-Curtis dissimilarity distance as a measure of β-diversity, by water filter use, using the vegan package in R.[144,183] For linear regression and PERMANOVA analysis, four models with increasing levels of confounders and interactions were run. Model 1 was unadjusted. Model 2 adjusted for demographics including age, gender, education, race, poverty, and urbanicity, as well as antibiotic use. Model 3 builds off of model 2 by adding adjustment for the additional environmental and physiological factors including age of housing, length of residence, home tap water consumption, county, and BMI. To test for effect modification, Model 4 adds interaction terms between water filter use and age, gender, income, and county. The QCAT_GEE test, using the miLineage package in R,[145] was used to identify which bacterial taxa were significantly different by water filter use, adjusting for significant confounders in PERMANOVA analysis, and correcting P-values using the FDR method for multiple comparisons. The QCAT_GEE is a three part test, including the zero-part that assesses differences in presence and absence of each taxa, the positive-part, which assesses differences in abundance for those taxa that are present, and the two-part that combines the zero and positive-part tests. This method is better suited to this analysis than similar tests, such similarity percentages (SIMPER) analysis, because it allows for the use of a continuous predictor variable, it can be adjusted for covariates, and the modeling approach better accounts for the skewed distribution of the OTU data with many zeros.

Sensitivity analysis was conducted using Shannon's diversity index, Chao-1 richness index, and Jaccard similarity index as alternate measures of α-diversity, richness, and β-diversity, respectively. Additional sensitivity analysis was conducted with missing values imputed using the missForest package in R.[146]

4.4. RESULTS

Of 466 participants, 138 reported using an in home water filter or treatment system. 170 (40.4%) of participants reported that their household water comes from a private well. Water filter use was associated with increased education level, residence in a rural area, and higher income level (Table 4.1), with 44% of private well owners and 23% of municipal water users reporting the use of household water treatment. Aerator use was the most frequently used type of water treatment, followed by ceramic or charcoal filter, and water softener (Table 4.2).

Table 4.1. Frequency of demographics and other covariates by water filter use.

		Water Filter: Yes		Water Filter: No		
		N	%	N	%	P-value
Age						0.462
	18-29	15	34.1	29	65.9	
	30-49	33	32.4	69	67.7	
	50-69	69	32.4	144	67.6	
	70+	21	23.9	67	76.1	
Gender						0.979
	Female	77	30.9	172	69.1	
	Male	61	30.8	137	69.2	
Race/Ethnicity						0.135
	Non- Hispanic White	123	32.0	261	68.0	
	Other	14	22.6	48	77.4	

Education						0.006
	≤ High School	29	24.4	90	75.6	
	Some college	40	26.1	113	73.9	
	≥ Bachelor's Degree	69	39.7	105	60.3	
Income						0.000
	Low	19	16.1	99	83.9	
	Middle	45	33.1	91	66.9	
	High	70	39.3	108	60.7	
Antibiotic Use						0.899
	Yes	47	30.5	107	69.5	
	No	84	31.1	186	68.9	
Urbanicity						0.001
	Urban	67	25.0	201	75.0	
	Rural	71	39.7	108	60.3	
Housing age						0.212
	Built before 1979	72	29.5	172	70.5	
	Bui8lt 1979 or later	56	35.4	102	64.6	
Length of Residence						0.698
	< 1 year	9	23.1	30	76.9	
	1-3 years	21	30.0	49	70.0	
	3-10 years	30	30.9	67	69.1	
	> 10 years	78	32.5	162	67.5	
Home Water Consumption (Servings)						0.823
	0	31	29.8	73	70.2	
	1-3	59	32.6	122	67.4	
	≥ 4	48	29.8	113	70.2	
BMI						0.391
	Underweight	0	0.0	4	100.0	
	Normal Weight	39	35.5	71	64.6	
	Overweight	41	29.3	99	70.7	
	Obese	58	30.7	131	69.3	
County						0.690
	Brown	27	30.3	62	69.7	
	Eau Claire	37	30.8	83	69.2	
	Milwaukee	29	27.1	78	72.9	
	Waushara	45	34.4	86	65.7	

Table 4.2. Frequency of types of water filter used. Responses are non-exclusive.		
Water Filter Type	N	%
Brita or Pitcher With Filter	7	1.5
Ceramic or Charcoal Filter	45	9.4
Water Softener	35	7.3
Aerator	61	12.8
Reverse Osmosis	1	0.2
None	10	2.1
Don't Know	4	0.8
Other	24	5.0

General gut microbial sequencing results for this population were reported in Chapter 2. The stacked bar graph in Figure 4.2.A, which shows the normalized abundance of bacterial phyla across all study samples, illustrates the variability in gut microbial composition between participants. The bar graph in Figure 4.2.B shows percent abundance of the 5 most prevalent phyla by water filter type, with Firmicutes dominating both groups, but slightly higher in those that do not use water filters. The heat map in Figure 4.3 shows the percent abundance of the top 20 bacterial genera, grouped by water filter use.

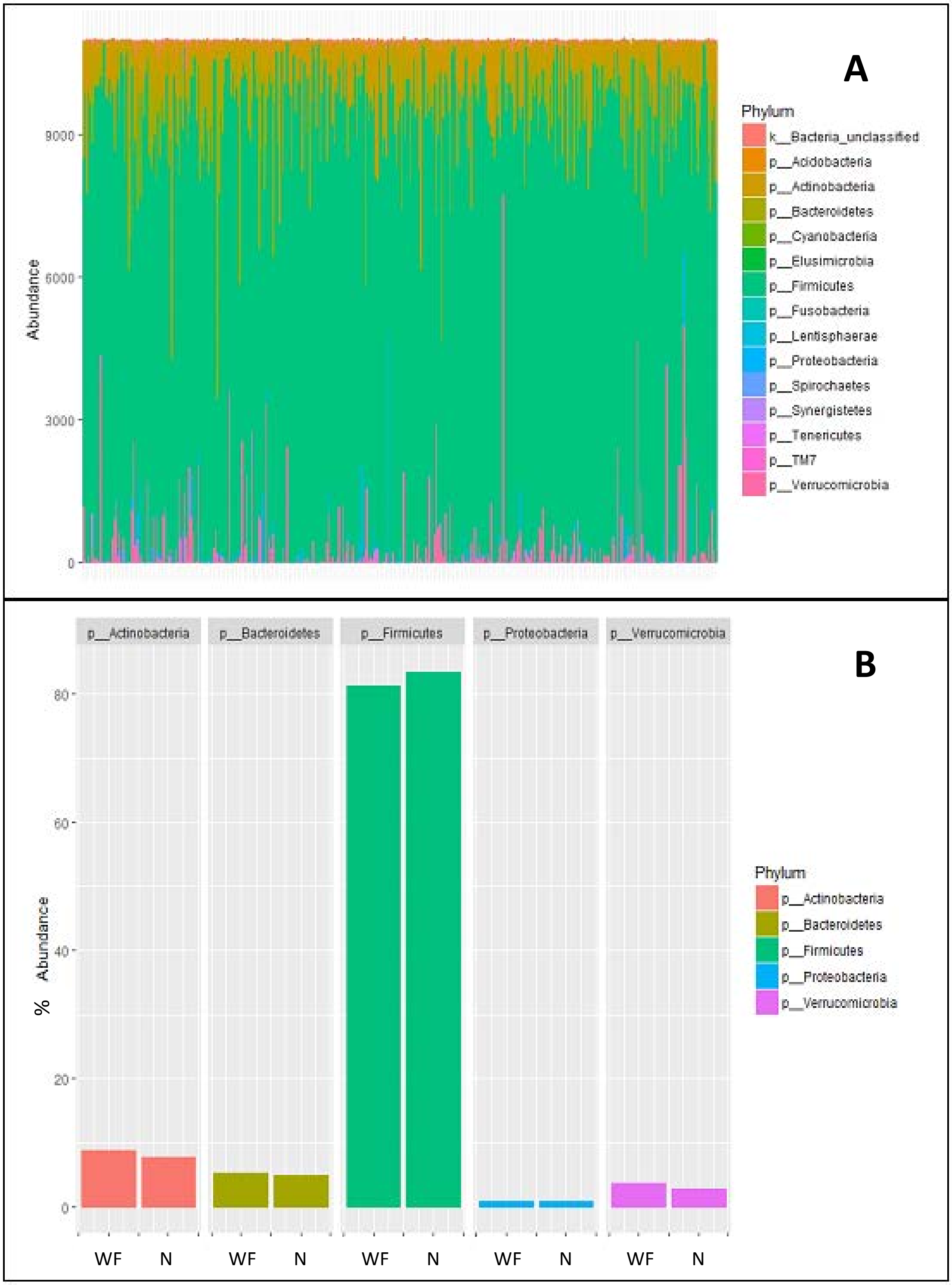

Figure 4.2. Bar charts displaying abundance of bacterial phyla within study samples. A) Stacked bar chart showing normalized abundance of all phyla across all participants. B) Bar chart

showing percent abundance of the top 5 phyla of bacteria found in the study samples, displayed by water filter use (WF = Water Filter/Treatment, N=None).

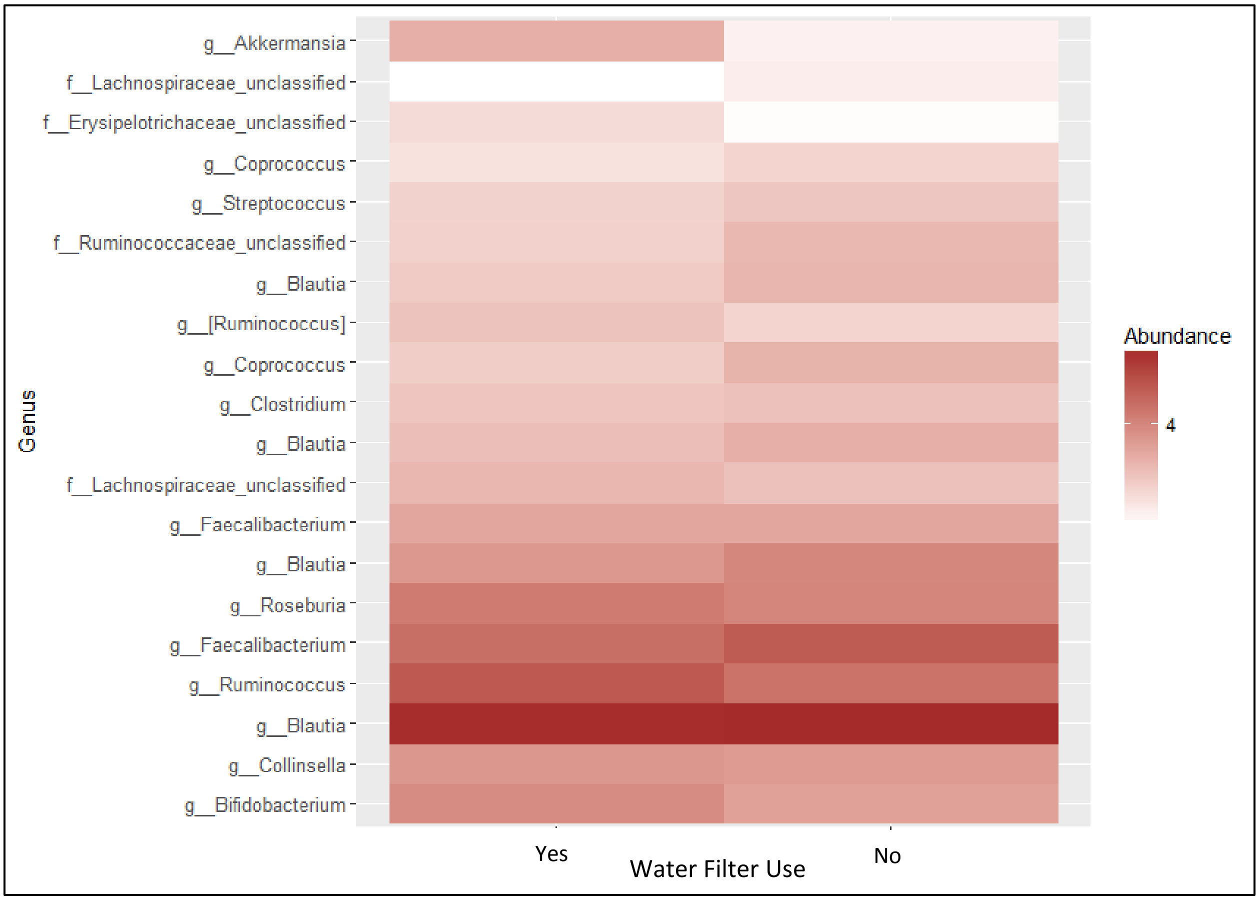

Figure 4.3. Heat map displaying percent abundance of the top 20 OTUs by genus of bacteria found in the study samples, grouped by water filter use.

In linear regression models of α-diversity (Table 4.3), a measure of the number and abundance of OTUs by individual, the main effect of using a water filter was slightly positive for all models except the interaction model (models 1-3), with the main effect of water filter in the interaction model being negative (model 4), although non-significant across all models. In model 4, the interaction between age and water filter use was significant, indicating increased diversity with increased age and use of a water filter. The interaction between using a water filter and living in Waushara County was also significant. Taken together with the main effects of Waushara County and water filter use, this interaction indicates that those living in Waushara County who use a water filter have a much larger decrease in α-diversity than those who use water filters in other counties. Other factors that were significantly associated with α-diversity were age, and education level.

Table 4.3. Linear regression estimates of α-diversity (Inverse-Simpson)								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
Intercept	14.3	0.000	10.1	0.000	10.2	0.000	11.3	0.000
Water Filter (Yes)	0.7	0.344	0.3	0.662	0.4	0.625	-4.2	0.192
Age			0.0	0.086	**0.1**	**0.005**	**0.0**	**0.209**
Gender (Female)			-0.1	0.899	-0.1	0.895	-0.9	0.255
Race (Non-White)			0.7	0.550	1.0	0.410	0.9	0.440
Education - Some College			**2.4**	**0.007**	**2.5**	**0.004**	**2.4**	**0.005**
Education - Bachelor's degree +			**2.6**	**0.004**	**2.4**	**0.006**	**2.2**	**0.013**
Income			0.2	0.201	0.1	0.361	0.3	0.111
Antibiotic Use (Yes)			-1.2	0.081	-1.3	0.058	-1.2	0.074
Urbanicity (Rural)			0.4	0.589	1.2	0.309	0.6	0.635
Housing Age (Built 1979 or later)					0.6	0.443	0.7	0.339
Length of Residence (1-3 years)					**3.1**	**0.048**	2.6	0.082
Length of Residence (3-10 years)					0.8	0.590	0.4	0.754
Length of Residence (>10 years)					-0.2	0.872	-0.5	0.703
Tap Water Consumption					0.1	0.338	0.2	0.124
BMI					0.0	0.373	0.0	0.460
County (Eau Claire)					-1.8	0.059	-1.8	0.122
County (Milwaukee)					-1.9	0.077	-1.5	0.240
County (Waushara)					-2.5	0.064	**-0.4**	**0.781**
Water Filter (Yes)*Age							**0.1**	**0.008**
Water Filter (Yes)*Gender (Female)							2.6	0.065
Water Filter (Yes)*Income							-0.4	0.169
Water Filter (Yes)*County (Eau Claire)							-0.2	0.925
Water Filter (Yes)*County (Milwaukee)							-1.2	0.605
Water Filter (Yes)*County (Waushara)							**-5.2**	**0.010**

Linear regression on richness (Table 4.4), the number of different OTUs present, also showed non-significant results across all 4 models that were positive in all models except the interaction model (models 1-3), with a negative main effect in the interaction model (4). Interactions between water filter use and income, as well as residence in Waushara County were significant. The effect of using a water filter results in decreasing richness as income increases. Residence in Waushara County is associated with a net decreased richness when using a water filter, and an increase when not using a water filter.

Table 4.4. Linear regression estimates of Richness (ACE).

	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
Intercept	287.3	0.000	220.9	0.000	247.7	0.000	238.3	0.000
Water Filter (Yes)	-4.5	0.736	-9.6	0.481	-9.1	0.509	23.7	0.699
Age			**0.8**	**0.035**	**1.0**	**0.030**	0.8	0.145
Gender (Female)			6.3	0.616	9.5	0.448	9.1	0.543
Race (Non-White)			14.1	0.509	21.0	0.347	16.1	0.331
Education (Some College)			15.1	0.363	18.1	0.278	15.6	0.362
Education (≥ Bachelor's degree)			24.3	0.149	25.2	0.142	19.0	0.391
Income			1.4	0.584	2.2	0.400	**7.7**	**0.015**
Antibiotic Use (Yes)			**-28.3**	**0.029**	**-26.8**	**0.041**	-25.4	0.050
Urbanicity (Rural)			13.6	0.295	11.2	0.617	-3.9	0.864
Housing Age (Built 1979 or later)					-23.7	0.092	-21.5	0.121
Length of Residence (1-3 years)					37.2	0.211	32.3	0.270
Length of Residence (3-10 years)					17.6	0.521	12.3	0.654
Length of Residence (>10 years)					6.1	0.821	0.0	1.000
Tap Water Consumption					-1.2	0.594	-0.3	0.892
BMI					-1.5	0.070	-1.4	0.085
County (Eau Claire)					7.4	0.691	0.1	0.995
County (Milwaukee)					-8.8	0.671	-8.7	0.714
County (Waushara)					13.2	0.613	53.6	0.075
Water Filter (Yes)*Age							0.8	0.361
Water Filter (Yes)*Gender (Female)							7.0	0.794
Water Filter (Yes)*Income							**-13.8**	**0.005**
Water Filter (Yes)*County (Eau Claire)							33.1	0.392
Water Filter (Yes)*County (Milwaukee)							1.9	0.965
Water Filter (Yes)*County (Waushara)							**-82.8**	**0.033**

The Bray-Curtis β-diversity distance, representing the difference in OTU composition between individuals, is displayed by water filter use in the NMSD graph (Figure 4.4). PERMANOVA analysis showed no significant differences by water filter use across all 4 models (Table 4.5). However, the interaction term between filter use and income was significant, indicating increased differences in β-diversity by water filter use with increasing income level. Other factors significantly associated with differences in β-diversity were age, gender, education level, antibiotic use, and BMI.

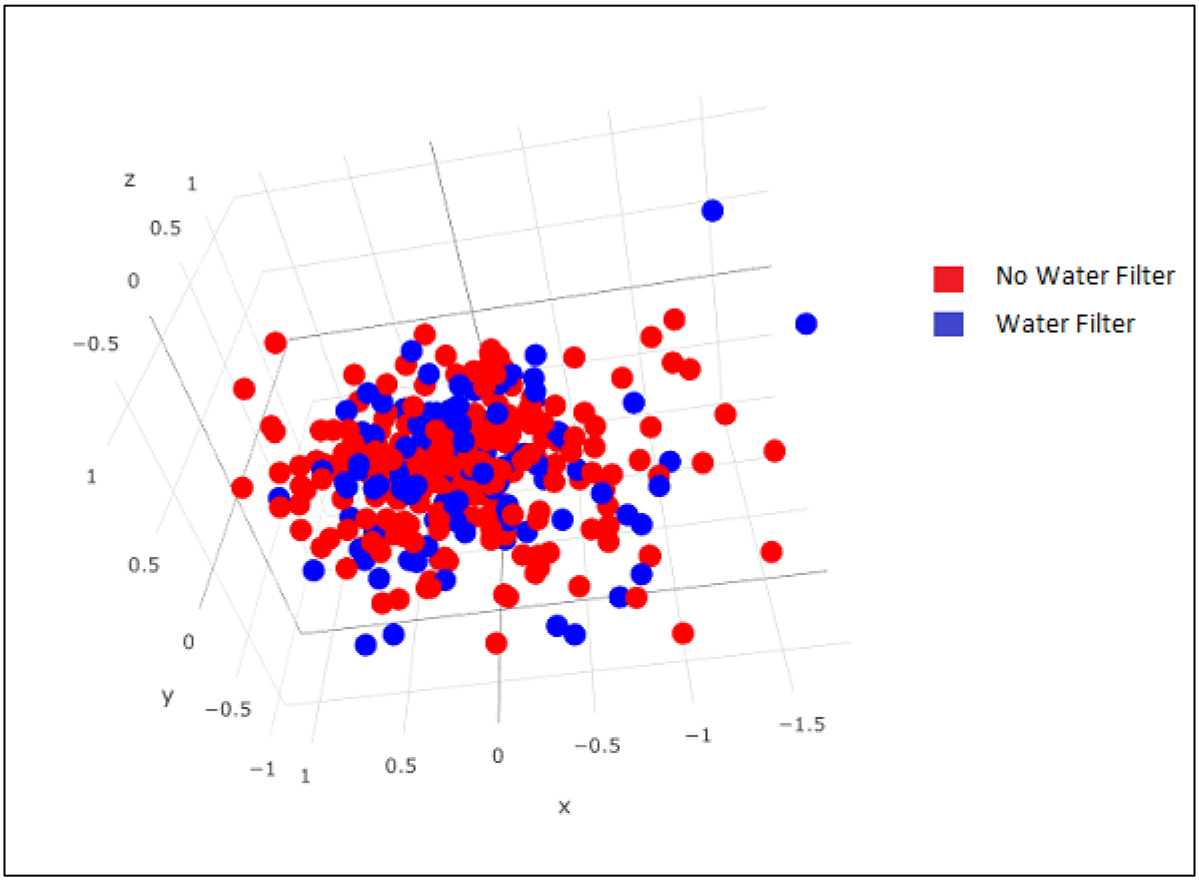

Figure 4.4. NMDS graph displaying Bray-Curtis distance graphically, by water filter use, with increasing distance between dots representing increasing differences between samples.

Table 4.5. PERMANOVA p-values of Bray-Curtis dissimilarity distances (β-diversity).				
Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Water Filter (Yes)	0.2717	0.224	0.254	0.245
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.001**	**0.001**	**0.001**
Race (Non-White)		0.552	0.511	0.463
Education (Some College)		0.451	0.444	**0.006**
Education (≥ Bachelor's degree)		**0.012**	**0.009**	0.615
Income		0.600	0.581	0.587
Antibiotic Use (Yes)		**0.002**	**0.001**	**0.002**
Urbanicity (Rural)		0.545	0.544	0.551
Housing Age (Built 1979 or later)			0.908	0.885
Length of Residence			0.104	0.127
Tap Water Consumption			0.426	0.435
BMI			**0.021**	**0.016**
County (Eau Claire)			0.291	0.308
County (Milwaukee)			0.329	0.310
County (Waushara)			0.117	0.111
Water Filter (Yes)*Age				0.302
Water Filter (Yes)*Gender (Female)				0.889
Water Filter (Yes)*Income				**0.003**
Water Filter (Yes)*County (Eau Claire)				0.384
Water Filter (Yes)*County (Milwaukee)				0.428
Water Filter (Yes)*County (Waushara)				0.486

The QCAT_GEE test from the MiLineage package, adjusted for age, gender, income, education, antibiotic use, BMI and county, produced a list of several genera, families, orders, classes, and phyla that were significantly different by water filter use before correction for multiple comparisons (Table 4.6). After FDR correction, phylum Tenericutes, class Clostridia, order Clostridiales, family Lachnospiraceae, Lactobacillaceae, Rikenellaceae, Ruminococcaceae, geneus *Collinsella*, *Coprobacillus*, *Coprococcus*, *Lactobacillus, Prevotela,* and *Ruminococcus* all remained significant at the <0.05 level. The Zero-Part tests differences in presence of the taxa, and the Positive-Part tests differences in abundance of the taxa. The Two-Part tests combined presence and abundance of the taxa. Presence only was significantly different for orders

Eubacteriaceae and Rikenellaceae, and orders Lachnospiraceae and Ruminococcaceae were only significantly different when presence and abundance were combined. All other significant taxa were significantly different in presence only and when presence and abundance were combined. Tenericutes, Rikenellaceae, *Ruminococcus*, and *Collinsella* decreased with water filter use, and all other significant taxa increased with filter use, based on linear and logistic regression analysis.

Table 4.6. Output from QCAT-GEE analysis displaying bacterial taxa that differ by water filter use. Results shown are p-values before correction for multiple comparisons.

	Uncorrected P-Value		
Phylum	Two-Part	Zero-Part	Positive-Part
Actinobacteria	0.0198	0.0842	0.0376
Firmicutes	0.0198	0.4673	0.0099
Tenericutes	**0.0014**	**0.0014**	0.9333
Class			
Actinobacteria	0.0396	0.2851	0.0149
Clostridia	**0.0056**	**0.0025**	0.3585
Order			
Bacteroidales	0.0149	0.0436	0.1079
Clostridiales	**0.0010**	**0.0005**	0.1236
Family			
Eubacteriaceae	0.0218	**0.0059**	0.7069
Lachnospiraceae	**0.0002**	0.0656	0.0008
Lactobacillaceae	**0.0017**	**0.0012**	0.5284
Leuconostocaceae	0.0495	0.0485	0.9703
Micrococcaceae	0.0257	0.3307	0.0257
Peptococcaceae	0.0287	0.0693	0.0287
Rikenellaceae	0.0129	**0.0030**	0.9871
Ruminococcaceae	**0.0046**	0.0304	0.0236
Streptococcaceae	0.0475	0.0713	0.2178
Veillonellaceae	0.0309	0.0235	0.4119
Genus			
Blautia	0.0485	0.1386	0.0733
Collinsella	**0.0004**	**0.0007**	0.3009
Coprobacillus	**0.0038**	**0.0057**	0.1493
Coprococcus	**0.0001**	**0.0008**	0.0446

Desulfovibrio	0.0158	0.0158	1.0000
Dorea	0.0084	0.0090	0.1746
Lactobacillus	**0.0002**	**0.0001**	0.8538
Moryella	0.0121	0.0121	1.0000
Prevotella	**0.0046**	**0.0048**	0.3610
Roseburia	0.0188	0.0089	0.5812
Ruminococcus	**0.0002**	**0.0001**	0.3958
Veillonella	0.0376	0.0327	0.7782
Bold values were significant at the <0.05 level after FDR correction. Analysis was adjusted for age, gender, education, income, antibiotic use, BMI, and county.			

Sensitivity analysis with the Shannon index had similar results to the Invers-Simpson analysis with effects in the same direction and similar significance (Appendix C: ST8). Analysis with Chao-1 instead of ACE, also resulted in similar effect size and significance, although the interaction between water filter use and Waushara County was only marginally significant (ST9). PERMANOVA analysis using Jaccard distance as the outcome showed similar significance levels to Bray-Curtis PERMANOVA (ST10). Sensitivity analysis using imputed data for linear regressions with Inverse-Simpson as the outcome showed very similar main effects across all models for water filter use, with the only notable difference being an increase in significance for the interaction between water filter use and gender (ST11). Linear regressions of ACE with imputed data showed similar effects for water filter use and its interactions, with increased significance for the main effects of income and antibiotic use (ST12). PERMANOVA analysis of Bray-Curtis distance with imputed data found the same variables and interactions to be significantly different, with similar levels of significance (ST13). Interpretations of study findings are unchanged after examination of these sensitivity analyses.

4.5. DISCUSSION

In this analysis of home water filter use and the gut microbiota, α-diversity and richness were associated with water filter most strongly for those sampled from Waushara County. Differences in β-diversity by filter use were significant with increasing income level. Differences in specific bacterial taxa by filter use were significant across phylum, class, order, family, and genus. The minimal results of α-diversity, richness, and β-diversity may seem inconsistent with the number of individual taxa that were associated with water filter use. However, because these diversity measures aim to capture overall composition metrics, they may not be sensitive enough to detect the level of changes captured for individual taxa, especially if the changes in individual taxa counteract each other in such a way as to not alter the evenness of abundance between OTUs. Taken together, these findings suggest that household drinking water treatment is one environmental factor contributing to the composition of the gut microbiota.

Waushara County differs from the other counties sampled in this study because it is entirely rural, with study participants distributed widely throughout the county. 92.3% percent of participants from Waushara County get their water from a private well, compared to 8.0%, 33.9%, and 2.2%, in Brown, Eau Claire, and Milwaukee Counties, respectively. Wisconsin's well water supplies vary based on different levels of chemical constituents and other properties, making some more or less vulnerable to contamination from natural mineral supplies and agricultural runoff.[119] Further, water quality varies by source water type and treatment. Not all private wells are created equal; location, water source, land use, and practices used to create and maintain the well can have large impacts on the composition and safety of the drinking water.[184] Thus, private well testing is an important component of prevention in public health. Wisconsin well owners are responsible for testing and maintaining their own water quality, but in previous analysis of SHOW data, only about 10% reported having their water testing in the past year.[25]

Water treatment and filtering can be effective ways of removing many kinds of water contaminants including minerals, xenobiotics and microbes, and their effects are likely strongest for those who do not use publicly treated and distributed municipal water. This likely explains the strong effects seen on gut microbial composition for residents of Waushara County, a county largely served by private wells.

In home water treatment systems come in many forms, and can be used either at the point of entry, treating all water that enters the home, or at the point of use, treating the water immediately prior to consumption.[120] Filtration is a common point of use treatment method, including filters in pitchers, on the faucet, or in the refrigerator. These filters can be composed of ceramic, fabric or carbon, and physically remove contaminants including metals and some biological agents. Commonly used point of entry systems include aerators and water softeners. Aerators remove contaminants with high volatility, such as radon, by blowing air through the water using jets. Water softeners use salt to replace calcium and magnesium ions with sodium or potassium ions, but do not remove most contaminants of concern for health. Some point of entry units combine methods to remove multiple types of contaminants.[120] Filters, aerators, and water softeners were the most commonly used water treatment systems in this study population, indicating that the contaminants treated in this population varied widely. Because these treatment systems have a limited life span and are available at a wide range of quality standards, our findings of differences in β-diversity by income and filter use are not surprising, as it would be expected that those with higher income would be able to access higher quality products, and maintain them more faithfully. Further analysis of specific types of treatments on water from different sources is needed to better understand the mechanisms by which the gut microbiota may be altered by water treatment.

While there is still uncertainty about how differences in gut microbial composition influence health, there is some indication that drinking water filtration does alter the distribution of some specific taxa. The bacterial taxa found at significantly different levels by water filter use in this population are all typical inhabitants of the human digestive tract. They perform a wide range of functions within our gut, but a few in particular may have notable effects on human health. For instance the Lachnospiraceae family are known producers of butyric acid, which helps maintain a healthy epithelial lining within the gut.[185] Those using water filters may have increased Lachnospiraceae which could reduce the risk of gut disease and inflammation. Similarly, the family Lactobacillaceae are lactic acid producing bacteria commonly found in probiotic products, and are thought to confer a wide range of health benefits including preventing and ameliorating symptoms of infection.[186–188] While these results suggest there may be added health benefits to water filter use, further investigation is needed to support causal associations, interventions to promote water filtration, human health impacts and future policy implications.

This study is among the first to explore how a modifiable environmental factor, use of household water filtration may alter gut microbial composition. Other strengths of this study include the use of a well characterized sample with diversity in water source type. The use of 16S rRNA V4 amplicon sequencing for characterization of the gut microbiota is also a strength, as it allows for more complete identification of bacterial taxa than other methods, with less risk of falsely inflating OTU reads due to sequencing error, compared to longer regions of the 16S gene. Statistical modeling approaches used allowed for the inclusion of many important covariates while still allowing for interpretation of results based on more parsimonious models. The various sensitivity analyses conducted also add strength to the findings of the main analysis. Use of the QCAT_GEE to explore differences in bacterial taxa is also a strength, as it allows for the use of continuous predictors and adjustment for covariates, and it is more statistically valid for the data structure than alternative methods.

Antibiotic use and body mass index (BMI) were included in some of the models, although not considered to be true confounders of the association under investigation. Because recent antibiotic use has strong effects on microbial composition, many microbiome studies exclude participants based on this criterion. The SHOW's microbiome study queried antibiotic use in the past year, but because the gut microbiome can get back to a normal (or new normal) state within a few weeks, and approximately 50% of participants answered yes, exclusion would have drastically reduced our sample size. Therefore, the variable was included in the model building process, instead of as an exclusion criterion. BMI is not thought to be associated with water filter use; however, it is included in models because it is so closely correlated with gut microbial composition. The vast majority of human gut microbiome studies are adjusted for BMI, thus not including it in models would limit the comparability of our findings to those of other studies.

Despite this study being the first of its kind to examine drinking water source and its relationship to human gut microbiome composition, this analysis includes some limitations to consider. The cross-sectional nature of the data limit findings to associations rather than causal relationships. This data set is also limited in racial and ethnic diversity, weakening its generalizability to other populations. There was a relatively large amount of missing data for some of the regression analyses. This could cause estimates to be biased if differentially associated with the predictor variables, however sensitivity analysis conducted with imputed data suggests that our findings are robust to the impacts of missing data. The use of self-reported exposure variables may also result in biased effect estimates due to misclassification of exposure.

Other limitations of the microbiota analysis specifically include the use 16s rRNA amplicon sequencing rather than metagenomic sequencing, and the limited information on antibiotic use. While 16s rRNA amplicon sequencing is a great place to begin examining the composition of the gut microbiota, it does not permit the functional analysis that metagenomics allow. Further analysis of metabolic functions within the microbiota give a better explanation of what is happening within the gut. A similarity in gene function may account for differences we see in colonization by differing taxa. The use of self-reported exposure to antibiotics in the previous year is not the ideal measure of exposure for several reasons. The effects of antibiotics on the gut microbiota are strong, but within a short time of finishing treatment, the microbiota either return to their previous state, or come to a new normal state that is different from the pre- and during-antibiotics state. For the purposes of this study, it would be most useful to know about antibiotic use in the past 2-4 weeks, and exclude participants whose microbiota are not in their usual state due to antibiotic use. Because we do not have this information, we account for the self-reported past year variable in our models, but do not exclude those participants from our analyses. This variable is also problematic however, because it is subject to recall bias.

This analysis leads to many natural directions for future research. Much further analysis can be done to examine the effects of specific types of treatment systems and specific sources of drinking water. Further analysis of these samples could also be done using metagenomic analysis for a more in depth view of metabolic pathways that may be altered within the microbiome. Differences in these bacterial taxa and metabolic pathways could also be linked to downstream health effects. Composition of other human microbiomes could also be examined, as water treatment practices likely also effect microbes living on the skin and in the mouth. Examining these effects in other populations with different age and other demographic distributions would also add greatly to our understanding of home water treatment and human microbiota.

4.6. CONCLUSION

This study found that household water treatment was associated with significant differences in gut microbial composition, in a population-based adult sample. Further examination of this association and its downstream health effects is warranted.

Chapter 5. Conclusion

5.1. SUMMARY OF RESULTS AND CONCLUSIONS

The gut microbiota plays an important role in both chronic and acute diseases, interacting with immune function, inflammation, and other important biological processes. This study is significant because understanding as much as possible about the gut microbiota, and how to prevent dysbiosis, is critical to the prevention of many adverse health effects, including infection. Colonization and subsequent infection by ARB is a major health crisis and effective treatment options are becoming increasingly rare. Therefore, effective strategies for prevention of ARB colonization are key to reducing antibiotic resistance in the future, and understanding the association between Pb exposure and ARB colonization is a first step in developing effective prevention strategies. Moreover, characterizing the role of household water treatment in altering microbial composition adds insight into possible mechanisms for prevention of dysbiosis. This dissertation provides evidence that Pb exposure may play an important role in this pathway, thus limiting exposure to Pb, even at levels previously considered safe, may be a significant step in limiting the various disease outcomes associated with dysbiosis of the gut microbiota.

The purpose of this dissertation was to evaluate Pb exposure in a general population of adults, and its association with the composition of the human gut microbiota, including colonization by ARB. This dissertation further examines the association between the use of household water treatment and gut microbial composition, as a potential prevention strategy to reduce Pb exposure, and exposure to other xenobiotics and bacterial contaminants of drinking water. This study is the first large scale human study to explore heavy metal exposures and its relationship with gut microbial composition, antibiotic resistance, and water filtration in a general adult population based study. Findings will add to a growing body of literature from animal studies that support the role of xenobiotic chemicals in gut homeostasis and immune function.

Chapter 2 showed a number of significant findings not previously reported in human populations. This sample is unique in that Pb exposure is lower among the study sample of randomly selected adults from a household based survey of Wisconsin residents (GM= 0.30 μg/L) than in the United States overall (GM=0.45 μg/L).[152] Firmicutes were the dominant bacterial phylum within the gut microbiota across all levels of Pb exposure in the study population. This is different than what was found in other humans studies,[29] and this is likely due to the high prevalence of BMI > 25 in this study population.

Factors that appear to increase susceptibility to Pb exposure and alter or modify gut dysbiosis and associations with Pb include gender and diet. Even among this general population based sample, with relatively low exposure levels, increasing Pb exposure was associated with decreasing gut microbial α-diversity, but not richness, among females only. These findings indicate that on average Pb exposure was associated with reduced the evenness of abundance but not the number of species present in each individual's gut microbiota. The finding that the effects of Pb on gut microbial composition differed by gender is consistent with previous findings that gender is an important covariate to consider in gut microbial analysis.[189,190] This association likely plays a role in mediating downstream health effects that are associated with gender, such as irritable bowel syndrom.[191]

Dietary fiber had significant effects on α-diversity, and a marginally significant interaction with Pb, indicating that increasing dietary fiber intake may reduce the negative impacts of Pb on microbial diversity. Increasing Pb exposure was also associated with significant differences in β-diversity, meaning that composition of the gut microbiota was increasingly different with increased Pb exposure. Marginally significant effects of the interaction between gender and Pb, indicate that this association may be stronger for women than men. Gut colonization by the genus *Desulfovibrio,* a common sulfur reducing bacteria, increased with Pb exposure. The finding of significant differences in colonization by *Desulfovibrio,* and its potential to increase tolerance to heavy metals and antibiotics within the gut microbiota adds an additional previously unconsidered pathway between Pb exposure and antibiotic resistance that is explored in Chapter 3.

This study was the first to examine the association between Pb and gut microbial composition in a large sample of human adults. This analysis is the first attempt at understanding this complex relationship, and it establishes a baseline for all future studies to compare. While some findings confirmed associations seen in other studies, such as the association between dietary fiber and gut microbial composition,[190] this study also contributed to the current state of the literature by translating some findings, including changes in α and β-diversity by Pb level, from animal models to a human population.[12] This study was also unique in its use of a randomly sampled population with a wide range of ages and urbanicity. The use of this unique population allows for broader generalizability of results, as well as adding the ability to examine the interaction of effects between these variables.

These findings provide important insights to the field of environmental health research because they reinforce and translate the findings from animal studies to humans, and illustrate that even at relatively low levels of exposure, Pb is associated with subtle changes in our microbiota. This knowledge is meaningful because even these small changes over an extended period of exposure can lead to tangible health impacts. Although the Pb is relatively well known for its detrimental health effects, our society is mostly concerned about its toxicity in children and adults who are highly occupationally exposed. These findings illustrate that there is cause for concern about Pb exposure for adults who are exposed to Pb even at relatively low levels. Given the importance of maintaining a healthy and diverse gut, reducing environmental exposures such as Pb by maintaining policies and programs for Pb reduction should remain important public health goals.

Chapter 3 examined the associations between Pb exposures and relationships with prevalence of ARB in the gut. Findings indicate a large number of individuals, 32% of participants, were colonized with ARB. This suggests that while not everyone is colonized by ARB, that these bacteria continue to be highly prevalent and detectable in a substantial number of human guts. It was also found that ARB colonization varied across demographic groups and by exposure to Pb. Pb exposure was associated with increased odds of ARB colonization for those in the highest income level. Gut microbial diversity did not appear to mediate the relationship between Pb exposure and ARB colonization. RGNB isolated from stool samples were much more resistant to Pb than MRSA or VRE, however, higher individual Pb exposure was not associated with increased level of Pb resistance.

This study is the first epidemiologic study to examine the association between Pb exposure and multiple ARB colonization (Chapter 3). Taken together with our previous study in NHANES examining MRSA colonization,[92] it is clear that an association between Pb exposure and ARB colonization exists, at least in some subsets of the population. Although the natural history of this association in humans is yet unknown, the findings of this study are important as they suggest that there may be opportunities for prevention and intervention to reduce Pb exposure and downstream ARB colonization and infection, especially for those at high risk. One possible mechanism of intervention to reduce the effects of Pb exposure is eating a healthy diet, high in dietary fiber. Analyses in Chapters 2 and 3 included several dietary variables, including fiber, which had significant impacts on the effects of Pb. While the idea that a healthy diet can ameliorate the toxicity of Pb is not novel,[155] this analysis adds new support to the argument, which may not have been previously considered.

Chapter 4 examined associations between household water filtration use and gut microbial composition. 30% of participants reported using a household water filter or treatment system. Aerators, charcoal filters, and water softeners were the most frequently reported treatments types. Aerators are most effective at reducing water contaminants with high volatility such as radon, while charcoal filters physically remove contaminant particulates, and can be certified for removal of many different types of contaminants. Water softeners make water "soft" by changing the acidity of water by exchanging sodium and potassium salts for magnesium and calcium ions.[120] These various treatment methods are effective at reducing a variety of different contaminants, however, of the three varieties most commonly used in this population, only charcoal filter would likely remove either Pb or bacterial contaminants. Furthermore, because filter materials and certifications can vary widely, and effectiveness depends on upkeep and regular replacement of the filter, it is likely that relatively few study participants are using treatment methods that would effectively reduce Pb contamination.

In general, gut microbial α-diversity decreased with water filter use, especially for those living in Waushara County. Gut microbial α-diversity increased with water filter use and increasing age. Similarly, gut microbial richness decreased overall with water filter use, and effects were strongest for those in Waushara County, and for those with the highest income. Participants from Waushara County, unlike the other counties samples, come entirely from rural communities, with 92% using private wells that draw from groundwater as their primary water source. These significant differences in diversity based on water treatment in Waushara County suggest that household water treatments have the most impact on gut microbial composition for people who are drinking otherwise untreated groundwater.

Differences in β-diversity were also significant with water filter use as income increased. Differences by income might be explained in that those with higher income are likely better able to properly maintain their filter and treatment systems, so they can be most effective at removing contamination. Phylum Tenericutes, class Clostridia, order Clostridiales, family Lachnospiraceae, Lactobacillaceae, Rikenellaceae, Ruminococcaceae, geneus *Collinsella*, *Coprobacillus*, *Coprococcus*, and *Lactobacillus* were significantly different by water filter use. These bacteria are all normal gut colonizers that play a role in metabolizing different nutrients, however, some of these bacteria, such as Lachnospiraceae and Lactobacillaceae, are known to have beneficial health properties, including ameliorating symptoms of irritable bowel disease and infection, and are commonly found in probiotic products.[46,185–187] Both of these families increased upon water treatment use, suggesting that the changes in gut microbial composition from water filter use likely lead to positive health outcomes.

This dissertation adds to the literature by newly examining the association between gut microbial composition and household water treatment, which was previously unexplored (Chapter 4). Understanding this association is important, as it helps us better understand the potential role of household water treatment in prevention of exposures that can alter gut microbial composition and its downstream health effects. While household water treatment offers direct health benefits by reducing exposure to chemical and biological contaminants, the findings of this study suggest additional health benefits may be gained through changes to the gut microbiota. Because different water treatment systems are effective against varying types of contaminants, further examination is needed to understand mechanisms of action, and to determine which type is most effective for altering the gut microbiota in different ways. However, this study is a crucial first step in examining this association as it confirms that relatively simple interventions to environmental exposures can have significant impacts on our gut microbes that may have health benefits.

Aim 3, examined in Chapter 4, was based on the hypothesis that water filtration would potentially alter Pb exposure and reduce any potentially adverse effects seen by Pb exposure, this hypothesis did not appear to hold true with these study findings. When comparing the findings from Chapters 2 and 4, Pb exposure and water treatment have different effects on gut microbial composition, and the types of treatments most commonly used suggest little Pb was likely removed from the drinking water by these mechanisms. This suggests that the mechanism of action for the measure of water treatment used in this analysis is not primarily through altered Pb exposure. Although Pb may play a part, the altered gut microbial composition associated with water filter use is likely affected by difference in a variety of chemical and biological exposures.

When findings from this dissertation are considered as a whole, it is clear that environmental exposures play a role in shaping the composition of the adult gut microbiota, including the presence of ARB. These shifts in microbial composition caused by Pb may lead to or amplify the adverse health outcomes caused by Pb. The relationships between environmental contamination, the gut microbiota, and human health are complex. Not only did this study address this by adding novel insight to the relationship between Pb and gut microbial composition in humans, but it used an innovative approach of additionally examining both an upstream predictor of exposure, and a downstream health outcome. Moreover, this study is unique in its multi-disciplinary approach to understanding these relationships. This study incorporates techniques and expertise from the fields of environmental toxicology, epidemiology, microbiology and infectious disease. The integration of these fields is critical to examining and understanding this complex problem in a holistic manner.

5.2. STRENGTHS AND LIMITATIONS

This project has some important strengths to consider. A key strength is that the aims examined here have not yet been examined in a real-world, adult, human population. While animal and in vitro models are critical to the understanding of the underlying biological mechanisms at play, epidemiological studies are necessary to determine if these mechanisms translate to humans on a population scale. The large scale, and use of a population sample for this project is also a strength. Few microbiota studies in humans use a sample size this large, and none of those studies have examined a link to environmental factors. Having the use of a large population-based sample not only added statistical power to detect effects, but gave a better depiction of how these associations work in the general population, as opposed to a population of sick, highly exposed, or highly motivated individuals. An additional strength of using this study population is leveraging a pre-existing, ongoing survey infrastructure by adding novel sample analytics to undertake a study that would otherwise have been quite cost-prohibitive.

Several study design elements also added to the strength of this project. The use of high throughput sequencing technology gives a much more accurate picture of the microbial content than culture methods. The use of the V4 hypervariable region of the 16S rRNA gene for sequencing allows for reliable identification of most bacterial taxa while reducing the possibility of falsely inflating the OTU reads due to sequencing error.[192] The comprehensive approach to examining the relationship between Pb exposure and gut microbial composition, by looking at the direct effects, a potential health outcome in ARB colonization, and a potential measure of exposure prevention in water filter use, is also a strength of this dissertation.

The statistical modeling approach of utilizing multiple models with different levels of confounding variables allows for inference with and without the potential of over and under adjusting. Sensitivity analyses with alternate outcome measures as well as imputed data adds greatly to the robustness of the findings as well. The use of the QCAT_GEE test,[145] a relatively novel procedure, also adds strength to the study by allowing for statistical analysis of differences in bacterial taxa with a continuous variable as the main predictor, which was not possible prior to the development of this test. The QCAT_GEE test also fits the OTU data structure of many zeros better than alternative testing methods.

While this study adds to the current knowledge about Pb exposure, the microbiota, and ARB colonization, there are some limitations to the analysis. The use of ARB colonization as an endpoint in Chapter 3 instead of infection is somewhat problematic. Not all bacteria that are resistant to antibiotics are also pathogenic, meaning that not every colonizing organism will lead to an infection. Antibiotic-resistant isolates identified in this study are not tested for the presence of pathogenicity genes. Colonization by ARB is, however, a strong risk factor for subsequent infection, because antibiotic-resistance genes and pathogenicity islands can be easily shared via horizontal gene transfer. [100,101,193,194] While ARB colonization is an imperfect surrogate measure of infection, it is useful in this population-based sample where prevalence of infection by ARB is likely to be low.

A limitation of using this study population is that it includes adults only. Studies on the detrimental effects of Pb typically use child populations as they are more highly exposed to Pb, and Pb is more readily absorbed in the body. For the purposes of this study, however, we want to know the level of Pb that enters the gut and to test the association between chronic lead exposure on gut microbiota, so differential absorption rate in children is not a concern. Urinary Pb level is perhaps not the best measure of exposure to the gut microbes because only 10% the Pb that enters the gut is absorbed into the blood stream, and less makes it into the urine.[130] There may be additional variability in measurement for post-menopausal females as addition bone structure changes are occurring that may release additional Pb into the blood stream.[195] Because the gut microbiota may play a role in metabolizing Pb, any association found between urine Pb level and gut microbial diversity may also be due to reverse causality. However, because urine also reflects chronic Pb that has been stored in bones and blood cells over the lifetime, and the differences in composition of the gut microbiota that we see are likely the result of long term exposure to Pb, urine may be a better matrix for measurement exposure than stool, for instance which measures only short-term exposure.

Self-reported household water filter/treatment use, used in Chapter 4, is also an imperfect exposure measurement. Some participants do not accurately report the treatment system used, and some participants use more than one treatment type, which is not captured in the dichotomous classification. Furthermore, grouping different treatment approaches that treat different types of contaminants in different ways, may not be appropriate to determine effects on the gut microbiota because biological pathways are likely different for different treatment types. However, our analysis was limited to this exposure grouping due to limited sample size within each treatment type.

Fecal samples are a useful matrix for examining the contents of the gut microbiota, however, some aspects of sample collection could have been improved upon in this study. For example, SHOW's ancillary microbiome study collects only one stool sample per participant, which gives a cross-sectional snapshot of microbiota, but may not accurately represent the usual composition. Contents of the fecal microbiota may also be subject to seasonal changes, therefore it is most accurate when samples from the entire population are collected at the same time, or at least within the same season. Samples from this study were collected from May, 2016 to January, 2017. The storage protocol for samples used for sequencing is important to consider, as different protocols can lead to differing results.[196] Rapid freezing at -80^{O}C is considered best practice, however, bacterial DNA can be damaged with each freeze-thaw cycle. Fecal samples from this study went through at least one freeze-thaw cycle before sequencing, which could potentially alter the bacterial DNA detected in sequencing.

5.3. FUTURE DIRECTIONS

This study establishes a foundation for many different studies in the future. More studies in populations with higher and lower Pb exposure levels would help establish a dose response relationship, and determine risks of Pb exposure at levels currently thought to be less dangerous. Including populations with different demographic distributions would also be useful in understanding the generalizability of these findings.

Another avenue of future study is to examine the effects of Pb on the microbiota and ARB colonization in children. Because Pb exposure is absorbed differently and leads to different health effects in children than adults, the relationship seen in this study population may not be the same in children. Future analysis of the role of the microbiota on Pb absorption rates, and neurotoxicity in children would also add greatly to the literature.

This study could also be replicated using many other environmental exposures, including other xenobiotics, mixtures of exposures, and macro-level variables, including other potential prevention techniques, in adult and child populations. Two additional ancillary SHOW studies of the microbiome began collecting samples in 2018. One will collect stool and nasal samples from young children, which could be used to examine some environmental exposures in that population. The other will collect a follow-up stool sample from many of the participants in this study, as well as additional environmental samples from within and around their homes. Future analysis could be done using these samples to examine longitudinal changes in Pb exposure, or combinations of exposure to Pb and other metals, and microbiota composition, as well as using the environmental samples as novel sources for exposure measurement.

Additional analysis on the current study population could also be done using other “omics” analysis. The use of metagenomics and metabolomics could add a great amount of insight into the metabolic pathways that are altered by the noted changes in the gut microbiota. Analysis of the metabolic pathways would allow for better understanding of the downstream health consequences caused by these shifts.

Further studies could also examine many other health outcomes, including interactions between Pb, the gut microbiota, and immune function. Perhaps such studies would be most effective in children because their immune systems are still under development, and Pb exposure would likely have much stronger effects. Additional examination of antibiotic resistance is another potential avenue in child and adult populations. Studies done examining a range of antibiotic resistance genes, rather than culturing for a specific few ARB as done here, would also add greatly to the literature. Moreover, analysis of abundance of ARB and antibiotic resistance genes, rather than presence or absence would allow for further assessment of the relationship between Pb and ARB.

References:

1. Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut Microbiota in Health and Disease. Physiol Rev. 2010 Jul 1;90(3):859–904. PMID: 20664075

2. Bull MJ, Plummer NT. Part 1: The Human Gut Microbiome in Health and Disease. Integr Med Clin J. 2014 Dec;13(6):17–22. PMCID: PMC4566439

3. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB. Decreased Diversity of the Fecal Microbiome in Recurrent Clostridium difficile—Associated Diarrhea. J Infect Dis. 2008 Feb 1;197(3):435–438. PMID: 18199029

4. Claus SP, Guillou H, Ellero-Simatos S. The gut microbiota: a major player in the toxicity of environmental pollutants? Npj Biofilms Microbiomes. 2016 May 4;2:16003.

5. Jarosławiecka A, Piotrowska-Seget Z. Lead resistance in micro-organisms. Microbiology. 2014;160(1):12–25.

6. Abadin H, Ashizawa A, Stevens Y-W, Llados F, Diamond G, Sage G, Citra M, Quinones A, Bosch SJ, Swarts SG. Toxicological Profile for Lead. U.S. Departmentn of Health and Human Services; 2007.

7. Breton J, Massart S, Vandamme P, De Brandt E, Pot B, Foligné B. Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. BMC Pharmacol Toxicol. 2013;14:62.

8. Wu J, Wen XW, Faulk C, Boehnke K, Zhang H, Dolinoy DC, Xi C. Perinatal Lead Exposure Alters Gut Microbiota Composition and Results in Sex-specific Bodyweight Increases in Adult Mice. Toxicol Sci. 2016 Jun 1;151(2):324–333. PMID: 26962054

9. Bisanz JE, Enos MK, Mwanga JR, Changalucha J, Burton JP, Gloor GB, Reid G. Randomized Open-Label Pilot Study of the Influence of Probiotics and the Gut Microbiome on Toxic Metal Levels in Tanzanian Pregnant Women and School Children. mBio. 2014 Oct 31;5(5):e01580-14. PMID: 25293764

10. Roane TM. Lead Resistance in Two Bacterial Isolates from Heavy Metal–Contaminated Soils. Microb Ecol. 37(3):218–224.

11. Tan TL. Effect of long-term lead exposure on the seawater and sediment bacteria from heterogeneous continuous flow cultures. Microb Ecol. 1980 Dec 1;5(4):295–311.

12. Gao B, Chi L, Mahbub R, Bian X, Tu P, Ru H, Lu K. Multi-Omics Reveals that Lead Exposure Disturbs Gut Microbiome Development, Key Metabolites, and Metabolic Pathways. Chem Res Toxicol. 2017 Apr 17;30(4):996–1005.

13. Zhai Q, Li T, Yu L, Xiao Y, Feng S, Wu J, Zhao J, Zhang H, Chen W. Effects of subchronic oral toxic metal exposure on the intestinal microbiota of mice. Sci Bull. 2017 Jun 30;62(12):831–840.

14. Xia J, Jin C, Pan Z, Sun L, Fu Z, Jin Y. Chronic exposure to low concentrations of lead induces metabolic disorder and dysbiosis of the gut microbiota in mice. Sci Total Environ. 2018 Mar 9;631–632:439–448. PMID: 29529432

15. Xia J, Lu L, Jin C, Wang S, Zhou J, Ni Y, Fu Z, Jin Y. Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish. Comp Biochem Physiol Part C Toxicol Pharmacol. 2018 Jul 1;209:1–8.

16. Keesing F, Belden LK, Daszak P, Dobson A, Harvell CD, Holt RD, Hudson P, Jolles A, Jones KE, Mitchell CE, Myers SS, Bogich T, Ostfeld RS. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature. 2010 Dec 2;468(7324):647–652.

17. Madan JC, Salari RC, Saxena D, Davidson L, O'Toole GA, Moore JH, Sogin ML, Foster JA, Edwards WH, Palumbo P, Hibberd PL. Gut microbial colonisation in premature neonates predicts neonatal sepsis. Arch Dis Child - Fetal Neonatal Ed. 2012 Nov 1;97(6):F456–F462. PMID: 22562869

18. Dillon RJ, Vennard CT, Buckling A, Charnley AK. Diversity of locust gut bacteria protects against pathogen invasion. Ecol Lett. 2005 Dec 1;8(12):1291–1298.

19. Center for Disease Control and Prevention. Antibiotic Resistance Threats in the United States, 2013. U.S. Department of Health and Human Services; 2013 Apr.

20. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol. 2006 Apr;14(4):176–182.

21. Aktan Y, Tan S, Icgen B. Characterization of lead-resistant river isolate Enterococcus faecalis and assessment of its multiple metal and antibiotic resistance. Environ Monit Assess. 2013 Jun;185(6):5285–93.

22. Calomiris JJ, Armstrong JL, Seidler RJ. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl Environ Microbiol. 1984 Jun 1;47(6):1238–1242. PMID: 6742841

23. Nisanian M, Holladay SD, Karpuzoglu E, Kerr RP, Williams SM, Stabler L, McArthur JV, Tuckfield RC, Gogal RM. Exposure of juvenile Leghorn chickens to lead acetate enhances antibiotic resistance in enteric bacterial flora. Poult Sci. 2014 Apr 1;93(4):891–897. PMID: 24706966

24. Ug A, Ceylan Ö. Occurrence of Resistance to Antibiotics, Metals, and Plasmids in Clinical Strains of Staphylococcus spp. Arch Med Res. 2003 Mar;34(2):130–136.

25. Malecki KMC, Schultz AA, Severtson DJ, Anderson HA, VanDerslice JA. Private-well stewardship among a general population based sample of private well-owners. Sci Total Environ. 2017 Dec 1;601–602:1533–1543. PMCID: PMC5662198

26. Pinto AJ, Xi C, Raskin L. Bacterial Community Structure in the Drinking Water Microbiome Is Governed by Filtration Processes. Environ Sci Technol. 2012 Aug 21;46(16):8851–8859.

27. Masten SJ, Davies SH, Mcelmurry SP. Flint Water Crisis: What Happened and Why? J - Am Water Works Assoc. 2016 Dec;108(12):22–34. PMCID: PMC5353852

28. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet. 2012 Apr;13(4):260–270.

29. The Human Microbiome Project Consortium. Structure, Function and Diversity of the Healthy Human Microbiome. Nature. 2012 Jun 13;486(7402):207–214. PMCID: PMC3564958

30. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the Human Intestinal Microbial Flora. Science. 2005 Jun 10;308(5728):1635–1638. PMID: 15831718

31. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. Curr Opin Immunol. 2014 Aug;29:16–22. PMCID: PMC4132187

32. Methé BA, Nelson KE, Pop M, Creasy HH, Giglio MG, Huttenhower C, Gevers D, Petrosino JF, Abubucker S, Badger JH, Chinwalla AT, Earl AM, FitzGerald MG, Fulton RS, Hallsworth-Pepin K, Lobos EA, Madupu R, Magrini V, Martin JC, Mitreva M, Muzny DM, Sodergren EJ, Versalovic J, Wollam AM, Worley KC, Wortman JR, Young SK, Zeng Q, Aagaard KM, Abolude OO, Allen-Vercoe E, Alm EJ, Alvarado L, Andersen GL, Anderson S, Appelbaum E, Arachchi HM, Armitage G, Arze CA, Ayvaz T, Baker CC, Begg L, Belachew T, Bhonagiri V, Bihan M, Blaser MJ, Bloom T, Vivien Bonazzi J, Brooks P, Buck GA, Buhay CJ, Busam DA, Campbell JL, Canon SR, Cantarel BL, Chain PS, Chen I-MA, Chen L, Chhibba S, Chu K, Ciulla DM, Clemente JC, Clifton SW, Conlan S, Crabtree J, Cutting MA, Davidovics NJ, Davis CC, DeSantis TZ, Deal C,

Delehaunty KD, Dewhirst FE, Deych E, Ding Y, Dooling DJ, Dugan SP, Dunne WM, Durkin AS, Edgar RC, Erlich RL, Farmer CN, Farrell RM, Faust K, Feldgarden M, Felix VM, Fisher S, Fodor AA, Forney L, Foster L, Di Francesco V, Friedman J, Friedrich DC, Fronick CC, Fulton LL, Gao H, Garcia N, Giannoukos G, Giblin C, Giovanni MY, Goldberg JM, Goll J, Gonzalez A, Griggs A, Gujja S, Haas BJ, Hamilton HA, Harris EL, Hepburn TA, Herter B, Hoffmann DE, Holder ME, Howarth C, Huang KH, Huse SM, Izard J, Jansson JK, Jiang H, Jordan C, Joshi V, Katancik JA, Keitel WA, Kelley ST, Kells C, Kinder-Haake S, King NB, Knight R, Knights D, Kong HH, Koren O, Koren S, Kota KC, Kovar CL, Kyrpides NC, La Rosa PS, Lee SL, Lemon KP, Lennon N, Lewis CM, Lewis L, Ley RE, Li K, Liolios K, Liu B, Liu Y, Lo C-C, Lozupone CA, Lunsford RD, Madden T, Mahurkar AA, Mannon PJ, Mardis ER, Markowitz VM, Mavrommatis K, McCorrison JM, McDonald D, McEwen J, McGuire AL, McInnes P, Mehta T, Mihindukulasuriya KA, Miller JR, Minx PJ, Newsham I, Nusbaum C, O'Laughlin M, Orvis J, Pagani I, Palaniappan K, Patel SM, Pearson M, Peterson J, Podar M, Pohl C, Pollard KS, Priest ME, Proctor LM, Qin X, Raes J, Ravel J, Reid JG, Rho M, Rhodes R, Riehle KP, Rivera MC, Rodriguez-Mueller B, Rogers Y-H, Ross MC, Russ C, Sanka RK, Pamela Sankar J, Sathirapongsasuti F, Schloss JA, Schloss PD, Schmidt TM, Scholz M, Schriml L, Schubert AM, Segata N, Segre JA, Shannon WD, Sharp RR, Sharpton TJ, Shenoy N, Sheth NU, Simone GA, Singh I, Smillie CS, Sobel JD, Sommer DD, Spicer P, Sutton GG, Sykes SM, Tabbaa DG, Thiagarajan M, Tomlinson CM, Torralba M, Treangen TJ, Truty RM, Vishnivetskaya TA, Walker J, Wang L, Wang Z, Ward DV, Warren W, Watson MA, Wellington C, Wetterstrand KA, White JR, Wilczek-Boney K, Wu YQ, Wylie KM, Wylie T, Yandava C, Ye L, Ye Y, Yooseph S, Youmans BP, Zhang L, Zhou Y, Zhu Y, Zoloth L, Zucker JD, Birren BW, Gibbs RA, Highlander SK, Weinstock GM, Wilson RK, White O. A framework for human microbiome research. Nature. 2012 Jun 13;486(7402):215–221. PMCID: PMC3377744

33. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto J-M, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalog established by metagenomic sequencing. Nature. 2010 Mar 4;464(7285):59–65. PMCID: PMC3779803

34. Huse SM, Ye Y, Zhou Y, Fodor AA. A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters. PLoS ONE [Internet]. 2012 Jun 13 [cited 2016 Nov 7];7(6). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3374614/ PMCID: PMC3374614

35. Morgan XC, Segata N, Huttenhower C. Biodiversity and Functional Genomics in the Human Microbiome. Trends Genet TIG. 2013 Jan;29(1):51–58. PMCID: PMC3534939

36. Morgan XC, Huttenhower C. Chapter 12: Human Microbiome Analysis. PLOS Comput Biol. 2012 Dec 27;8(12):e1002808.

37. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R. Human gut microbiome viewed across age and geography. Nature. 2012 Jun 14;486(7402):222–227.

38. Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, Björkstén B, Engstrand L, Andersson AF. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. Gut. 2014 Apr 1;63(4):559–566. PMID: 23926244

39. Dominianni C, Sinha R, Goedert JJ, Pei Z, Yang L, Hayes RB, Ahn J. Sex, body mass index, and dietary fiber intake influence the human gut microbiome. PloS One. 2015;10(4):e0124599. PMCID: PMC4398427

40. Markle JGM, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, Bergen M von, McCoy KD, Macpherson AJ, Danska JS. Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. Science. 2013 Mar 1;339(6123):1084–1088. PMID: 23328391

41. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012 Sep 13;489(7415):220–230.

42. Khachatryan ZA, Ktsoyan ZA, Manukyan GP, Kelly D, Ghazaryan KA, Aminov RI. Predominant Role of Host Genetics in Controlling the Composition of Gut Microbiota. PLOS ONE. 2008 Aug 26;3(8):e3064.

43. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. Nature. 2009 Jan 22;457(7228):480–484.

44. Wang J, Kurilshikov A, Radjabzadeh D, Turpin W, Croitoru K, Bonder MJ, Jackson MA, Medina-Gomez C, Frost F, Homuth G, Rühlemann M, Hughes D, Kim H, Spector TD, Bell JT, Steves CJ, Timpson N, Franke A, Wijmenga C, Meyer K, Kacprowski T, Franke L, Paterson AD, Raes J, Kraaij R, Zhernakova A. Meta-analysis of human genome-microbiome association studies: the MiBioGen consortium initiative. Microbiome. 2018 Jun 8;6:101.

45. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across

multiple body habitats in newborns. Proc Natl Acad Sci. 2010 Jun 29;107(26):11971–11975. PMID: 20566857

46. Butel M-J. Probiotics, gut microbiota and health. Med Mal Infect. 2014 Jan;44(1):1–8. PMID: 24290962

47. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiol Read Engl. 2010 Nov;156(Pt 11):3216–3223. PMID: 20705661

48. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology. 2004 May;126(6):1620–1633.

49. Serre CB de L, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol - Gastrointest Liver Physiol. 2010 Aug 1;299(2):G440–G448. PMID: 20508158

50. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan 23;505(7484):559–63.

51. Maukonen J, Saarela M. Human gut microbiota: does diet matter? Proc Nutr Soc. 2015 Feb;74(01):23–36.

52. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, Almeida M, Quinquis B, Levenez F, Galleron N, Gougis S, Rizkalla S, Batto J-M, Renault P, consortium AM, Doré J, Zucker J-D, Clément K, Ehrlich SD, Blottière H. Dietary intervention impact on gut microbial gene richness. Nature. 2013 Aug 29;500(7464):585–588.

53. Claesson MJ, Jeffery IB, Conde S, Power SE, O’Connor EM, Cusack S, Harris HMB, Coakley M, Lakshminarayanan B, O’Sullivan O, Fitzgerald GF, Deane J, O’Connor M, Harnedy N, O’Connor K, O’Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012 Aug 9;488(7410):178–184.

54. Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen Y, Knight R, Ahima RS, Bushman F, Wu GD. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. Gastroenterology. 2009 Nov;137(5):1716-1724.e2.

55. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Sci Transl Med. 2009 Nov 11;1(6):6ra14. PMCID: PMC2894525

56. Filippo CD, Cavalieri D, Paola MD, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci. 2010 Aug 17;107(33):14691–14696. PMID: 20679230

57. Lu K, Mahbub R, Fox JG. Xenobiotics: Interaction with the Intestinal Microflora. ILAR J. 2015 Aug 31;56(2):218–227. PMID: 26323631

58. Logan AC. Dysbiotic drift: mental health, environmental grey space, and microbiota. J Physiol Anthropol. 2015;34:23.

59. Hoisington AJ, Brenner LA, Kinney KA, Postolache TT, Lowry CA. The microbiome of the built environment and mental health. Microbiome. 2015;3:60.

60. Biedermann L, Zeitz J, Mwinyi J, Sutter-Minder E, Rehman A, Ott SJ, Steurer-Stey C, Frei A, Frei P, Scharl M, Loessner MJ, Vavricka SR, Fried M, Schreiber S, Schuppler M, Rogler G. Smoking Cessation Induces Profound Changes in the Composition of the Intestinal Microbiota in Humans. PLOS ONE. 2013 Mar 14;8(3):e59260.

61. Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, Flores SC, Fontenot AP, Ghedin E, Huang L, Jablonski K, Kleerup E, Lynch SV, Sodergren E, Twigg H, Young VB, Bassis CM, Venkataraman A, Schmidt TM, Weinstock GM. Comparison of the Respiratory Microbiome in Healthy Nonsmokers and Smokers. Am J Respir Crit Care Med. 2013 Mar 14;187(10):1067–1075.

62. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, Caporaso JG, Knights D, Clemente JC, Nakielny S, Gordon JI, Fierer N, Knight R. Cohabiting family members share microbiota with one another and with their dogs. eLife. 2013 Apr 16;2:e00458. PMID: 23599893

63. Hooper LV, Midtvedt T, Gordon JI. How Host-Microbial Interactions Shape the Nutrient Environment of the Mammalian Intestine. Annu Rev Nutr. 2002;22(1):283–307. PMID: 12055347

64. Diaz-Bone RA, Van de Wiele TR. Biovolatilization of Metal(loid)s by Intestinal Microorganisms in the Simulator of the Human Intestinal Microbial Ecosystem. Environ Sci Technol. 2009 Jul 15;43(14):5249–5256.

65. Breton J, Daniel C, Dewulf J, Pothion S, Froux N, Sauty M, Thomas P, Pot B, Foligné B. Gut microbiota limits heavy metals burden caused by chronic oral exposure. Toxicol Lett. 2013 Oct 24;222(2):132–138.

66. Dietert RR, Silbergeld EK. Biomarkers for the 21st century: listening to the microbiome. Toxicol Sci Off J Soc Toxicol. 2015 Apr;144(2):208–216. PMID: 25795652

67. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. J Physiol. 2009 Sep 1;587(Pt 17):4153–4158. PMCID: PMC2754355

68. Gueimonde M, Jalonen L, He F, Hiramatsu M, Salminen S. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. Food Res Int. 2006 May;39(4):467–471.

69. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components. J Nutr. 2011 May 1;141(5):769–776. PMID: 21430248

70. Hooper LV, Littman DR, Macpherson AJ. Interactions Between the Microbiota and the Immune System. Science. 2012 Jun 8;336(6086):1268–1273. PMID: 22674334

71. Lee YK, Mazmanian SK. Has the Microbiota Played a Critical Role in the Evolution of the Adaptive Immune System? Science. 2010 Dec 24;330(6012):1768–1773. PMID: 21205662

72. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009 May;9(5):313–323.

73. Cénit MC, Matzaraki V, Tigchelaar EF, Zhernakova A. Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Biochim Biophys Acta BBA - Mol Basis Dis. 2014 Oct;1842(10):1981–1992.

74. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res. 2013 Sep;54(9):2325–2340. PMCID: PMC3735932

75. Thomas VM, Socolow RH, Fanelli JJ, Spiro TG. Effects of Reducing Lead in Gasoline: An Analysis of the International Experience. Environ Sci Technol. 1999 Nov 1;33(22):3942–3948.

76. US EPA O. EPA History [Internet]. US EPA. 2016 [cited 2018 Jul 16]. Available from: https://www.epa.gov/history

77. Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. Heavy Metals Toxicity and the Environment. EXS. 2012;101:133–164. PMCID: PMC4144270

78. WHO | Lead poisoning and health [Internet]. WHO. [cited 2016 Aug 19]. Available from: http://www.who.int/mediacentre/factsheets/fs379/en/

79. WHO | Arsenic [Internet]. WHO. [cited 2016 Aug 19]. Available from: http://www.who.int/mediacentre/factsheets/fs372/en/

80. Chi L, Bian X, Gao B, Ru H, Tu P, Lu K. Sex-Specific Effects of Arsenic Exposure on the Trajectory and Function of the Gut Microbiome. Chem Res Toxicol. 2016 Jun 20;29(6):949–951. PMID: 27268458

81. Winther G, Pyndt Jørgensen BM, Elfving B, Nielsen DS, Kihl P, Lund S, Sørensen DB, Wegener G. Dietary magnesium deficiency alters gut microbiota and leads to depressive-like behaviour. Acta Neuropsychiatr. 2015 Jun;27(3):168–176. PMID: 25690713

82. Guo X, Liu S, Wang Z, Zhang X, Li M, Wu B. Metagenomic profiles and antibiotic resistance genes in gut microbiota of mice exposed to arsenic and iron. Chemosphere. 2014 Oct;112:1–8.

83. Dheer R, Patterson J, Dudash M, Stachler EN, Bibby KJ, Stolz DB, Shiva S, Wang Z, Hazen SL, Barchowsky A, Stolz JF. Arsenic induces structural and compositional colonic microbiome change and promotes host nitrogen and amino acid metabolism. Toxicol Appl Pharmacol. 2015 Dec 15;289(3):397–408.

84. Lapanje A, Zrimec A, Drobne D, Rupnik M. Long-term Hg pollution-induced structural shifts of bacterial community in the terrestrial isopod (Porcellio scaber) gut. Environ Pollut. 2010 Oct;158(10):3186–3193.

85. Newell RG, Rogers K. The US experience with the phasedown of lead in gasoline. Resour Future Wash DC. 2003;2.

86. US EPA O. National Ambient Air Quality Standards (NAAQS) for Lead (Pb) [Internet]. [cited 2016 Nov 12]. Available from: https://www.epa.gov/lead-air-pollution/national-ambient-air-quality-standards-naaqs-lead-pb

87. US EPA O. Basic Information about Lead in Drinking Water [Internet]. [cited 2016 Nov 21]. Available from: https://www.epa.gov/ground-water-and-drinking-water/basic-information-about-lead-drinking-water

88. Commission UCPS. Ban of lead-containing paint and certain consumer products bearing lead-containing paint. 16 CFR 1303. Fed Reg. 1977;42:44199.

89. Tong S, Schirnding YE von, Prapamontol T. Environmental lead exposure: a public health problem of global dimensions. Bull World Health Organ. 2000 Jan;78(9):1068–1077.

90. Lidsky TI, Schneider JS. Lead neurotoxicity in children: basic mechanisms and clinical correlates. Brain. 2003 Jan 1;126(1):5–19. PMID: 12477693

91. Dietert RR, Piepenbrink MS. Lead and Immune Function. Crit Rev Toxicol. 2006 Apr;36(4):359–385.

92. Eggers S, Safdar N, Malecki KM. Heavy metal exposure and nasal Staphylococcus aureus colonization: analysis of the National Health and Nutrition Examination Survey (NHANES). Environ Health Glob Access Sci Source. 2018 Jan 5;17(1):2. PMCID: PMC5756436

93. Krueger WS, Wade TJ. Elevated blood lead and cadmium levels associated with chronic infections among non-smokers in a cross-sectional analysis of NHANES data. Environ Health [Internet]. 2016 Feb 11 [cited 2016 Sep 22];15. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4750187/ PMCID: PMC4750187

94. WHO | Antimicrobial resistance [Internet]. WHO. [cited 2016 Nov 14]. Available from: http://www.who.int/mediacentre/factsheets/fs194/en/

95. Huang H, Flynn NM, King JH, Monchaud C, Morita M, Cohen SH. Comparisons of Community-Associated Methicillin-Resistant Staphylococcus aureus (MRSA) and Hospital-Associated MSRA Infections in Sacramento, California. J Clin Microbiol. 2006 Jul 1;44(7):2423–2427. PMID: 16825359

96. Vysakh PR, Jeya M. A Comparative Analysis of Community Acquired and Hospital Acquired Methicillin Resistant Staphylococcus Aureus. J Clin Diagn Res JCDR. 2013 Jul;7(7):1339–1342. PMCID: PMC3749631

97. Tosas Auguet O, Betley JR, Stabler RA, Patel A, Ioannou A, Marbach H, Hearn P, Aryee A, Goldenberg SD, Otter JA, Desai N, Karadag T, Grundy C, Gaunt MW, Cooper BS, Edgeworth JD, Kypraios T. Evidence for Community Transmission of Community-Associated but Not Health-Care-Associated Methicillin-Resistant Staphylococcus Aureus Strains Linked to Social and Material Deprivation: Spatial Analysis of Cross-sectional Data. PLoS Med. 2016 Jan 26;13(1):1–24.

98. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal Carriage as a Source of Staphylococcus aureus Bacteremia. N Engl J Med. 2001 Jan 4;344(1):11–16. PMID: 11136954

99. Wenzel RP, Perl TM. The significance of nasal carriage of Staphylococcus aureus and the incidence of postoperative wound infection. J Hosp Infect. 1995 Sep;31(1):13–24.

100. Longfield JN, Townsend TR, Cruess DF, Stephens M, Bishop C, Bolyard E, Hutchinson E. Methicillin-Resistant Staphylococcus aureus (MRSA): Risk and Outcome of Colonized vs. Infected Patients. Infect Control. 1985;6(11):445–450.

101. Safdar N, Bradley EA. The Risk of Infection after Nasal Colonization with Staphylococcus Aureus. Am J Med. 2008 Apr;121(4):310–315.

102. Pacio GA, Visintainer P, Maguire G, Wormser GP, Raffalli J, Montecalvo MA. Natural History of Colonization With Vancomycin-Resistant Enterococci, Methicillin-Resistant Staphylococcus aureus, and Resistant Gram-Negative Bacilli Among Long-Term–Care Facility Residents. Infect Control Hosp Epidemiol. 2003;24(4):246–250.

103. VRE in Healthcare Settings | HAI | CDC [Internet]. [cited 2016 Nov 14]. Available from: https://www.cdc.gov/HAI/organisms/vre/vre.html

104. CDC - Gram-negative Bacteria Infections in Healthcare Settings - HAI [Internet]. [cited 2016 Oct 27]. Available from: https://www.cdc.gov/hai/organisms/gram-negative-bacteria.html

105. Kluytmans J, Belkum A van, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev. 1997 Jul 1;10(3):505–520. PMID: 9227864

106. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, Jensen BJ, Killgore G, Tenover FC, Kuehnert MJ. Changes in the Prevalence of Nasal Colonization with Staphylococcus aureus in the United States, 2001–2004. J Infect Dis. 2008 May 1;197(9):1226–1234.

107. Graham I Philip L, Lin SX, Larson EL. A U.S. Population-Based Survey of Staphylococcus aureus Colonization. Ann Intern Med. 2006 Mar 7;144(5):318–325.

108. Gadd GM, Griffiths AJ. Microorganisms and heavy metal toxicity. Microb Ecol. 1977 Dec;4(4):303–317.

109. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics [Internet]. 2015 Nov 17 [cited 2016 Oct 13];16. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4650350/ PMCID: PMC4650350

110. Becerra-Castro C, Machado RA, Vaz-Moreira I, Manaia CM. Assessment of copper and zinc salts as selectors of antibiotic resistance in Gram-negative bacteria. Sci Total Environ. 2015 Oct 15;530–531:367–372.

111. Chen S, Li X, Sun G, Zhang Y, Su J, Ye J. Heavy Metal Induced Antibiotic Resistance in Bacterium LSJC7. Int J Mol Sci. 2015 Sep 29;16(10):23390–23404. PMCID: PMC4632705

112. Summers AO, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, Bennett S, Billard L. Mercury released from dental “silver” fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob Agents Chemother. 1993 Apr 1;37(4):825–834. PMID: 8280208

113. Wireman J, Liebert CA, Smith T, Summers AO. Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates. Appl Environ Microbiol. 1997 Nov 1;63(11):4494–4503. PMID: 9361435

114. Davis IJ, Richards H, Mullany P. Isolation of silver- and antibiotic-resistant Enterobacter cloacae from teeth. Oral Microbiol Immunol. 2005 Jun 1;20(3):191–194.

115. Jacobs DE, Clickner RP, Zhou JY, Viet SM, Marker DA, Rogers JW, Zeldin DC, Broene P, Friedman W. The prevalence of lead-based paint hazards in U.S. housing. Environ Health Perspect. 2002 Oct;110(10):A599–A606. PMCID: PMC1241046

116. Hanna-Attisha M, LaChance J, Sadler RC, Champney Schnepp A. Elevated Blood Lead Levels in Children Associated With the Flint Drinking Water Crisis: A Spatial Analysis of Risk and Public Health Response. Am J Public Health. 2015 Dec 21;106(2):283–290.

117. Lead Awareness and Drinking Water Safety [Internet]. [cited 2016 Nov 13]. Available from: http://city.milwaukee.gov/2015water/WaterQuality/Lead-Awareness-and-Drinking-Water-Safety.htm#.WCjk7OErKYU

118. Christensen K, Coons M, Walsh R. 2014 Report on Childhood Lead Poisoning in Wisconsin. 2016.

119. Knobeloch L, Gorski P, Christenson M, Anderson H. Private drinking water quality in rural Wisconsin. J Environ Health. 2013 Mar;75(7):16–20. PMID: 23505770

120. Water Health Series: Filtration Facts. The Environmental Protection Agency; 2005 p. 9. Report No.: 816-K-05–002.

121. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. Nature. 2007 Oct 18;449(7164):804–810.

122. Prideaux L, Kang S, Wagner J, Buckley M, Mahar JE, De Cruz P, Wen Z, Chen L, Xia B, van Langenberg DR, Lockett T, Ng SC, Sung JJY, Desmond P, McSweeney C, Morrison M, Kirkwood CD, Kamm MA. Impact of ethnicity, geography, and disease on the microbiota in health and inflammatory bowel disease. Inflamm Bowel Dis. 2013 Dec;19(13):2906–2918. PMID: 24240708

123. Yadav D, Ghosh TS, Mande SS. Global investigation of composition and interaction networks in gut microbiomes of individuals belonging to diverse geographies and age-groups. Gut Pathog. 2016;8:17.

124. Wang Y, Kasper LH. The role of microbiome in central nervous system disorders. Brain Behav Immun. 2014 May;38:1–12.

125. Eggers S, Malecki KM, Peppard P, Mares J, Shirley D, Shukla SK, Poulsen K, Gangnon R, Duster M, Kates A, Suen G, Sethi AK, Safdar N. Wisconsin microbiome study, a cross-sectional investigation of dietary fibre, microbiome composition and antibiotic-resistant organisms: rationale and methods. BMJ Open. 2018 Mar 1;8(3):e019450. PMID: 29588324

126. Rosenfeld CS. Gut Dysbiosis in Animals Due to Environmental Chemical Exposures. Front Cell Infect Microbiol [Internet]. 2017 Sep 8;7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5596107/ PMCID: PMC5596107

127. Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJM. Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. Appl Environ Microbiol. 2001 Oct;67(10):4399–4406. PMCID: PMC93182

128. Kamada N, Chen GY, Inohara N, Núñez G. Control of Pathogens and Pathobionts by the Gut Microbiota. Nat Immunol. 2013 Jul;14(7):685–690. PMCID: PMC4083503

129. Nieto FJ, Peppard PE, Engelman CD, McElroy JA, Galvao LW, Friedman EM, Bersch AJ, Malecki KC. The Survey of the Health of Wisconsin (SHOW), a novel infrastructure for population health research: rationale and methods. BMC Public Health. 2010 Dec 23;10:785. PMCID: PMC3022857

130. Sakai T. Biomarkers of Lead Exposure. Ind Health. 2000;38(2):127–142.

131. Simpson EH. Measurement of diversity. Nature. 1949;

132. Gotelli NJ, Colwell RK. Estimating species richness.

133. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr. 1957;27(4):325–349.

134. Shannon CE. A mathematical theory of communication. Bell Syst Tech J. 1948;(27):379–423.

135. Levandowsky M, Winter D. Distance between Sets. Nature. 1971 Nov 5;234(5323):34–35.

136. Diet History Questionnaire (DHQ-II) and Canadian Diet History Questionnaire (C-DHQ II) [Internet]. [cited 2017 Jun 17]. Available from: https://epi.grants.cancer.gov/dhq2/about/

137. Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Sears MR, Becker AB, Scott JA, Kozyrskyj AL. Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. Allergy Asthma Clin Immunol Off J Can Soc Allergy Clin Immunol. 2013;9(1):15. PMCID: PMC3655107

138. Geography UCB. 2010 Census Urban and Rural Classification and Urban Area Criteria [Internet]. [cited 2015 May 14]. Available from: https://www.census.gov/geo/reference/ua/urban-rural-2010.html

139. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol. 2013 Sep;79(17):5112–5120. PMCID: PMC3753973

140. MiSeq SOP - mothur [Internet]. [cited 2016 Nov 14]. Available from: https://www.mothur.org/wiki/MiSeq_SOP

141. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 2007;35(21):7188–7196. PMCID: PMC2175337

142. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011 Aug 15;27(16):2194–2200. PMID: 21700674

143. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol. 2006 Jul;72(7):5069–5072. PMCID: PMC1489311

144. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. vegan: Community Ecology Package [Internet]. 2016 [cited 2016 Nov 14]. Available from: https://cran.r-project.org/web/packages/vegan/index.html

145. Tang Z-Z, Chen G, Alekseyenko AV, Li H. A general framework for association analysis of microbial communities on a taxonomic tree. Bioinforma Oxf Engl. 2017 May 1;33(9):1278–1285. PMCID: PMC5408811

146. Stekhoven DJ, Bühlmann P. MissForest—non-parametric missing value imputation for mixed-type data. Bioinformatics. 2012 Jan 1;28(1):112–118.

147. Rey FE, Gonzalez MD, Cheng J, Wu M, Ahern PP, Gordon JI. Metabolic niche of a prominent sulfate-reducing human gut bacterium. Proc Natl Acad Sci U S A. 2013 Aug 13;110(33):13582–13587. PMCID: PMC3746858

148. Figliuolo VR, Dos Santos LM, Abalo A, Nanini H, Santos A, Brittes NM, Bernardazzi C, de Souza HSP, Vieira LQ, Coutinho-Silva R, Coutinho CMLM. Sulfate-reducing bacteria stimulate gut immune responses and contribute to inflammation in experimental colitis. Life Sci. 2017 Nov 15;189:29–38. PMID: 28912045

149. Finegold SM. Desulfovibrio species are potentially important in regressive autism. Med Hypotheses. 2011 Aug;77(2):270–274. PMID: 21592674

150. Rowan F, Docherty NG, Murphy M, Murphy B, Calvin Coffey J, O'Connell PR. Desulfovibrio bacterial species are increased in ulcerative colitis. Dis Colon Rectum. 2010 Nov;53(11):1530–1536. PMID: 20940602

151. Shukla P, Khodade VS, SharathChandra M, Chauhan P, Mishra S, Siddaramappa S, Pradeep BE, Singh A, Chakrapani H. "On demand" redox buffering by H2S contributes to antibiotic resistance revealed by a bacteria-specific H2S donor †Electronic supplementary information (ESI) available. See DOI: 10.1039/c7sc00873b Click here for additional data file. Chem Sci. 2017 Jul 1;8(7):4967–4972. PMCID: PMC5607856

152. Buser MC, Ingber SZ, Raines N, Fowler DA, Scinicariello F. Urinary and blood cadmium and lead and kidney function: NHANES 2007–2012. Int J Hyg Environ Health. 2016 May;219(3):261–267.

153. Eggers S, Remington PL, Ryan K, Nieto FJ, Peppard P, Malecki K. Obesity Prevalence and Health Consequences: Findings From the Survey of the Health of Wisconsin. WMJ. 2016;115(5):238–243.

154. Sirard JR, Patnode CD, Hearst MO, Laska MN. Dog Ownership and Adolescent Physical Activity. Am J Prev Med. 2011 Mar;40(3):334–337. PMCID: PMC3395162

155. Zhai Q, Narbad A, Chen W. Dietary Strategies for the Treatment of Cadmium and Lead Toxicity. Nutrients. 2015 Jan;7(1):552–571.

156. Center for Disease Control and Prevention. Antibiotic Use in the United States, 2017: Progress and Opportunities. Atlanta, Georgia: U.S. Department of Health and Human Services; 2017.

157. Seiler C, Berendonk TU. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol [Internet]. 2012 Dec 14;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3522115/ PMCID: PMC3522115

158. Hemme CL, Green SJ, Rishishwar L, Prakash O, Pettenato A, Chakraborty R, Deutschbauer AM, Van Nostrand JD, Wu L, He Z, Jordan IK, Hazen TC, Arkin AP, Kostka JE, Zhou J. Lateral Gene Transfer in a Heavy Metal-Contaminated-Groundwater Microbial Community. mBio. 2016 Apr 5;7(2):e02234-02215. PMCID: PMC4817265

159. Cani PD. Gut microbiota and obesity: lessons from the microbiome. Brief Funct Genomics. 2013 Jul;12(4):381–387. PMID: 23616309

160. Alam MZ, Alam Q, Kamal MA, Abuzenadah AM, Haque A. A possible link of gut microbiota alteration in type 2 diabetes and Alzheimer's disease pathogenicity: an update. CNS Neurol Disord Drug Targets. 2014 Apr;13(3):383–390. PMID: 24059311

161. Yazdanbakhsh M, Kremsner PG, Ree R van. Allergy, Parasites, and the Hygiene Hypothesis. Science. 2002 Apr 19;296(5567):490–494. PMID: 11964470

162. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 26th informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute; 2016. Report No.: M100-S26.

163. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—10th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2006. Report No.: CLSI M07–A10.

164. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009 Dec;75(23):7537–7541. PMCID: PMC2786419

165. Kafilzadeh F, Afrough R, Johari H, Tahery Y. Range determination for resistance/tolerance and growth kinetic of indigenous bacteria isolated from lead contaminated soils near gas stations (Iran). Eur J Exp Biol. 2012;2(1):62–69.

166. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR, Sievert DM. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. Infect Control Hosp Epidemiol. 2016;37(11):1288–1301. PMID: 27573805

167. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, Heeg P, Ilschner C, Kramer A, Larson E, Merkens W, Mielke M, Oltmanns P, Ross B, Rotter M, Schmithausen RM, Sonntag H-G, Trautmann M. Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria? GMS Hyg Infect Control. 2017;12:Doc05. PMCID: PMC5388835

168. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev. 2011 Sep;35(5):820–855. PMID: 21564142

169. Evans GW, Kantrowitz E. Socioeconomic Status and Health: The Potential Role of Environmental Risk Exposure. Annu Rev Public Health. 2002;23(1):303–331. PMID: 11910065

170. Forastiere F, Stafoggia M, Tasco C, Picciotto S, Agabiti N, Cesaroni G, Perucci CA. Socioeconomic status, particulate air pollution, and daily mortality: Differential exposure or differential susceptibility. Am J Ind Med. 50(3):208–216.

171. US EPA O. Drinking Water Contaminants – Standards and Regulations [Internet]. US EPA. 2015 [cited 2018 Jun 24]. Available from: https://www.epa.gov/dwstandardsregulations

172. Fawell JK, Stanfield G. Drinking water quality and health. Pollut Causes Eff Control. 2001;4.

173. Rehman K, Fatima F, Waheed I, Akash MSH. Prevalence of exposure of heavy metals and their impact on health consequences. J Cell Biochem. 2018;119(1):157–184. PMID: 28643849

174. Li M, Wang M, Donovan SM. Early development of the gut microbiome and immune-mediated childhood disorders. Semin Reprod Med. 2014 Jan;32(1):74–86. PMID: 24390924

175. Ghaisas S, Maher J, Kanthasamy A. Gut microbiome in health and disease: Linking the microbiome–gut–brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. Pharmacol Ther. 2016 Feb;158:52–62.

176. Zapata HJ, Quagliarello VJ. The Microbiota and Microbiomein Aging: Potential Implications in Health and Age-related Diseases. J Am Geriatr Soc. 2015 Apr;63(4):776–781. PMCID: PMC4406803

177. Lax S, Smith DP, Hampton-Marcell J, Owens SM, Handley KM, Scott NM, Gibbons SM, Larsen P, Shogan BD, Weiss S, Metcalf JL, Ursell LK, Vázquez-Baeza Y, Van Treuren W, Hasan NA, Gibson MK, Colwell R, Dantas G, Knight R, Gilbert JA. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science. 2014 Aug 29;345(6200):1048–1052. PMCID: PMC4337996

178. Brooks JP, Adeli A, McLaughlin MR. Microbial ecology, bacterial pathogens, and antibiotic resistant genes in swine manure wastewater as influenced by three swine management systems. Water Res. 2014 Jun 15;57:96–103.

179. Ruiz-Calderon JF, Cavallin H, Song SJ, Novoselac A, Pericchi LR, Hernandez JN, Rios R, Branch OH, Pereira H, Paulino LC, Blaser MJ, Knight R, Dominguez-Bello MG. Walls talk: Microbial biogeography of homes spanning urbanization. Sci Adv. 2016 Feb;2(2):e1501061. PMCID: PMC4758746

180. The National Academies of Sciences, Engineering, and Medicine. Microbiomes of the Built Environment: A Research Agenda for Indoor Microbiology, Human Health and Buildings. [Internet]. Washington (DC): The National Academies Press; 2017 p. 297. Available from: https://doi.org/10.17226/23647

181. Wolf KJ, Daft JG, Tanner SM, Hartmann R, Khafipour E, Lorenz RG. Consumption of Acidic Water Alters the Gut Microbiome and Decreases the Risk of Diabetes in NOD Mice. J Histochem Cytochem. 2014 Jan 22;0022155413519650. PMID: 24453191

182. USGS Water Use Data for Wisconsin [Internet]. [cited 2018 Jul 12]. Available from: https://waterdata.usgs.gov/wi/nwis/wu

183. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001 Feb 1;26(1):32–46.

184. Wells - Wisconsin DNR [Internet]. [cited 2018 Jul 2]. Available from: https://dnr.wi.gov/topic/wells/

185. Van Immerseel F, Ducatelle R, De Vos M, Boon N, Van De Wiele T, Verbeke K, Rutgeerts P, Sas B, Louis P, Flint HJ. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. J Med Microbiol. 2010;59(2):141–143.

186. Kemgang TS, Kapila S, Shanmugam VP, Kapila R. Cross-talk between probiotic lactobacilli and host immune system. J Appl Microbiol. 2014 Aug;117(2):303–319. PMID: 24738909

187. Eggers S, Barker AK, Valentine S, Hess T, Duster M, Safdar N. Effect of Lactobacillus rhamnosus HN001 on carriage of Staphylococcus aureus: results of the impact of probiotics for reducing infections in veterans (IMPROVE) study. BMC Infect Dis. 2018 Mar 14;18(1):129. PMID: 29540160

188. Barker AK, Duster M, Valentine S, Hess T, Archbald-Pannone L, Guerrant R, Safdar N. A randomized controlled trial of probiotics for Clostridium difficile infection in adults (PICO). J Antimicrob Chemother. 2017 Aug 23; PMID: 28961980

189. Takagi T, Naito Y, Inoue R, Kashiwagi S, Uchiyama K, Mizushima K, Tsuchiya S, Dohi O, Yoshida N, Kamada K, Ishikawa T, Handa O, Konishi H, Okuda K, Tsujimoto Y, Ohnogi H, Itoh Y. Differences in gut microbiota associated with age, sex, and stool consistency in healthy Japanese subjects. J Gastroenterol. 2018 Jun 20; PMID: 29926167

190. Dominianni C, Sinha R, Goedert JJ, Pei Z, Yang L, Hayes RB, Ahn J. Sex, Body Mass Index, and Dietary Fiber Intake Influence the Human Gut Microbiome. PLoS ONE [Internet]. 2015 Apr 15 [cited 2017 Jan 3];10(4). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4398427/ PMCID: PMC4398427

191. Chang L, Heitkemper MM. Gender differences in irritable bowel syndrome. Gastroenterology. 2002 Nov 1;123(5):1686–1701.

192. Yang B, Wang Y, Qian P-Y. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics [Internet]. 2016 Mar 22 [cited 2018 Jul 5];17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4802574/ PMCID: PMC4802574

193. Jenkins A, Diep BA, Mai TT, Vo NH, Warrener P, Suzich J, Stover CK, Sellman BR. Differential Expression and Roles of Staphylococcus aureus Virulence Determinants during Colonization and Disease. mBio [Internet]. 2015 Feb 17 [cited 2017 Feb 6];6(1).

Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4337569/ PMCID: PMC4337569

194. Juhas M. Horizontal gene transfer in human pathogens. Crit Rev Microbiol. 2015 Feb;41(1):101–108. PMID: 23862575

195. Lee B-K, Kim Y. Association between bone mineral density and blood lead level in menopausal women: analysis of 2008-2009 Korean National Health and Nutrition Examination Survey data. Environ Res. 2012 May;115:59–65. PMID: 22480535

196. Choo JM, Leong LEX, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. 2015 Nov 17;5:16350. PMCID: PMC4648095

Appendix A

ST1. Linear regression estimates of α-diversity (Shannon)

	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	3.3	<2e-16	238.7	0.000	251.1	0.000	234.4	0.003
Log Pb^{+}	**0.1**	**0.012**	15.2	0.140	11.4	0.279	10.4	0.828
Age			0.6	0.126	0.7	0.164	1.6	0.062
Gender (Female)			-0.8	0.947	1.6	0.895	-27.8	0.243
Race (Non-White)			23.3	0.214	28.6	0.134	26.6	0.173
Income			0.6	0.815	0.7	0.771	1.8	0.715
Education - Some college			17.1	0.273	17.5	0.263	14.6	0.357
Education - ≥ Bachelor's degree			19.4	0.251	15.3	0.373	17.5	0.311
Smoking (Former)			12.2	0.567	15.2	0.480	10.9	0.615
Smoking (Never)			15.9	0.428	13.8	0.497	9.5	0.643
Antibiotic Use (Yes)			-18.6	0.133	-17.3	0.163	-19.0	0.128
Dietary Iron			-1.3	0.208	-1.4	0.174	-2.7	0.149
Dietary Vitamin C			0.0	0.604	0.0	0.603	-0.1	0.487
Dietary Fiber			1.8	0.062	1.9	0.051	**3.9**	**0.027**
Dietary Calcium			0.0	0.504	0.0	0.449	0.0	0.472
Indoor Pet					-4.1	0.757	-2.5	0.854
BMI					**-1.7**	**0.036**	-1.6	0.053
Urbanicity (Rural)					18.6	0.142	17.9	0.161
Length of Residence (1-3 years)					23.5	0.345	23.9	0.340
Length of Residence (3-10 years)					4.8	0.842	11.0	0.650
Length of Residence (>10 years)					1.6	0.945	4.4	0.851
Log Pb^{+}*Age							0.7	0.232
Log Pb^{+}*Gender (Female)							-28.7	0.131
Log Pb^{+}*Income							0.9	0.823
Log Pb^{+}*Dietary Iron							-1.5	0.348

Log Pb^{+}*Dietary Vitamin C		-0.1	0.605
Log Pb^{+}*Dietary Fiber		2.2	0.149
Log Pb^{+}*Dietary Calcium		0.0	0.636
$^{+}$ Creatinine adjusted. **Bold** values are significant at the <0.05 level.			

ST2. Linear regression estimates of Richness (Chao)								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	279.0	< 2e-16	195.9	0.000	206.8	0.000	190.5	0.003
Log Pb^{+}	**21.9**	**0.002**	13.6	0.103	10.1	0.233	7.1	0.855
Age			**0.8**	**0.025**	**0.9**	**0.037**	**1.7**	**0.016**
Gender (Female)			-2.1	0.833	-0.4	0.967	-24.1	0.210
Race (Non-White)			3.2	0.832	5.2	0.734	4.6	0.770
Income			0.6	0.751	0.8	0.701	0.1	0.985
Education - Some college			21.6	0.087	21.3	0.092	19.0	0.137
Education - ≥ Bachelor's degree			13.4	0.325	9.9	0.471	11.6	0.403
Smoking (Former)			12.8	0.454	16.2	0.351	12.1	0.490
Smoking (Never)			23.8	0.140	23.4	0.153	20.5	0.216
Antibiotic Use (Yes)			-14.3	0.151	-13.0	0.196	-14.4	0.151
Dietary Iron			-1.0	0.214	-1.1	0.186	-2.7	0.080
Dietary Vitamin C			0.0	0.637	0.0	0.667	-0.1	0.611
Dietary Fiber			**1.7**	**0.034**	**1.8**	**0.027**	**3.7**	**0.010**
Dietary Calcium			0.0	0.512	0.0	0.429	0.0	0.565
Indoor Pet					-1.5	0.887	0.1	0.993
BMI					-1.2	0.077	-1.1	0.118
Urbanicity (Rural)					6.6	0.516	5.5	0.593
Length of Residence (1-3 years)					22.6	0.261	22.3	0.270
Length of Residence (3-10 years)					-2.8	0.884	1.5	0.937
Length of Residence (>10 years)					3.2	0.864	4.5	0.812
Log Pb^{+}*Age							0.6	0.181
Log Pb^{+}*Gender (Female)							-22.7	0.139
Log Pb^{+}*Income							-0.8	0.792
Log Pb^{+}*Dietary Iron							-1.7	0.188
Log Pb^{+}*Dietary Vitamin C							0.0	0.764

Log Pb[+]*Dietary Fiber		2.1	0.090
Log Pb[+]*Dietary Calcium		0.0	0.767

[+] Creatinine adjusted. **Bold** values are significant at the <0.05 level.

ST3. PERMANOVA p-values of Bray-Curtis dissimilarity distances (β-diversity).

Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Log Pb+	**0.002**	**0.003**	**0.003**	**0.005**
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.002**	**0.002**	**0.002**
Race (Non-White)		**0.008**	**0.010**	**0.006**
Income		0.098	0.119	0.105
Education - Some College		0.189	0.180	0.188
Education - ≥ Bachelor's degree		0.057	**0.057**	0.052
Smoking (Former)		0.083	0.068	0.083
Smoking (Never)		0.088	0.087	0.086
Antibiotic Use (Yes)		**0.001**	**0.001**	**0.001**
Dietary Iron		0.503	0.479	0.451
Dietary Vitamin C		0.518	0.469	0.506
Dietary Fiber		**0.001**	**0.001**	**0.001**
Dietary Calcium		0.059	0.051	0.065
Indoor Pet			0.171	0.183
BMI			**0.008**	**0.012**
Urbanicity (Rural)			0.348	0.337
Length of Residence			**0.022**	**0.019**
Log Pb+*Age				0.207
Log Pb+*Gender (Female)				**0.049**
Log Pb+*Income				0.544
Log Pb+*Dietary Iron				0.217
Log Pb+*Dietary Vitamin C				0.530
Log Pb+*Dietary Fiber				0.765
Log Pb+*Dietary Calcium				0.198

+ Creatinine adjusted. **Bold** values are significant at the <0.05 level.

ST4. Linear regression estimates of α-diversity (inverse Simpson) on imputed data.

	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	15.127	<2e-16	10.550	0.000	8.694	0.001	9.103	0.013
Log Pb^{+}	0.832	**0.043**	0.715	0.137	0.601	0.219	1.474	0.497
Age			0.024	0.231	**0.050**	**0.031**	**0.081**	**0.025**
Gender (Female)			-0.835	0.164	-0.708	0.236	**-2.783**	**0.009**
Race (Non-White)			1.010	0.261	1.082	0.236	0.932	0.311
Income			0.220	0.073	0.202	0.103	0.356	0.105
Education - Some College			**2.017**	**0.007**	**2.194**	**0.003**	**2.085**	**0.005**
Education - ≥ Bachelor's degree			**1.918**	**0.017**	**2.002**	**0.013**	**2.089**	**0.010**
Smoking (Former)			1.954	0.059	**2.047**	**0.049**	1.759	0.091
Smoking (Never)			**2.302**	**0.017**	**2.365**	**0.015**	**2.177**	**0.025**
Antibiotic Use (Yes)			-0.998	0.100	-0.971	0.110	-1.093	0.070
Dietary Iron			-0.095	0.062	-0.095	0.061	**-0.222**	**0.015**
Dietary Vitamin C			-0.001	0.866	-0.001	0.860	-0.003	0.694
Dietary Fiber			**0.097**	**0.043**	**0.101**	**0.035**	**0.257**	**0.002**
Dietary Calcium			0.000	0.933	0.000	0.968	0.000	0.787
Indoor Pet					0.971	0.122	0.986	0.117
BMI					-0.040	0.315	-0.040	0.306
Urbanicity (Rural)					0.768	0.205	0.692	0.252
Length of Residence (1-3 years)					1.403	0.233	1.340	0.254
Length of Residence (3-10 years)					-0.724	0.533	-0.363	0.755
Length of Residence (>10 years)					-0.940	0.400	-0.763	0.494
Log Pb^{+}*Age							0.023	0.363
Log Pb^{+}*Gender (Female)							**-2.081**	**0.014**
Log Pb^{+}*Income							0.134	0.443
Log Pb^{+}*Dietary Iron							-0.139	0.075

Log Pb[+]*Dietary Vitamin C		-0.003	0.678
Log Pb[+]*Dietary Fiber		**0.170**	**0.018**
Log Pb[+]*Dietary Calcium		0.000	0.714

[+] Creatinine adjusted. **Bold** values are significant at the <0.05 level.

ST5. Linear regression estimates of Richness (ACE) on imputed data.								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	306.1	< 2e-16	225.6	0.000	235.7	0.000	245.2	0.001
Log Pb^+	**20.1**	**0.009**	12.8	0.163	9.0	0.332	23.5	0.572
Age			0.5	0.155	0.6	0.170	0.9	0.183
Gender (Female)			4.5	0.693	6.7	0.558	-18.9	0.355
Race (Non-White)			23.2	0.177	29.2	0.094	26.4	0.135
Income			2.5	0.289	2.3	0.330	4.6	0.273
Education - Some College			13.5	0.339	14.7	0.295	13.4	0.343
Education - ≥ Bachelor's degree			13.7	0.370	11.5	0.453	13.2	0.394
Smoking (Former)			16.0	0.417	17.4	0.380	13.2	0.509
Smoking (Never)			19.5	0.289	16.6	0.370	13.1	0.482
Antibiotic Use (Yes)			-22.7	0.050	-20.5	0.077	-22.3	0.054
Dietary Iron			-1.2	0.210	-1.2	0.199	-3.1	0.073
Dietary Vitamin C			0.0	0.794	0.0	0.700	-0.1	0.566
Dietary Fiber			**1.8**	**0.045**	**1.9**	**0.040**	**4.5**	**0.005**
Dietary Calcium			0.0	0.333	0.0	0.287	0.0	0.283
Indoor Pet					-0.7	0.951	-1.2	0.918
BMI					**-1.8**	**0.016**	**-1.8**	**0.015**
Urbanicity (Rural)					**26.9**	**0.020**	**26.2**	**0.024**
Length of Residence (1-3 years)					13.8	0.539	12.6	0.575
Length of Residence (3-10 years)					-2.3	0.916	3.0	0.893
Length of Residence (>10 years)					-4.5	0.834	-1.8	0.933
Log Pb^+*Age							0.2	0.648
Log Pb^+*Gender (Female)							-26.2	0.107
Log Pb^+*Income							2.1	0.532
Log Pb^+*Dietary Iron							-2.1	0.156
Log Pb^+*Dietary Vitamin C							-0.1	0.623

Log Pb[+]*Dietary Fiber		**2.9**	**0.037**
Log Pb[+]*Dietary Calcium		0.0	0.509
[+] Creatinine adjusted. **Bold** values are significant at the <0.05 level.			

ST6. PERMANOVA p-values of Bray-Curtis dissimilarity distances (β-diversity) on imputed data.

Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Log Pb^{+}	**0.001**	**0.001**	**0.001**	**0.001**
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.001**	**0.001**	**0.001**
Race (Non-White)		**0.003**	**0.005**	**0.007**
Income		**0.007**	**0.006**	**0.010**
Education - Some College		0.164	0.155	0.163
Education - ≥ Bachelor's degree		**0.019**	**0.017**	**0.022**
Smoking (Never)		0.069	0.085	0.077
Smoking (Former)		0.057	0.051	**0.049**
Antibiotic Use (Yes)		**0.001**	**0.001**	**0.001**
Dietary Iron		0.510	0.490	0.498
Dietary Vitamin C		0.625	0.594	0.613
Dietary Fiber		**0.001**	**0.001**	**0.001**
Dietary Calcium		**0.009**	**0.007**	**0.011**
Indoor Pet			0.161	0.160
BMI			**0.003**	**0.002**
Urbanicity (Rural)			0.224	0.229
Length of Residence			**0.040**	**0.034**
Log Pb^{+}*Age				0.175
Log Pb^{+}*Gender (Female)				**0.047**
Log Pb^{+}*Income				0.215
Log Pb^{+}*Dietary Iron				0.348
Log Pb^{+}*Dietary Vitamin C				0.543
Log Pb^{+}*Dietary Fiber				0.723
Log Pb^{+}*Dietary Calcium				0.149

$^{+}$ Creatinine adjusted. **Bold** values are significant at the <0.05 level.

<u>Appendix B</u>

Lead MIC Testing SOP

Broth Microdilution for aerobic organisms

Adapted from CLSI document M07-A9; 2012

Day 1:

Sub isolates to be tested from freezer onto a blood agar plate (BAP).

Day 2:

Inoculate organism to be tested into 4 mL of Luria Broth (LB).

Day 3:

<u>Solution Preparation:</u>

<u>Lead (II) Acetate:</u> 38 mg/mL is equivalent to a 0.1M solution. A 0.2M solution should be prepared for the first well of each plate. 600 microliters is used for each plate. Prepare enough lead (II) acetate to inoculate the number of plates prepared for the day. For example, 3 plates uses 1.8 mL consider preparing at least 2.0 mL for the day. For 2.0 mL weigh out 152 mg into 0.4M EDTA solution. This yields a 0.2M solution of lead (II) acetate.

<u>Cefoxitin Working Solution:</u> Make a 1:100 dilution of cefoxitin stock solution. Cefoxitin stock solution concentration is 6,400 micrograms/mL. Thaw the stock vial before use; please note repeated freeze thaw cycles are detrimental to antibiotic. If you notice a creep in MIC then discard stock solution and make new. Pipet 100 microliters of cefoxitin stock solution

into 900 microliters of 1X PBS. Prepare enough cefoxitin working solution for as many plates needed. For example, 3 plates uses 600 microliters, consider preparing 1 mL of cefoxitin working solution. To do this pipet 100 microliters of the 1:10 dilution made previously into 900 microliters of Luria Broth. This yields a 64 microgram/mL solution of cefoxitin.

Plate Preparation:

100 µL of a 200 mmol/L solution of lead (II) acetate was pipetted into column 1 Rows A-F. G1 and H1 contained 100 µL of 64 µg/mL solution of cefoxitin each. Columns 2-11 were filled with 50 µL of Luria Broth (LB) (Sigma Aldrich St. Louis, MO). Column 12 was filled with 100 µL of LB and served as a negative control. Serial dilutions were made by taking 50 µL from column 1 and dispensing into column 2. The solution was gently mixed with the pipet. Tips were changed and the procedure was repeated until column 10. Upon completion of column 10, 50 µL was dumped into a waste container. Column 11 served as a growth control and no compound was placed into it.

Inoculum Preparation and Addition:

A 1:50 dilution (0.1 mL OB into 4.9 mL of LB) of an overnight broth suspension that had been adjusted to a 0.5 McFarland was made for each organism to achieve a final concentration of ~1 x 10^6 CFU/mL. 50 µL of the 1:50 suspension was pipetted into the appropriate wells. The microtiter plate was incubated at 36°C aerobically for 24 hours and read visually using a mirror apparatus.

An assessment of the inoculum concentration was done by diluting 10 microliters of solution from column 11 into 990 microliters of 1X PBS. 100 microlites was plated onto Blood Agar (Remel, Lenexa, KS). Plates were incubated overnight aerobically. Growth of 30-300 colonies was considered an acceptable inoculum.

Plates (both BAP and microtiter) were read after an overnight incubation. MIC end points were determined by following CLSI document M07-A9; Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Briefly, the MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye.

ST7. Logistic regression estimates and relevant odds ratios for RGNB colonization.

	Model 1			Model 2			Model 3			Model 4	
Variable	Estimate	OR	95% CI	Estimate	OR	95% CI	Estimate	OR	95% CI	Estimate	P-value
(Intercept)	-1.45			-3.21			-3.00			-3.58	0.258
Log Pb^{+}	-0.02	0.98	0.70 - 1.37	-0.17	0.85	0.55 - 1.31	-0.16	0.85	0.55 - 1.32	-0.48	0.670
Age				**0.02**	1.02	1.00 - 1.04	**0.02**	1.02	1.00 - 1.04	0.02	0.358
Gender (Female)				0.03	1.03	0.61 - 1.76	0.04	1.04	0.61 - 1.78	0.25	0.655
Race (Non-White)				-0.07	0.87	0.39 - 1.97	-0.06	0.89	0.39 - 2.04	-0.22	0.369
Education - Some College				0.10	1.50	0.75 - 2.99	0.10	1.52	0.76 - 3.07	-0.07	0.835
Education - Bachelor's degree +				0.20	1.65	0.79 - 3.45	0.22	1.72	0.81 - 3.63	0.33	0.419
Income - Middle				0.28	1.18	0.59 - 2.33	0.28	1.16	0.58 - 2.31	0.01	0.980
Income - High				**-0.40**	0.60	0.29 - 1.25	**-0.42**	0.57	0.27 - 1.21	0.06	0.864
Smoking (Former)				-0.06	1.08	0.41 - 2.90	-0.04	1.17	0.43 - 3.17	-0.14	0.603
Smoking (Never)				0.20	1.40	0.55 - 3.52	0.23	1.53	0.59 - 3.96	0.18	0.447
Antibiotic Use (Yes)				-0.12	0.79	0.46 - 1.34	-0.14	0.76	0.44 - 1.30	-0.19	0.222
Dietary Vitamin C				0.00	1.00	1.00 - 1.01	0.00	1.00	1.00 - 1.01	0.00	0.228
Dietary Fiber				-0.01	0.99	0.95 - 1.03	-0.01	0.99	0.95 - 1.03	0.02	0.688
Dietary Iron				0.01	1.01	0.97 - 1.05	0.01	1.01	0.97 - 1.06	-0.01	0.890
Dietary Calcium				0.00	1.00	1.00 - 1.00	0.00	1.00	1.00 - 1.00	0.00	0.351
Indoor Pet							0.11	1.25	0.72 - 2.19	-0.01	0.974
Urbanicity (Rural)							-0.19	0.83	0.48 - 1.43	-0.09	0.777
Inverse Simpson							0.01	1.01	0.96 - 1.05	0.02	0.471
ACE							0.00	1.00	1.00 - 1.00	0.00	0.433
Infection with drug-resistant germ										0.06	0.954
Proton pump inhibitor use										-0.29	0.456
Live or work on a farm										0.26	0.639
Log Pb^{+}*Age										0.00	0.934
Log Pb^{+}*Gender (Female)										0.26	0.551

Log Pb^{+}*Education (some College)		-0.14	0.617
Log Pb^{+}*Education (Bachelor's degree +)		0.08	0.814
Log Pb+*Income - Middle		-0.30	0.329
Log Pb+*Income - High		0.40	0.234
Log Pb^{+}*Dietary Fiber		0.04	0.300
Log Pb^{+}*Dietary Calcium		0.00	0.343
Log Pb^{+}*Dietary Iron		-0.03	0.522
Log Pb^{+}*Dietary Vitamin C		0.00	0.739

Appendix C

ST8. Linear regression estimates of α-diversity (Shannon)

Variable	Model 1 Estimate	Model 1 P-value	Model 2 Estimate	Model 2 P-value	Model 3 Estimate	Model 3 P-value	Model 4 Estimate	Model 4 P-value
(Intercept)	3.22	<2e-16	2.90	<2e-16	3.02	< 2e-16	3.11	< 2e-16
Water Filter (Yes)	0.03	0.581	0.01	0.909	0.00	0.964	-0.32	0.168
Age			**0.00**	**0.027**	**0.01**	**0.002**	0.00	0.148
Gender (Female)			0.01	0.830	0.01	0.839	-0.03	0.658
Race (Non-White)			-0.02	0.761	-0.01	0.942	-0.01	0.865
Education - Some College			**0.15**	**0.021**	**0.16**	**0.012**	**0.16**	**0.013**
Education - Bachelor's degree +			**0.16**	**0.014**	**0.15**	**0.021**	**0.13**	**0.043**
Income			0.01	0.136	0.01	0.241	**0.02**	**0.043**
Antibiotic Use (Yes)			**-0.12**	**0.012**	**-0.12**	**0.013**	**-0.12**	**0.020**
Urbanicity (Rural)			0.01	0.904	0.11	0.179	0.07	0.431
Housing Age (Built 1979 or later)					-0.01	0.902	0.00	0.944
Length of Residence (1-3 years)					0.16	0.164	0.13	0.243
Length of Residence (3-10 years)					-0.03	0.744	-0.06	0.565
Length of Residence (>10 years)					-0.06	0.556	-0.08	0.411
Tap Water Consumption					0.00	0.708	0.01	0.320
BMI					0.00	0.254	0.00	0.295
County (Eau Claire)					-0.12	0.090	-0.15	0.073
County (Milwaukee)					-0.15	0.062	-0.15	0.107
County (Waushara)					**-0.23**	**0.022**	-0.09	0.414
Water Filter (Yes)*Age							**0.01**	**0.009**
Water Filter (Yes)*Gender (Female)							0.12	0.232
Water Filter (Yes)*Income							-0.03	0.084
Water Filter (Yes)*County (Eau Claire)							0.10	0.512
Water Filter (Yes)*County (Milwaukee)							0.01	0.956
Water Filter (Yes)*County (Waushara)							**-0.32**	**0.030**

ST9. Linear regression estimates of Richness (Chao)								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	260.08	<2e-16	194.49	0.000	220.37	0.000	221.02	0.000
Water Filter (Yes)	-7.96	0.464	-10.55	0.342	-10.23	0.360	-8.51	0.864
Age			**0.92**	**0.005**	**1.03**	**0.006**	**0.79**	**0.062**
Gender (Female)			4.77	0.638	6.18	0.544	2.39	0.843
Race (Non-White)			-5.79	0.737	0.28	0.988	-1.06	0.953
Education - Some College			16.35	0.224	19.36	0.153	18.66	0.163
Education - Bachelor's degree +			16.04	0.240	17.02	0.220	9.48	0.494
Income			1.91	0.347	2.30	0.268	**6.58**	**0.010**
Antibiotic Use (Yes)			**-23.23**	**0.027**	**-21.87**	**0.039**	**-21.09**	**0.045**
Urbanicity (Rural)			4.70	0.656	6.74	0.709	-6.03	0.741
Housing Age (Built 1979 or later)					-18.07	0.112	-16.61	0.139
Length of Residence (1-3 years)					39.01	0.105	34.70	0.144
Length of Residence (3-10 years)					10.47	0.637	6.14	0.782
Length of Residence (>10 years)					9.49	0.662	4.16	0.847
Tap Water Consumption					-0.46	0.796	0.33	0.856
BMI					-1.11	0.099	-1.05	0.115
County (Eau Claire)					-6.92	0.646	-18.19	0.309
County (Milwaukee)					-22.77	0.173	-23.92	0.216
County (Waushara)					-5.68	0.788	23.97	0.324
Water Filter (Yes)*Age							0.81	0.260
Water Filter (Yes)*Gender (Female)							16.03	0.462
Water Filter (Yes)*Income							**-10.66**	**0.008**
Water Filter (Yes)*County (Eau Claire)							43.01	0.169
Water Filter (Yes)*County (Milwaukee)							4.91	0.889
Water Filter (Yes)*County (Waushara)							-56.56	0.071

ST10. PERMANOVA p-values of Jaccard dissimilarity distances (β-diversity).

Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Water Filter (No)	0.266	0.226	0.236	0.240
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.002**	**0.002**	**0.001**
Race (Non-White)		0.533	0.518	0.434
Education - Some College		0.427	0.427	**0.006**
Education - Bachelor's degree +		**0.019**	**0.012**	0.637
Income		0.584	0.571	0.590
Antibiotic Use (No)		**0.001**	**0.001**	**0.002**
Urbanicity (Rural)		0.557	0.565	0.557
Housing Age (Built 1979 or later)			0.913	0.880
Length of Residence			0.093	0.095
Tap Water Consumption			0.467	0.436
BMI			**0.019**	**0.026**
County (Eau Claire)			0.258	0.280
County (Milwaukee)			0.327	0.310
County (Waushara)			0.092	0.106
Water Filter (No)*Age				0.304
Water Filter (No)*Gender (Female)				0.894
Water Filter (No)*Income				**0.003**
Water Filter (No)*County (Eau Claire)				0.396
Water Filter (No)*County (Milwaukee)				0.413
Water Filter (No)*County (Waushara)				0.472

ST11. Linear regression estimates of α-diversity (inverse Simpson) with Imputed Data								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	14.07	<2e-16	9.72	0.000	10.98	0.000	11.89	0.000
Water Filter (Yes)	0.80	0.215	0.33	0.611	0.31	0.634	-3.88	0.175
Age			**0.04**	**0.019**	**0.06**	**0.001**	**0.04**	**0.100**
Gender (Female)			-0.56	0.339	-0.55	0.345	-1.25	0.064
Race (Non-White)			0.49	0.569	0.80	0.390	0.57	0.544
Education - Some College			**2.12**	**0.005**	**2.34**	**0.002**	**2.25**	**0.002**
Education - Bachelor’s degree +			**2.52**	**0.001**	**2.61**	**0.001**	**2.47**	**0.002**
Income			0.21	0.079	0.16	0.196	0.26	0.078
Antibiotic Use (Yes)			-0.97	0.109	-0.97	0.111	-0.93	0.119
Urbanicity (Rural)			0.75	0.223	1.96	0.066	1.47	0.171
Housing Age (Built 1979 or later)					0.43	0.516	0.46	0.487
Length of Residence (1-3 years)					1.77	0.134	1.58	0.180
Length of Residence (3-10 years)					-0.19	0.869	-0.34	0.766
Length of Residence (>10 years)					-0.73	0.514	-0.90	0.417
Tap Water Consumption					0.05	0.618	0.08	0.410
BMI					-0.04	0.358	-0.03	0.421
County (Eau Claire)					**-2.25**	**0.010**	-1.93	0.054
County (Milwaukee)					**-2.15**	**0.023**	**-1.36**	**0.205**
County (Waushara)					**-3.16**	**0.012**	**-1.08**	**0.442**
Water Filter (Yes)*Age							**0.12**	**0.002**
Water Filter (Yes)*Gender (Female)							**2.58**	**0.041**
Water Filter (Yes)*Income							-0.29	0.230
Water Filter (Yes)*County (Eau Claire)							-0.82	0.654
Water Filter (Yes)*County (Milwaukee)							-2.15	0.275
Water Filter (Yes)*County (Waushara)							**-5.57**	**0.003**

ST12. Linear regression estimates of Richness (ACE) with Imputed Data								
	Model 1		Model 2		Model 3		Model 4	
Variable	Estimate	P-value	Estimate	P-value	Estimate	P-value	Estimate	P-value
(Intercept)	288.70	<2e-16	215.35	<2e-16	266.78	0.000	259.00	0.000
Water Filter (Yes)	-8.45	0.482	-15.62	0.204	-15.48	0.210	31.01	0.569
Age			**0.72**	**0.031**	**0.82**	**0.030**	**0.63**	**0.131**
Gender (Female)			9.01	0.412	10.18	0.356	10.31	0.423
Race (Non-White)			19.97	0.222	23.84	0.182	16.09	0.251
Education - Some College			16.43	0.243	19.05	0.180	14.95	0.320
Education - Bachelor’s degree +			26.41	0.074	24.43	0.105	23.88	0.180
Income			2.79	0.220	3.29	0.161	8.64	**0.002**
Antibiotic Use (Yes)			**-24.70**	**0.031**	-22.58	0.051	-22.72	**0.047**
Urbanicity (Rural)			**25.46**	**0.029**	27.36	0.177	14.76	0.469
Housing Age (Built 1979 or later)					-17.40	0.170	-16.00	0.202
Length of Residence (1-3 years)					18.76	0.404	9.43	0.673
Length of Residence (3-10 years)					2.89	0.895	-4.25	0.847
Length of Residence (>10 years)					-1.01	0.962	-10.44	0.621
Tap Water Consumption					-0.42	0.821	-0.13	0.944
BMI					**-1.86**	**0.011**	**-1.78**	**0.014**
County (Eau Claire)					-0.06	0.997	-1.18	0.951
County (Milwaukee)					-8.31	0.645	-6.08	0.767
County (Waushara)					3.17	0.895	44.32	0.100
Water Filter (Yes)*Age							0.81	0.280
Water Filter (Yes)*Gender (Female)							3.15	0.896
Water Filter (Yes)*Income							**-14.54**	**0.001**
Water Filter (Yes)*County (Eau Claire)							16.74	0.631
Water Filter (Yes)*County (Milwaukee)							-9.25	0.805
Water Filter (Yes)*County (Waushara)							**-96.70**	**0.006**

ST13. PERMANOVA p-values of Bray-Curtis dissimilarity distances (β-diversity) with Imputed data.

Variable	Model 1 P-value	Model 2 P-value	Model 3 P-value	Model 4 P-value
Water Filter (Yes)	0.264	0.244	0.239	0.237
Age		**0.001**	**0.001**	**0.001**
Gender (Female)		**0.002**	**0.002**	**0.001**
Education - Some College		**0.030**	**0.023**	0.120
Education - Bachelor's degree +		0.147	0.140	**0.002**
Race (Non-White)		**0.004**	**0.002**	0.061
Income		0.292	0.280	0.304
Antibiotic Use (Yes)		**0.001**	**0.001**	**0.001**
Urbanicity (Rural)		0.553	0.582	0.578
Housing Age (Built 1979 or later)			0.868	0.870
Length of Residence			0.089	0.094
Tap Water Consumption			0.447	0.423
BMI			**0.006**	**0.007**
County (Eau Claire)			0.064	0.080
County (Milwaukee)			0.128	0.134
County (Waushara)			0.094	0.098
Water Filter (Yes)*Age				0.449
Water Filter (Yes)*Gender (Female)				0.742
Water Filter (Yes)*Income				**0.003**
Water Filter (Yes)*County (Eau Claire)				0.311
Water Filter (Yes)*County (Milwaukee)				0.506
Water Filter (Yes)*County (Waushara)				0.372