GUT MICROBES – HEAVY METAL(LOID) INTERACTIONS

by

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Bolan, S., Seshadri, B., Talley, N.J. and Naidu, R. (2016). Bio-banking gut microbiome samples. EMBO Reports, 17(7), 929-930.

Bolan, S., Kunhikrishnan, A., Chowdhury, S., Seshadri, B., Naidu, R., Ok, Y.S. (2017). Comparative analysis of speciation and bioaccessibility of arsenic in rice grains and complementary medicines. Chemosphere, 182, 433-440.

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Bolan, S. et al. Differential toxicity of toxic metal(loid) species on gut microbes (in preparation).

Bolan, S. et al. Speciation and bioaccessibility of toxic metal(loid) species as impacted by gut microbes (in preparation).

Bolan, S. et al. Interactive effect of chelating agents and gut microbes on the bioaccessibility of toxic metal(loid) species (in preparation).

Bolan, S. et al. Interactive effect of chelating agents and gut microbes on the bioavailability of toxic metal(loid) species using Caco-2 cell technique (in preparation).

Conference papers/proceedings:

Bolan, S., Seshadri, B., Naidu, R. and Talley, J.N. (2016). The Microbiome – Guardians of gut galaxy. *55th ASMR National Scientific Conference*, Newcastle, Australia.

Bolan, S., Seshadri, B., Naidu, R. and Talley, N. (2016). Heavy metals – Gut microbiome interactions. *18th International Conference on Heavy Metals in the Environment*. 12 to 15 September 2016, Ghent, Belgium.

Bolan, S.S., Seshadri, B., Wijayawardena, A.M.A., Grainge, I., Naidu, R. and Nicholas, J.T. (2017). Differential toxicity effect of arsenic species on gut microbiome. In *7th International Contaminated Site Remediation Conference*. Melbourne, Australia: CRC CARE Pty LTD.

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GLOSSARY OF TERMINOLOGIES AND ABBREVIATIONS

Absolute bioavailability: The absolute bioavailability (ABA) refers to the fraction of the metal(loid) which, following ingestion is absorbed and reaches systemic circulation.

Aqua regia: A digestion mixture consisting of concentrated nitric and hydrochloric acid at a ratio of 3:1. This mixture is used for measuring total heavy metal(loid)s in complementary medicines.

BET surface area: Brunauer, Emmet and Teller surface area.

Bioaccessibility: Bioaccessibility refers to potential bioavailability and is often used to assess bioavailability which is an '*in-vitro*' test and represents the fraction of metal(loid) that becomes soluble following gastrointestinal extraction and is therefore assumed accessible for absorption.

Bioactivity: Response of tissue resulting from heavy metal(loid) toxicity.

Bioavailability: Bioavailability of heavy metal(loid)s refers to the biologically available chemical fraction that can be taken up by an organism and can react with its metabolic machinery.

Blank: Refers to control samples; for example, in the case of total heavy metal(loid) analysis of complementary medicines the *aqua regia* used for digesting samples was used as the control or blank sample to measure the contribution of heavy metal(loid)s from *aqua regia* to the total metal(loid) analysis.

Caco 2 Cell: Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells. Although derived from a colon (large intestine) carcinoma, when cultured under specific conditions the cells become differentiated and polarized such that their phenotype, morphologically and functionally, resembles the enterocytes lining the small intestine.

Certified reference material: Standard material used to verify analytical results; in this study Montana Soil (SRM 271) was used as a reference material to verify the total heavy metal(loid) concentration in various sources.

Chelating agent: A chelating agent is a substance whose molecules can form several bonds to a single metal ion, thereby resulting in the formation of a stable, water-soluble metal complex.

Complementary medicines: Complementary medicines include herbal medicines, vitamin and dietary health supplements, and traditional Ayurvedic, Chinese and homoeopathic medicines (WHO 2005).

Correlation: Statistical relationship between two variables – not necessarily gives the causal relationship.

Fish feed: Fish feed consists of a range of ingredients nutritionally formulated to provide fish all the correct nutrients in the form of protein, fat, carbohydrate, vitamins and minerals.

Fractionation: Fractionation is defined as the process of classification of analyte or a group of analytes from a certain sample according to physical (e.g., size, solubility) or chemical (e.g., bonding, reactivity) properties.

FTIR: Fourier transform infrared spectrometer; FTIR data provide functional groups and their role in the interactions with heavy metal(loid)s.

Gastrointestinal bioaccessibility test: It is an *in vitro* bioaccessibility test which involves a two-step sequential extraction: a gastric [0.15 M NaCl and 1% porcine pepsin (pH 1.8)] followed by an intestinal [0.15 M NaCl and bovine and porcine pancreatin (pH 5.8)] extraction.

Gut microbe: Family of microorganisms including bacteria, archea and fungi that reside in the intestinal track.

Gut microbiome: The microorganisms, the gene reserve, the genes coding for proteins and the metabolites in the 'human ecosystem' are collectively termed as "microbiome."

Heavy metal(loid)s: Include metals and metalloids with an atomic density greater than 6g/cm^3 (with the exception of arsenic, boron and selenium).

ICP-MS: Inductively coupled plasma mass spectrometry; ICP-MS was used for the analysis of heavy metal(loid)s in various samples.

Intestinal permeability: Intestinal permeability is a term describing the control of material passing from inside the gastrointestinal tract through the cells lining the gut wall, reaching the blood circulation into the rest of the body.

LD_{50} value: It is the heavy metal(loid) concentration at which the microbial growth is inhibited by 50% of the maximum growth in the absence of metal(loid) input.

Minimum inhibition concentration (MIC): It is the minimum heavy metal(loid) concentration above which the microbial growth is inhibited by metal(loid) input.

Offal: Offal refers to any of the internal organs and entrails of an animal often used as a source of pet food.

PAMPA test: Parallel artificial membrane permeability assay (PAMPA) is a method which determines the permeability of substances from a donor compartment, through a lipid-infused artificial membrane into an acceptor compartment.

Regression relationship: Gives the statistical relationship between two variables (independent and dependent variables); in the current study it is aimed to obtain a relationship between speciation (independent variable) and bioavailability (dependent variable) of heavy metal(loid)s.

Relative bioavailability: Relative bioavailability (RBA) refers to the ratio of the absorbed fraction from an exposure media (for example, lead in this case complementary medicines) to the absorbed fraction from a reference dose (for example, lead acetate when examining the bioavailability of lead).

SEM: Environmental scanning electron microscope (SEM); in this study SEM is used to visualize the interactions between heavy metal(loid) and gut microbes.

Speciation: Speciation of an element is also defined as distribution of an element amongst defined chemical species in a system. Often fractionation and speciation terms are used interchangeably.

Therapeutic ingredient: Ingredients used as a beneficial medicinal value; for example, arsenic has been used as a therapeutic ingredient in Ayurvedic medicines

WHO: World Health Organisation.

XRD: X-ray diffraction (XRD). XRD is used to identify crystalline mineral phases in a substance.

ABSTRACT

Specific microorganisms in the human gut (i.e., gut microbes) provide positive benefits to the host such as fermenting unused energy substrates, training the immune system, preventing growth of pathogenic microbes, and producing vitamins for the host. The intake of contaminants including heavy metal(loid)s can occur through food, air, water and some medicines. The gut microbes not only can be affected by environmental contaminants but they themselves can alter the speciation and bioavailability of these contaminants. Chelation therapy is an important clinical treatment for managing metal(loid) toxicity in human. Chelating agents are organic or inorganic compounds capable of binding metal(loid) ions to form complex ring-like structure called 'chelates'. Chelating agents can affect metal(loid) toxicity by mobilizing the toxic metal(loid)s and their subsequent excretion mainly through urine.

This thesis provides a greater understanding of the interactions of selected gut microbes and heavy metal(loid)s in relation to metal(loid) toxicity to gut microbes, and gastrointestinal bioaccessibility and bioavailability of heavy metal(loid)s as impacted by gut microbes and chelating agents. In this work, gastrointestinal bioaccessibility is defined as the amount of metal(loid)s that become solubilized in the gastric and intestinal system, and bioavailability as the amount of metal(loid)s that passes through the intestinal epithelial cells, thereby reaching the blood circulation (Naidu et al., 2008).

The overall objective of the thesis is to examine the interactions between gut microbes and heavy metal(loid)s in relation to metal(loid) toxicity to gut microbes, and bioaccessibility and bioavailability of metal(loid)s. The specific objectives of the study include: (i) to demonstrate the effect of arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) on the growth of selected gut microbes; (ii) to examine the effect of selected gut microbes on the bioaccessibility of these heavy metal(loid)s; (iii) to examine the effect of selected chelating therapeutic agents on the bioaccessibility of heavy metal(loid)s; and (iv) to examine the effect of gut microbes and chelating therapeutic agents on the bioavailability of heavy metal(loid)s.

Chapter 1 (*Introduction)* gives an overall outline of gut microbiome, environmental contaminants, the effect of heavy metal(loid)s on gut microbes, and bioaccessibility and bioavailability of heavy metal(loid)s as impacted by gut microbes. **Chapter 2** (*Literature review)* covers the role of gut microbes in human health and the effects of environmental contaminants on gut microbes.

Chapter 3 (*Heavy metal(loid) toxicity to gut microbe)* demonstrates the relationship between increasing concentration of selected heavy metal(loid)s and growth of gut microbes. The toxicity of four heavy metal(loid)s including As, Cd, Hg and Pb to three gut bacteria (*Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Escherichia coli*) was examined. While the toxicity of all the cationic metal(loid)s (Cd, Pb and Hg) to gut bacteria decreased with pH, the anionic As species exhibited an opposite effect. The order of toxicity was Hg>Cd>Pb>As(III)>As(V) for *E. coli*; and Hg>Cd>As(III)>Pb>As(V) for the two *Lactobacillus sp*. Arsenite (AsIII) is more toxic than arsenate (AsV) to gut bacteria. The toxicity of these metal(loid)s to the bacteria depends on their speciation and bioavailability.

Chapter 4 *(Bioaccessibility of heavy metal(loid)s)* investigated the gastrointestinal bioaccessibility of heavy metal(loid)s in selected orally ingested sources. The bioaccessibility of As (rice grain), Cd (offal pet food), Hg (fish feed) and Pb (complementary medicines) was examined by measuring gastrointestinal bioaccessibility test. The gastric bioaccessibility of As, Cd, Pb and Hg was less than that of intestinal bioaccessibility of these metals. Majority of the metal(loid)s extracted in gastric and intestinal extracts was present as metal(loid) complexes. The distribution of metal(loid)s in the gastric and intestinal extracts will have implications on their bioavailability.

Chapter 5 *(Bioaccessibility of heavy metal(loid)s as impacted by gut microbes)* describes the influence of gut microbes on the bioaccessibility of heavy metal(loid)s. The bioaccessibility of As, Cd, Pb and Hg as impacted by three gut bacteria (*L. rhamnosus, L. acidophilus* and *E. coli*) was examined by measuring gastrointestinal bioaccessibility. This study demonstrated that gut microbes decreased bioaccessibility of metal(loid)s, which is likely to impact their bioavailability. The effect of gut microbes on bioaccessibility may be attributed to bioimmobilization of metal(loid)s through adsorption, precipitation, and complexation reactions.

Chapter 6 (G*ut microbes on bioaccessibility of heavy metal(loid)s as impacted by chelating agents)* describes the impact of gut bacteria on chelate-induced bioaccessibility of heavy metal(loid)s. Firstly, the effect of three chelating agents (EDTA, DMSA and DMPS) on the solubility and bioaccessibility of As, Cd, Hg and Pb sources was examined. The results indicated that all the three chelating agents increased both gastric and intestinal

bioaccessibility of As, Cd, Hg and Pb. The increase in chelate-induced bioaccessibility of heavy metal(loid)s is attributed to the complexation of metal(loid)s by the chelating agents. Secondly, the effect of two gut microbes (*E. coli* and *L. acidophilus*) on the bioaccessibility of As, Cd, Hg and Pb sources as impacted by two chelating agents (EDTA and DMPS) was examined. The results indicated that, in the presence of both gut microbes and chelating agents, there was a net increase in the bioaccessibility of heavy metal(loid)s indicating that chelate-mediated complexed metal(loid) species are not readily adsorbed by gut bacteria.

Chapter 7 (*Bioavailability of heavy metal(loid)s as measured by permeability test) c*ompares the bioavailability of heavy metal(loid)s as measured by intestinal permeability. The intestinal permeability of As, Cd, Hg and Pb in the gastric and intestinal extracts as impacted by gut microbes (*E. coli and L. rhamnosus*) and chelating agents (EDTA and DMPS) was measured using Caco-2 test. The results demonstrated that the P_{app} (apparent permeability coefficient value), which measures the velocity with which a solute crosses the cell monolayer, was lower in the presence of chelating agents indicating low intestinal absorption. Similarly, the P_{app} value was markedly reduced in the presence of gut bacteria for all the metal(loid)s indicating low intestinal absorption in the presence of gut bacteria. The results may be attributed to a direct protection of the intestinal barrier against the metal(loid)s or indirect intestinal metal(loid) sequestration by the gut bacteria.

Chapter 8 *(Summary and conclusions)* provides overall research conclusions and a summary of the major research findings. This chapter proposes possible future directions in research. This study demonstrated the toxicity of heavy metal(loid)s on the gut bacteria and also the effect of gut bacteria on the bioaccessibility and bioavailability of these heavy metal(loid)s. It is important to point out that the human gut hosts a large number of microbial species including bacteria, fungi, and archaea. In this study, the effect of only selected bacterial species on bioaccessibility and bioavailability of heavy metal(loid)s was examined. Future studies should focus on the effect of composite gut microbial consortia on bioaccessibility and subsequent bioavailability of toxic metal(loid)s.

DECLARATION

I hereby certify that the work embodied in the thesis is my own work. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Signature of candidate

Shiv Shankar Bolan
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University of Newcastle
Date: May 2019

I hereby certify that the work embodied in this thesis contains published paper work of which I am a joint author.

Signature of Supervisor

Professor Ravi Naidu
Global Centre for Environmental Remediation
The University of Newcastle
Date: May 2019

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Finally, I thank my parents for their constant support and unconditional love, which helped me continue my work to completion.

Chapter 1

GENERAL INTRODUCTION

1.1 Gut microbiome and human health

Understanding human associated microorganisms is an emerging area of research as recent studies have shown the influence of microorganisms towards improved health outcomes and overall homeostasis (the body's usual healthy equilibrium) in humans. Microorganisms that live both inside and outside the human system play vital roles in health via the functioning of physiology, metabolic activities and immune system. Among the various microbial ecosystems, gut microbiota or the microorganisms present in the digestive tract outnumber the entire microbial population present in human system. Hence, the human gut microbiota is considered a 'hidden organ'. It is an amalgamation of up to 1000 individual bacterial species that occupy the human digestive tract (Li et al., 2012; Pflughoeft and Versalovic, 2012; Weinstock, 2012). Fungi, protozoa and archaea are found in lesser numbers and constitute the rest of the gut microbiota. In general, a single human being is comprised of approximately $3.0x10^{13}$ human cells. This is matched by our estimated count of $3.8x10^{13}$ microbial cells that cohabitate humans, resulting in a ratio of almost 1:1 human to microbial cells (Sender et al., 2016a,b). There are around 8 million unique protein coding genes of the gut microbiota and their metabolites that are crucial for normal human homeostasis and survival (Strachan and Read, 1999). The microorganisms, the gene reserve, the genes coding for proteins and the metabolites in the 'human ecosystem' are collectively termed as "microbiome."

Specific microorganisms in the human gut provide benefits to the host such as fermenting unused energy substrates, training the immune system, preventing growth of harmful, pathogenic bacteria, regulating the development of the gut, and producing vitamins for the host (such as biotin and vitamin K) (Fujimura et al., 2010; Panda et al., 2014; D'Argenio and Salvatore, 2015). Gut microbiota are involved in metabolic, protective and trophic functions in the human host. In terms of metabolic functions, they aid in the digestion and utilization of energy. Protective functions include the development of immunity against harmful organisms. In relation to trophic functions, they promote cellular growth, differentiation, and survival.

1.2 Gut microbiome and environmental contaminant interactions

The intake of environmental contaminants including heavy metal(loid)s and organic compounds can occur through food, air, water and some traditional medicines (Naidu et al., 2008; Bolan et al., 2017a). The gut microbes not only can be affected by environmental contaminants but they themselves can alter the speciation and bioavailability of these contaminants (Breton et al., 2013a). These interactions can have both positive and negative consequences for the host. Gut microbes can impact the way toxic compounds react with the human host. Experiments conducted using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), a system that mimics digestive processes in the gut showed that microbes can impact the bioavailability of toxic compounds. For example, Van de Wiele et al. (2010) showed that gut microbes change the bioavailability of arsenic (As) to increase its toxicity profile. It was found that the gut microbes could contribute to the transformation of arsenate (AsV) to arsenite (AsIII), which is more toxic than its predecessor. The gut microbes could add a methyl group (CH_3) actively methylating 1-10% of the As(V) to produce monomethyl arsenate and monomethyl arsenite. Monomethyl arsenate is less toxic than As(V). Therefore, while certain gut microbes can increase the risks associated with human exposure to environmental contaminants, it is evident that certain gut microbes can help to detoxify some of these environmental compounds. The effect of metal(loid)s on microbiome composition is impacted by both the nature and level of metal(loid) exposure.

1.3 Gut microbiome dysbiosis

The concept of ‘gut microbiome dysbiosis’ is known as the imbalance between the putative species of “protective” bacteria versus the “harmful” intestinal bacterial species. The term ‘dysbiosis’ was originally coined by Metchnikoff (1907) to describe altered pathogenic bacteria in the gut. Gut dysbiosis has been defined by others as “qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution” (Carding et al., 2015; Claus et al., 2016). Diet and environment factors are considered as the main factors for the dysbiosis of human gut microbiome. Other possible causes for the human gut microbiome dysbiosis include physiological and physical stress, certain medications, and the exposure of gut to environmental pollutants including heavy metal(loid)s and toxic substances.

Not only are intra-intestinal illnesses such as ulcerative colitis and Crohn's disease associated with changes in the microbiome, variations in the microbiome have also been associated with extra intestinal conditions such as diabetes mellitus, heart disease and neurological conditions such as autism and Alzheimer's disease. Therefore, it is increasingly understood that the gut microbiome plays an important role in intestinal homeostasis, and when the balance is tipped away from the 'healthy microbiome' there can be a negative outcome on human health (Clemente et al., 2012). Re-constructing the gut microbiome by means of probiotic intake and faecal microbial transfer (Bolan et al., 2016a) are being practiced but the long-term effects of such practices are little known.

1.4 Treatment of heavy metal(loid) toxicity in human

Metal(loid)s are an integral part of many structural and functional components in the body, and play a critical role in physiological and pathological processes (Jaishankar et al., 2014). There have been increasing research interests in the fields of metallotoxicology and metallopharmacology, which cover therapeutic strategies, based on alteration of the metal(loid) concentrations and bioavailability in specific body organs. Metallopharmacology deals with the application of metal(loid)s to restore the normal healthy physiology of the body using various approaches including the direct administration of essential metal(loid)s, removing excess or toxic metal(loid)s through chelation, using metal(loid)s as carriers for targeted drug delivery, and tagging biomolecules with metal(loid)s for diagnostics (Flora and Pachauri, 2010). Metal(loid) toxicity may occur due to the excess uptake of essential metal(loid)s or exposure to toxic heavy metal(loid)s from various sources. Most metal(loid)s form covalent bonds with carbon in biota, resulting in metal(loid)-organic compounds (Egorova and Ananikov, 2017). While essential metal(loid)s are involved in various metabolic functions, excess of essential metal(loid)s and toxic heavy metal(loid)s interfere with various functions of organ systems like the central nervous system, the haematopoietic system, liver, kidneys, *etc.* Diagnostic testing for the presence of heavy metal(loid)s, and subsequently decreasing the body's burden of these substances, should be an integral part of the overall treatment regimen for individuals with a metal(loid) poisoning symptomatology or a known exposure to these substances (Jaishankar et al., 2014).

Chelation therapy is an important tool for modifying metal(loid) concentrations in the body (Flora et al., 2007; Ferrero, 2016). Chelating agents are organic or inorganic compounds capable of binding metal(loid) ions to form complex ring-like structure called 'chelates' (Sears,

2013). Chelating agents can affect metal(loid) toxicity by mobilizing the toxic metal(loid) mainly into urine. A chelating agent forming a stable complex with a toxic metal(loid) may shield biological targets from the metal(loid) ion, thereby reducing the local toxicity (Aaseth et al., 2015). Some of the essential characteristics of chelating compounds in relation to treatment of metal(loid) toxicity include (Williams and Halstead, 1982; Sear, 2013): (1) ready transport across physiological barriers into compartments where a toxic metal(loid) ion is concentrated; (2) form a stable complex with the metal(loid) after separating it from the biological chelating agents; and (3) form a chelation complex whose properties render it non-toxic and facilitate its excretion both from the site of deposition and body.

This thesis provides a greater understanding of the interactions of certain gut microbes and the major toxic heavy metal(loid)s in relation to metal(loid) toxicity to gut microbes, and bioaccessibility and bioavailability of heavy metal(loid)s by carrying out the following experiments:

(i) Metal(loid) toxicity experiment: demonstrates the threshold values of various heavy metal(loid)s by investigating gut microbial growth. This experiment also demonstrates the relationship between metal(loid) concentration and gut microbial growth.
(ii) Bioaccessibility of heavy metal(loid)s: provides an understanding of the effect of gut microbes on the bioaccessibility of heavy metal(loid)s as measured by *in vitro* gastrointestinal bioaccessibility tests.
(iii) Bioaccessibility as impacted by chelating agents: provides an understanding of the effect of chelating agents on gut microbe-induced bioaccessibility of heavy metal(loid)s as measured by *in vitro* gastrointestinal bioaccessibility tests.
(iv) Bioavailability of heavy metal(loid)s: compares bioavailability of heavy metal(loid)s as impacted by gut microbes and chelating agents using Caco-2 permeability assay tests.

1.5 Thesis objectives

The overall objective of the thesis is to examine the interactions between gut microbiome and heavy metal(loid)s in relation to metal(loid) toxicity to gut microbes and bioaccessibility of metal(loid)s. The specific objectives of the study include:

i) To demonstrate the effect of arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) on the growth of selected gut microbes.

ii) To examine the effect of selected gut microbes on the bioaccessibility of these heavy metal(loid)s.
iii) To examine the effect of chelating therapeutic agents on bioaccessibility of heavy metal(loid).
iv) To examine the effect of gut microbes and chelating therapeutic agents on the bioavailability of heavy metal(loid)s.

1.6 Thesis hypothesis

A number of hypotheses relating to metal(loid) toxicity to gut bacteria, and the bioaccessibility and bioavailability of metal(loid)s as impacted by gut bacteria and chelating agents will be tested in the study. The major hypotheses include:

(i) The metal(loid) toxicity to gut bacteria is dependent of metal(loid) species and pH conditions of the growing media
(ii) Bioaccessibility of metal(loid)s varies between gastric and intestinal extractions, and depends on the nature of metal(loid)s and its sources.
(iii) Gut bacteria modulate bioaccessibility of metal(loid)s through their interactions with metal(loid)s via adsorption and speciation processes.
(iv) Chelating agents influences bioaccessibility of metal(loid)s through their effects on the solubilisation of metal(loid) sources and subsequent interactions with metal(loid)s, and the effect depends on the nature of chelating agents and metal(loid)s sources.
(v) Bioavailability of heavy metal(loid)s as measured by intestinal permeability is impacted by metal(loid) binding with compounds or gut microbes that reduce their solubility (i.e., bioaccessibility) or their passage through the epithelium.

1.7 Thesis structure

An overview of thesis chapters is given in Figure 1.1.

Chapter 1 *Introduction:* gives an overall outline of gut microbiome, environmental contaminants, the effect of heavy metal(loid)s on gut microbiome, and bioaccessibility of heavy metal(loid)s as impacted by gut microbiome. This chapter also lists the research objectives and how the thesis is structured.

Chapter 2 *Literature review:* describes the literature review covering the role of gut microbes in human health, processes relating to gut microbe dysbiosis, effects of environmental contaminants on gut microbes, and treatment of heavy metal(loid) toxicity.

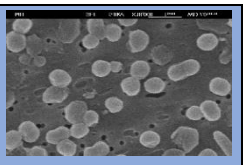
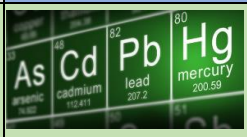
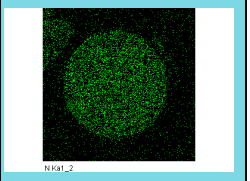
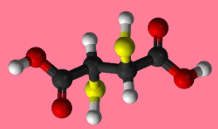
Chapter 1	**Introduction** • Gut microbes and human health. • Treatment of heavy metal(loid) toxicity in humans • The main objectives of research experiments in this thesis	
Chapter 2	**Review of Literature** • Microbiome (definition, number, diversity, functions) • Microbiome dysbiosis and human health • Source of contaminants • Environmental contaminants and gut microbiome interactions • Enhancing gut microbiome homeostasis • Summary and conclusions	
Chapter 3	**Heavy metal(loid) toxicity to gut bacteria** • Bacterial growth study: • Bacterial toxicity study: 4 metal(loid) sources • Speciation of metal(loid)s: 4 metal(loid) sources, 3 gut bacterial species, metal(loid) speciation in gastric and intestinal extracts	
Chapter 4	**Bioaccessibility of orally ingested heavy metal(loid) sources** • Bioaccessibility of metal(loid)s: 4 metal(loid) sources, gastric and intestinal bioaccessibility tests • Speciation of metal(loid)s: 4 metal(loid) sources, metal(loid) speciation in gastric and intestinal extracts	
Chapter 5	**Bioaccessibility of orally ingested heavy metal(loid) sources as impacted by gut bacteria** • Bioaccessibility of metal(loid)s: 4 metal(loid) sources, 3 gut bacteria, gastric and intestinal bioaccessibility tests • Speciation of metal(loid)s: 4 metal(loid) sources, metal(loid) speciation in gastric and intestinal extracts • Adsorption of metal(loid)s: 4 metal(loid) sources, 3 gut bacteria,	
Chapter 6	**Bioaccessibility of orally ingested heavy metal(loid) sources as impacted by gut bacteria and chelating agents.** • Effect of chelates on gut bacteria: 3 gut bacteria species, 3 chelating agents, bacterial growth • Bioaccessibility of metal(loid)s: 4 metal(loid) sources, 2 gut bacteria species, 2 chelating agents, gastric and intestinal bioaccessibility tests • Speciation of metal(loid)s: 4 metal(loid) sources, 2 gut bacteria species, 2 chelating agents, metal(loid) speciation in gastric and intestinal extracts	EDTA 
Chapter 7	**Bioavailability of orally ingested heavy metal(loid) sources as measured by intestinal permeability tests.** • Bioavailability of metal(loid)s: 4 metal(loid) sources, 2 gut bacteria species, 2 chelating agents, intestinal permeability test • Speciation of metal(loid)s: 4 metal(loid) sources, 2 gut bacteria species; 2 chelating agents, metal(loid) speciation in gastric and intestinal extracts	
Chapter 8	**Summary, conclusions and future research needs** • Summary of the research findings. • Main conclusions and future research needs.	

Figure 1.1 An overview of thesis chapters

Chapter 3 *Heavy metal(loid) toxicity to gut microbe:* demonstrates the relationship between concentration of selected heavy metal(loid)s and growth of gut microbes.

Chapter 4 *Bioaccessibility of heavy metal(loid)s:* evaluates the gastrointestinal bioaccessibility heavy metal(loid)s in selected sources including rice grain (As), fish meal (Hg), complementary medicine (Pb), and pet food (Cd).

Chapter 5 *Bioaccessibility of heavy metal(loid)s as impacted by gut microbes:* evaluates the influence of gut microbes on the gastrointestinal bioaccessibility heavy metal(loid)s in selected sources including rice grain (As), fish meal (Hg), complementary medicine (Pb), and pet food (Cd).

Chapter 6 *Chelating agents on bioaccessibility of heavy metal(loid) as impacted by selected gut microbes:* evaluates the impact of chelating agents on gut microbe-induced gastrointestinal bioaccessibility of heavy metal(loid)s in selected sources including rice grain (As), fish meal (Hg), complementary medicine (Pb), and pet food (Cd).

Chapter 7 *Bioavailability of heavy metal(loid)s as measured by permeability test:* compares the bioavailability of heavy metal(loid)s as impacted by gut microbes and chelating agents using Caco-2 permeability assay tests.

Chapter 8 *Summary and conclusions:* provides overall research conclusions and a summary of the major research findings. This chapter suggests possible future directions in research which can provide in-depth knowledge in the field of gut microbes and heavy metal(loid) interactions.

Since each research chapter (Chapter 3 – 7) has been written as a separate and independent future publication, there will be some repetition of Materials and Methods, and Results and Discussion sections in these chapters.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Microorganisms (bacteria, fungi and archaea) form a complex symbiotic relationship with humans to facilitate functions that improve the life of the human host (Hooper et al., 2002; HMPC, 2012; DeWeerdt, 2015). These microorganisms are found in specific sites in humans providing various roles for optimum functioning (Costello et al., 2009; HMPC, 2012; Blekhman et al., 2015). The specific sites include: skin, teeth and gum, saliva and oral mucosa, conjunctiva, vagina and gastrointestinal track (Ursell et al., 2012; Young, 2012; Hattori and Prakash, 2015).

In general, a single human being is comprised of approximately $3x10^{13}$ cells (30 trillion) (Senders et al., 2016a,b) which is higher than the earlier estimate of around 10 trillion cells (Strachan and Read, 1999; Hafen and Stocker, 2003; Goodsell, 2009; Bianconi et al., 2013). These human cells are almost matched by approximately $3.8x10^{13}$ microbial cells (38 trillion) that cohabitate humans, leading to a ratio of approximately 1:1 human to microbial cells (Senders et al., 2016a,b). These approximately 38 trillion microorganisms in and on the human body, the majority of which reside in the gastrointestinal tract play a key role in human functioning, metabolism, physiology, nutrition, immune function and gut homeostasis. Hence the gut microbiome is considered to be a hidden metabolic 'organ' (Hooper et al., 2002).

Until recent improvements in DNA sequencing, there had been little knowledge about the diversity of the human microbiota and the functions they play in human systems (Qin et al., 2010). High throughput DNA sequencing studies, for example the American Gut study (http://americangut.org/) and the Human Microbiome study (http://hmpdacc.org/), have improved our understanding of the morphology and functions of the bacteria that make up our own indigenous microbiota (Gray et al., 2015).

The human gut microbiota are a composite structure of up to 1000 distinct bacterial species that reside in the human digestive tract (Li et al., 2012; Pflughoeft and Versalovic, 2012; Weinstock, 2012). Fungi, protozoa and archaea are found in lesser numbers and constitute the rest of the gut microbiota (Jandhyala et al., 2012). The normal gastrointestinal

microbiota aid and facilitate a multitude of functions within the gut and in exogenous sites, they aid in digestion; synthesis and modification of vitamin K and vitamin B12; synthesis of serotonin; they out compete pathogen species for nutrients and binding sites, stimulate normal tissue, train the immune system and help in the synthesis of some antibiotics (HMPC, 2012; Pflughoeft and Versalovic, 2012).

A variety of internal and external factors can impact and alter gut microbiota including age, gender, diet, antibiotic treatment, infection, exposure to smoking, and exposure to environmental contaminants such as heavy metal(loid)s (Walter and Ley, 2011; Borer et al., 2013; Faith et al., 2013). Humans can affect the distribution and abundance of gut microbes through many channels such as changes in diet, exposure to antibiotics and use of probiotics. Large fluctuations in gut microbiota appear in the first year of life; the specific microorganisms being dependent on similar factors to changes in the adult gut microbiome, but it is also affected by family size, nutrition and water quality (Palmer et al., 2007; Turroni et al., 2012).

More recently, studies have shown that environmental factors may play a role in shaping the gut microbiota, such as exposure to pollution, water quality and environmental contaminants such as heavy metal(loid)s (Nicholson et al., 2012). For example, heavy metal(loid) exposure has been shown to have a direct impact on the diversity of gut microbiota of mice (Breton et al., 2013b). Until recently there has been very little knowledge on the functions of the gut microbiota and their interactions with environmental contaminants including heavy metal(loid)s (Dave et al., 2012; Holtcamp, 2012; Monachese et al., 2012; Lu et al., 2014; Potera, 2014a and b; Ninkov et al., 2015). The interaction between environmental factors such as heavy metal(loid)s and the gut microbiota provides an interesting and dynamic approach into examining the influence of environmental factors on human health (Dave et al., 2012).

In the following section, a review of current literature will establish an introduction to the makeup of the gut microbiota, their functions and the factors affecting them. Then it will discuss the interaction between gut microbes and the environment with a focus on heavy metal(loid) interaction.

2.2 Definition of the gut microbiota

The word ‘Microbiome’ refers to the entire collection of microorganisms, their complete genetic makeup (genomes), and the interactions of these in a specifically defined environment (Ursell et al., 2012). For example, the microorganisms found in the human digestive tract are often collectively referred to as the ‘gut microbiome’ (HMPC, 2012; Ursell et al., 2012)). The

environment in this case is the human digestive tract, which includes the upper gastrointestinal tract (oral cavity, oesophagus, stomach and small intestine up to the ileum) and lower digestive tract (cecum to anus) (Figure 2.1).

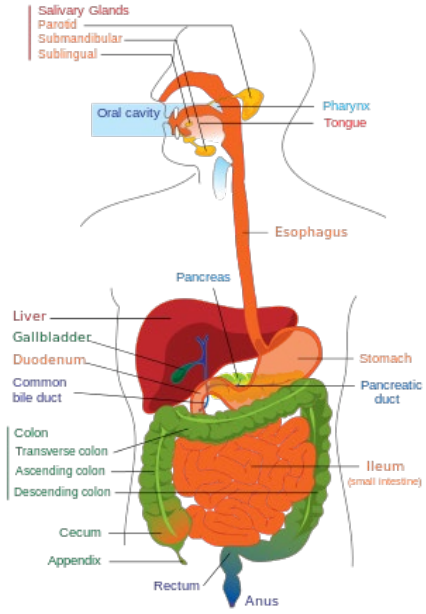


Figure 2.1 Human digestive system and gastrointestinal tract

Microorganisms are microscopic single or multicellular organisms that include bacteria, fungi, archaea and protozoa. These microorganisms can form mutualistic, commensal, parasitic or pathogenic relationships in human systems (Ghosh, 2013). The interaction between humans and microbes takes on four different modalities; namely Mutualism, Commensalism, Parasitism and Pathogenicity (Bradford and Schwab, 2012). Mutualism is the most common form of interaction between humans and microbes. For example, the intestinal environment providing nutrition to the microbiota from the food that host consumes and the microbes providing services to the host. Certain *E. coli* strains produce vitamin K as a vital element of haemostasis. Commensal organisms make up part of the microbiome, with some of the smaller microbial species providing no currently known benefit or harm to humans, although it could be argued these species provide mutualism by outcompeting pathogen microbial species. Parasitic microbial species that benefit at the hosts expense can exist in the gut microbial population. These parasites are usually ectoparasites and include protozoan species such as cryptosporidium, *Entamoeba histolytica* and *Giardia duodenalis*. Pathogenic gut microbes include opportunistic pathogens, for example, many intestinal microbial species

produce local and systemic infection when there is a breach in the gut wall immune systems (Rescigno et al., 2001; Meyer-Hoffert et al., 2008; Hooper and Macpherson, 2010).

The gut microbiota includes all the microorganisms that reside in the gastrointestinal tract, encompassing the anatomical structures between the mouth and anus and all the other related organs associated with digestion of food for normal human homeostasis (O'Hara and Shanahan, 2006; Furusawa et al., 2013). When discussing the gut microbiota, oral microbes residing in teeth and gum are generally not included (Ursell et al., 2012). Although fungi, archaea and protozoa are found in the human gastrointestinal tract, bacteria predominate and are the most common organism referred to when discussing gut microbiota (Cénit et al., 2014). A major proportion of gut microbiota is considered mutualistic organisms with humans (Qin et al., 2010; Ursell et al., 2012).

2.3 Acquisition of the human gut microbiota

Recent developments with the ability to sequence bacterial species with molecular techniques have increased our understanding of the acquisition and transitional relationship of gut microbes *in utero*, in the neonate and in the infant gut (Madan et al., 2012). The microbial species acquired early in life most likely alters adult biology (Rodríguez et al., 2015).

2.3.1 *In utero* transfer

Until recently, *in utero* bacterial ecology was thought to be non-existent, with the first baby-bacterial interaction being via the mechanism for delivery (Jiménez et al., 2005; Perez-Muñoz et al., 2017; Walker et al., 2017). Intrauterine microbial studies were conducted only in the case of suspected intrauterine infection (Jiménez et al., 2005; Bright and Bulgheresi, 2010; Funkhouser and Bordenstein, 2013), and there was a correlation between preterm (premature) deliveries and intrauterine bacterial infections. This had led earlier researches to conclude that the presence of intrauterine microbiota was only pathogenic (Goldenberg et al., 2008; Perez-Muñoz et al., 2017).

The placental microbiome has recently been described as a unique community of microorganisms. For example, Aagaard et al. (2014) in their study of 320 subjects found that the placental microbiome composed of low abundance of nonpathogenic commensal bacteria from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes and Fusobacteria phyla. When compared to other human microbial profiles, they found the placental profile most resembled the non-pregnant human microbiome profile.

Recent studies have shown that maternal gut microbiota can be transferred to fetuses *in utero*. For example, Jiménez et al. (2005) and Jiménez et al. (2008) showed that orally administered and genetically tagged *Enterococcus faecium* could be found in the maternal amniotic fluid and meconium of mice born by sterile cesarean (C) section. They also showed that blood samples taken from the umbilical cords of healthy neonates born via C-section contained bacterial species. They identified *Enterococcus faecium, Propionibacterium acnes, Staphylococcus epidermidis, and Streptococcus sanguinis* species. Postulating that the oral bacteria could enter the uterine environment through the blood stream, these studies and others have overturned the dogma of the sterile fetal environment. These studies show that the fetus may not occupy a sterile environment and that an intrauterine microbial environment may be the first exposure pathway for intestinal microbes.

Microbiome analysis in humans have identified bacterial species in infant meconium (Ardissone et al., 2014) and bacterial DNA sequences in cord blood (Jiménez et al., 2005). Meconium is a dark, viscus material that is normally present in the neonatal intestine at birth and passed as the first faeces after birth. Meconium microbiome in mice studies have shown that exposure to bacteria occurs *in utero* (Jiménez et al., 2008), confirming the non-sterile conditions of *in utero*. For example, Hu et al. (2013a) examined the microbial diversity of meconium samples from 23 newborns and noticed that they were not sterile and found to contain a diversity of microbiota. Furthermore, they found that maternal diabetes mellitus status effected the overall bacterial content. The diabetes mellitus groups in the study had greater Bacteroidetes (phyla) and *Parabacteriodes* (genus) in meconium samples than samples of infant meconium from non-diabetic mothers. This shows the significance of the transfer of prenatal microbial status to neonates.

Bacterial species have been detected in increased numbers in the meconium of premature infants. For example, Ardissone et al. (2014) found increased evidence of bacterial inter-uterine colonization was correlated with premature birth and lead to the hypothesis that the fetal intestinal microbiome that may be involved in the inflammatory response leading to premature birth is derived from swallowed amniotic fluid (Martinez et al., 2018). Foetal intestinal microbiome derived from amniotic fluid ingested *in utero* could be implicated in the inflammatory process that leads to premature birth (Gardella et al., 2004). Therefore, *in utero* acquisition of non-pathogenic microbes during gestation is important for healthy childbirth (Perez-Muñoz et al., 2017).

2.3.2 Neonatal acquisition of microbes

Following birth, the infant starts developing its own microbiome with the journey towards microbial equilibrium, with an adult-like microbial profile developing by the first 3-5 years of life (Rodríguez et al., 2015). At the time of birth the baby is exposed to a 'seed' ecology of microbes that depends on the mechanism of birth. The majority of the initial neonatal microbial gut colonisers are introduced via the mode of delivery (Azad et al., 2013b).

Infants that are born via vaginal delivery have a different gut microbiota spectrum compared to infants born via C-section. For example, newborns delivered via C-section have gut microbiota more similar to the microbial profile of maternal skin, whereas babies born by natural vaginal delivery have gut microbial species more similar to the maternal vaginal and faecal microbiota profile (Salminen et al., 2004; Dominguez-Bello et al., 2010; Dogra et al., 2015). The mode (C-section versus vaginal delivery) and location of delivery is a determining factor towards the configuration of the infant gut microbiota, along with type of infant feeding, gestational age, infant hospitalisation and antibiotic use. In one study of 1032 Dutch infants, Penders et al. (2006) noticed that infants born via C-section had different microbial gut species at 3-6 weeks compared vaginally born infants. Comparing hospital C-section deliveries to vaginal home deliveries, they found hospital C-section births resulted in lower gut colonisation rates of bifidobacteria and *Bacteroides*, whereas *C difficile* prevalence was higher. van Nimwegen et al. (2011) compared gastrointestinal microbiota composition of babies delivered at various locations (home vs hospital), and noticed that C-section-delivered hospital born children had higher rates of *C difficile* colonisation than other birth type and location groups. Furthermore, *C difficile* colonisation of gastrointestinal tract of babies was associated with an increased risk of further atopic complications, including asthma at 6-7 years of age.

Likewise, vaginally born infants generally have a rapid in-flux of Proteobacteria and a higher proportion of *Bifidobacteria*, particularly *Bifidobacter catenulatum* and *Bifidobacter longum* than C-section birth infants (Biasucci et al., 2010). Another study which examined the infant gut microbiome at four months of age of 24 term infants showed that infants born via C-section delivery had lower bacterial diversity and were under-represented in *Escherichia, Shigella* and *Bacteroides* species and species under the phylum Bacteroides than vaginally born infants (Azad et al., 2013b).

The infant gut continues to be influenced by the mechanisms of delivery well after the neonatal stages. Jakobsson et al. (2014) found in a study of 24 infants that C-section births were associated with a total lower gut microbial diversity during the first 2 years of life compared with vaginally born infants. This study also showed that C-section birthed infants'

intestines were less often colonised with Bacteroidetes phylum and had less abundance and diversity of this phylum.

During the first few weeks of life, the microbial gut community develops and shifts rapidly, being moulded mostly by babies' dietary exposure to breast milk (Wold and Adlerberth, 2000; Biagi et al., 2017; Murphy et al., 2017; Timmerman et al., 2017; Toscano et al., 2017). Not as dramatic but these shifts in normal microbial flora are seen across a human's lifespan, throughout childhood and into adult life and into old age with the microbial community of retirees shown to be different from those of midlife and early adults (Biagi et al., 2010). This dynamic developmental route that the microbiome takes throughout life likely has a great influence over the human host (Rodríguez et al., 2015).

Dietary changes are the main drivers preceding shifts in microbial gut populations after initial colonization, with initial feeding patterns (i.e. breast or formula milk) having persistent effects. Bäckhed et al. (2015) found that during the first year of life, the infant gut microbiome is shaped by mode of delivery and feeding. The major driving factor for the development of the infant gut microbiota was found to be nutrition with the cessation of breast-feeding causing the rapid maturation of gut microbiota to resemble adult profiles.

Penders' et al. (2006) study of 1032 Dutch infants found that the key determinants of the gut microbiota configuration in infants were the mode of delivery, infant feeding, gestational age, infant hospitalisation and antibiotic use. They found that infants who were born at gestational term via vaginal delivery at home and were exclusively breastfed, developed the most optimum gut microbiota at 1 month of age, with highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli*.

2.3.3 Microbes acquired at infant stage

Anaerobes are well represented members of the gut microbiota within several days of birth. The major shifts in the gut microbial populations in infants are preceded by dietary changes. Initial feeding mechanism (breast milk or formula) has persistent effects on the gut microbiota (Rodríguez et al., 2015). Gram negative bacteria are present at higher concentration than other groups of microbes in the stools of older children and adults. Convergence to an adult population of gut microbes does not occur until two years of age coinciding with the introduction of solid food (Marques et al., 2010) (Table 1).

The *in utero* environment provided by the maternal body habitus may influence infant microbial gut content. For example, the body mass index (BMI) of the mother can influence the communities of infant microbes. Faecal *Bacteroides* and *Staphylococcus* concentrations have

been found to be higher in infants of overweight mothers during the first 6 months (Collado et al., 2010). In this study higher maternal BMIs were related to higher concentrations of *Bacteroides, Clostridium*, and *Staphylococcus* and lower concentrations of the Bifidobacterium group. Prevalence of *Akkermansia muciniphila*, *Staphylococcus sp.*, and *C. difficile* groups was lower in infants of normal-weight mothers and of mothers with normal weight gains during pregnancy.

Logistic challenges exist in studying the sequential phases of bacterial colonization in infants. For example, high frequency stool samples are challenging to obtain from community based infants, sampling methods and bacterial identification differs between studies. Studies have related to the early colonisation of commensal gut microbiota with medical pathologies in later life (Tlaskalova-Hogenova et al., 2011; Collado et al., 2012; Romano-Keeler and Weitkamp, 2015). Sjogren et al. (2009) showed that there was an association with allergies in children at age 5 to the amount of lactobacilli in their infant stool. Other studies have shown that *C. difficile* colonization in the first month of life is related to atopy and asthma at six years of age (Penders et al., 2006; van Nimwegen et al., 2011). White et al. (2013) found that *Staphylococcus, E. coli* and *Bacteroides* species in stools of Norwegian infants in the initial months of life were associated with expected childhood body mass index at up to 24 months of age.

Table 2.1 Acquisition of the human gut microbiota in infants

Countries	No. of subjects (age(s) at sampling)	Samples per subject	Enumeration technology (16S rRNA sequenced)	Reference	Conclusions
USA	14 (0-1 yr)	26	Microarray	Palmer et al. (2007)	Colonisation process of the gut flora is individual specific; Gut microbiota converges to adult-like profile at 1 year of age
Finland and Spain	42 (1 and 6 months old)	2	qPCR	Collado et al. (2010)	Infant gut microbes are affected by maternal BMI and BMI gain during pregnancy
USA	1 (0-2.5 yr)	60	454 FLX pyrosequencing (V1-2)	Koenig et al. (2011)	Microbial succession associated with diet and other life events; gut bacteria start to stabilise at 1 year of age
Africa, USA and American Indians	146 (0.3 yr)	1	Illumina HiSeq 2000 (V4)	Yatsunenko et al. (2012)	Gut microbiome varies by age and geography, but becomes adult-like at the age of 3
Switzerland	7 (4-30 d)	3	Sanger (V1-9), culture of 454	Jost et al. (2012)	Anaerobes are pioneer colonisers, and their

			pyrosequencing (V4-5)		abundances are similar as adults in the first week of life.
Sweden	65 (1-8 wk)	4	Culture	Lundell et al. (2012)	Early gut microbiota including *E. coli* and *Bifidobacteria* contribute to B cell activation and memory differentiation
USA	12 (<1 yr)	1	Illumina GAIIx (V2)	Song et al. (2013)	Pronounced changes in gut microbiome occur in a protracted timeframe
Canada	24 (4 mo)	1	High throughput sequencing (V5-7)	Azad et al. (2013b)	Formula-fed infants have higher richness than breast-fed infants. *C. difficile* is more abundant in formula-fed babies. *Escherichia* and *Bacteroides* were less abundant in babies born by C-section

2.3.4 Stabilisation of infant gut microbes

From the early colonisation stage, the gut microbiota of infants undergo stabilisation and are transformed into a mature microbial population similar to an adult profile. The gut microbiome in infants is highly dynamic, and depends on early life changes in the composition of microbes as affected by environmental changes (Lim et al., 2015). The microbial composition in infants depends both on the mode of delivery and diet in the first year of establishment. In a metagenomics study, Bäckhed et al. (2015) sampled the microbiome at different stages of an infant gut and found that infant nutrition had a major effect on maturation of the gut microbiome, where the microbiota composition and ecological network were found to be distinctive at each stage. However, stabilisation of the gut microbes takes longer than one year of birth. Significant differences in taxonomic and functional microbiota composition between one year olds and mothers was detected in a study of 13 infants (Vallès et al., 2014). However, succession of core genera of gut microbes from maternal transfer and positive interactions among core genera during community assembly contribute to ensure their permanence within the gut.

2.4 Composition and structure of the adult gut microbiota

The gastrointestinal tract (Figure 2.1) is an important organ system that functions to consume and digest foodstuffs, absorb nutrients, and expel waste. It includes the upper gastrointestinal tract (mouth, oesophagus, stomach and small intestine to the terminal ileum) and lower digestive tract (cecum to anus), including the gall bladder, liver and pancreas (Yamada et al.,

2009). An increasingly important functional part of the gastrointestinal tract is the bacterial communities that reside in it.

The vast majority of gut microbes are bacteria and reside in the distal intestinal tract in the large intestine (DeWeerdt, 2015). The microbial communities are distinct between humans, and the variety and configuration of these microorganisms that make up the gut microbiota is influenced by the location these microbes reside in the human body (Figure 2.2). For example, many bacteria carve out specific niches in human systems, indicating that there are distinct regional areas in the intestines where specific bacteria are more likely to colonize (Wang et al., 2003). Archea, fungi, Protists and viruses are also resident microbiota in the human gastrointestinal tract.

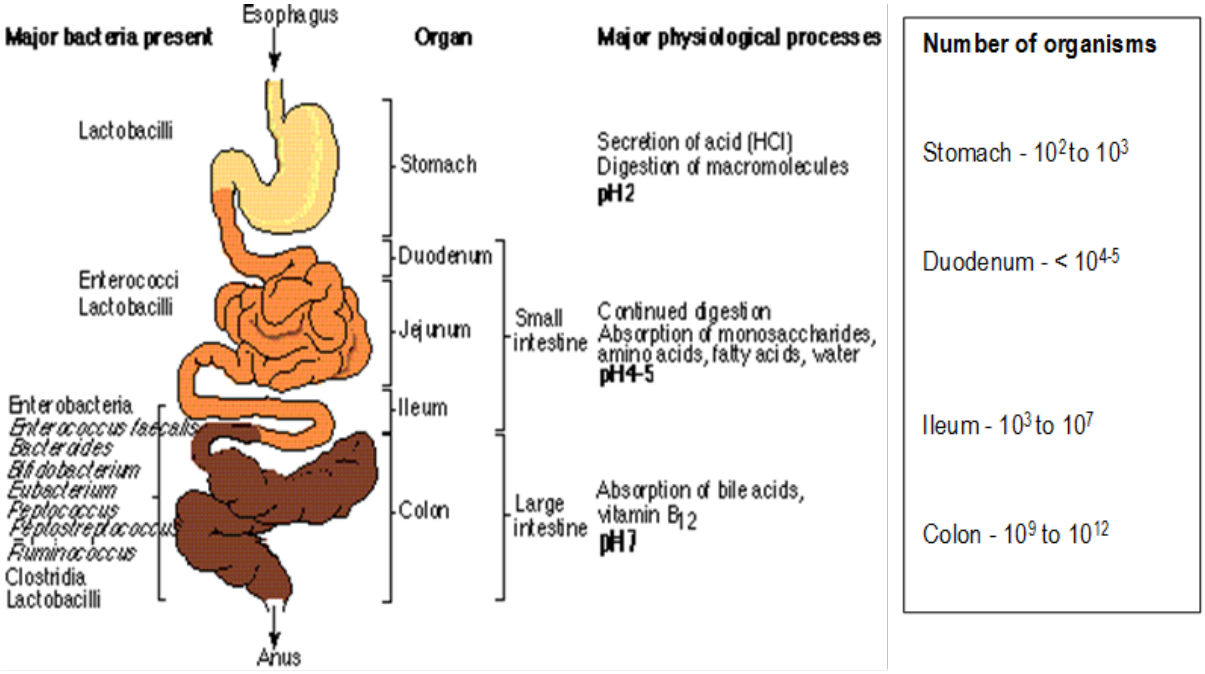


Figure 2.2 Major bacteria present in various organs in human digestive track

Recently, there have been many studies focusing on characterising the residents of the gut microbial populations in humans (Peterson et al., 2009). This has been facilitated by the advent of high throughput gene sequencing and increased computer performance to allow for the sequencing and analysis of large amounts of genetic data (Gray et al., 2015).

2.4.1 Stomach

The upper gastrointestinal tract includes the stomach, duodenum, jejunum, and upper ileum. It contains a sparse resident microbiota when compared to the large bowel. Microbial concentrations of 10^4 organisms/mL of stomach and intestinal secretions have been reported (Baron 1996). The stomach environment is generally considered hostile to microorganisms, thus very few bacteria colonize the stomach when compared to the small and large intestines (Yang et al., 2013). The pH of the stomach varies from 1.0-2.0 up to 4.0-5.0 depending on which phase of digestion it is going through. In response to food, the stomach releases hydrochloric acid which decreases the pH to 1.0-2.0 allowing the best environment for protease enzymes that are also released by the stomach. After digestion, buffers cause the pH to raise back to the resting level of 4-5 (Fordtran and Walsh, 1973; Duquette and Wray, 2014).

Organisms in the stomach are mostly thought to be transient and fluctuate. Their populations are lower (10^1 to 10^3 microbes/mLof contents) than the small and large bowel due to the relatively low pH which selects for small amounts of acid tolerant bacteria (Penders et al., 2007). Until recently, the only microbial presence that was thought to colonise this environment was the pathogenic *Helicobacter pylori* species. *H. pylori* stomach infection is part of the gastric microbiota in up to 70% of the population in developing countries and up to 40% in developed countries (Brown, 2000). Long-term *H. pylori* infection has been identified to have a role in the development of peptic ulcer (duodenal or gastric) and gastric cancer in approximately 15% of people infected (Logan and Walker, 2001).

Minimal commensal bacterial numbers are found in the stomach relative to the large intestine due to its low pH environment (Ghosh, 2013). Harsh conditions select for acid-tolerant species of *lactobacillus* and *streptococcus* which colonize the walls of the stomach (Bik et al., 2006). Where previously thought this environment was too hostile of a microbial presence, recent studies using advanced sequencing techniques have shown the colonisation of pathogenic and non-pathogenic bacterial species in this area (Thursby and Juge, 2017). The stomach is exposed to bacteria from the oral cavity and bacteria back tracking from the duodenum. Bacteria identified in oral cavity are common with more than 65% of the phylotypes in the stomach. Hence, bacterial species such as *Veillonella*, *Lactobacillus* and *Clostridium* that are found in gastric juice may be transient species (Nardone and Compare, 2015).

In a study by Bik et al. (2006), endoscopic gastric biopsies samples were taken from 23 subjects and subjected to bacterial PCR and bacterial species characterised via 16S rDNA (Table 2). They found sequences assigned to the Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria phyla. Thus, studies have shown that in healthy subjects,

there is a ‘core microbiome’ in the stomach. This is dominated by *Prevotella, Streptococcus, Veillonella, Rothia and Haemophilus*. When *H. pylori* infection is present or in cases of gastric cancer, there can be an associated shift in the microbial colony to changes in the abundance of Firmicutes phylum and *Streptococcus* and *Prevotella* genera (Nardone and Compare, 2015).

2.4.2 Small intestine

The small intestine starts from the gastro-duodenal valve at the juncture of the stomach and the duodenum and ends at the ileocecal junction. It includes all sections of the small intestine namely; the duodenum, the jejunum and the ileum. This tightly folded approximately 6m long tube is the longest portion of the digestive system and is tasked with the digestion of fats, proteins and carbohydrates in consumed food (Bloom et al., 2011). The nutrients produced are absorbed through the lining of the small intestine into the bloodstream.

The gastroduodenal junction contains a sharp pH gradient, from the strong acidic stomach to the circumneutral duodenum. The pH of the duodenum increases from the acidic stomach pH of 1.0 – 5.0 to approximately pH 6 in the duodenum. This gradient increases across the small intestine to pH 7.4 in the terminal ileum. This is important in relation to heavy metal(loid) exposure as aspects of metal(loid) function such as solubility are affected by pH gradients.

After receiving the partially digested acidic stomach content, duodenal cells produce bicarbonate to increase the pH and neutralize the contents; release of cholecystokinin helps in the stimulation of the gall bladder to release bile. Bile consists of bile salts and acids, bilirubin, cholesterol, fatty acids and lecithin. Large fat molecules are broken down by bile, which consists of acids, salts, pigments, cholesterol, and phospholipids. Lipase then breaks them down further into fatty acids which are absorbed across the intestinal wall into the lymphatic system (Bloom et al., 2011).

Duodenal cells also produce secretin which inhibits the secretion of gastric acid from parietal cells of the stomach and thereby regulate duodenal pH. Secretin also stimulates the pancreatic centroacinar cells and intercalated ducts to produce bicarbonate, further neutralising the pH.

Table 2.2 Sampling and identification methods used for studying stomach microbiota

Study	Sampling Method	Number of subjects	Identification method	Major Phyla/Genera	Observations

Zilberstein et al. (2007)	1 mL stomach mucus, jejunum and proximal ileum sampled via radioscopy probe	20 - 8 male 12 Female	Selective Culture media	*Veillonella Lactobacillus Clostridium Corynebacteriu m Bacteroides*	The presence of "non-pathogenic" anaerobic bacteria indicates the existence of a low oxidation-reduction potential environment, which suggests the possibility of adoption of these bacteria as biological markers of total digestive tract health.
Bik et al. (2006)	Single gastric mucosal biopsy	23 – 22 male 1 – female	16S rRNA sequences clone library approach	*Proteobacteria Firmicutes Bacteroides Actinobacteria Fusobacteria*	Gastric bacterial rDNA data set was significantly different from sequence collections of the human mouth and oesophagus described elsewhere, indicating distinction of human stomach ecosystem.
Aviles-Jimenez et al. (2014)	Endoscopic biopsy - from antrum and corpus. (NAG + IM) Surgical tumour removal. (GC)	15 total subjects 5-Non-atrophic gastritis	16s rRNA	Firmicutes Proteobacteria Bacteroidetes Actinobacteria Fusobacteria	Gastric microbiota of patients with non-atrophic gastritis (NAG), intestinal metaplasia (IM) and intestinal-type gastric cancer (GC)
Andersson et al. (2008)	Upper endoscopy - Stomach biopsies	6 subjects- 3- *H. pylori* positive and 3 negative	16S rRNA sequences	Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fusobacteria	Stomach displays diverse microbiota when *H. pylori* is absent
Li et al. (2009)	Endoscopy - Body and antrum biopsies	10 subjects – 5 healthy and 5 antral gastritis female subjects	16S rRNA and urease activity	Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria	Significantly higher abundance of the Firmicutes phylum and the *Streptococcus* genus within the Firmicutes phylum was observed in patients with antral gastritis, compared with normal controls
Engstrand and Lindberg (2013)	Endoscopy - Body and antrum biopsies	13 healthy subjects	Pyrosequenc ing	*Prevotella, Streptococcus, Veillonella, Rothia,*	They did not differ by comparing antrum versus body.

Limited studies have focused on categorising the microbial ecology of the small intestine. This is due to the difficultly in access, the proximity to the acidity of the stomach and the low bacterial population in comparison to the large intestine. The microbial distribution density across the small intestine is sparse when compared to the large intestine. The duodenal flora is also minimal (0 to 10^3 cells/gram of contents). Microbial contents of the ileum is higher (10^6 to10^8cells per gram of contents) with the majority of them being anaerobes (Zoetendal et al., 2012).

Proximity to the stomach influences the quantity of microbial species in the small intestine. The areas of the small intestine in close proximity to the pyloric sphincter have the least microbial density due to the relative low pH from stomach acidity (Kerchhoffs et al., 2006). Rapid peristalsis and the presence of bile could also help to explain the lower numbers of bacteria in the upper gastro intestinal (GI) tract. The small intestine contains small numbers of *Streptococci, lactobacilli* and yeasts species particularly *Candida albicans*. The majority of the microbial numbers of the small intestine are found at the terminal ileum, the small intestinal section that is of closest proximity to the large bowel (von Rosenvinge et al., 2013a). The small intestinal tract contains an incomplete mucus barrier adapted to exchange with the lumen contents. Bacterial interaction with the cell wall structures are impeded by epithelial-derived antimicrobial factors such as defensins and REGIIIg proteins (Moran et al., 2015).

2.4.3 Large bowel

In contrast to the small intestinal microbiota, the large bowel contains a larger number and diversity of microbes. The largest population of the human microbiome resides in the large intestines. Concentrations of 10^9 to 10^{12} bacterial cells/g of contents are found in the human colon and faeces (Savage, 1977; Sender et al., 2016a,b). More than 400 different species of bacteria have been identified in faecal samples. The majority of these microbes (95-99%) belong to the anaerobic genera *Bacteroides*, *Bifidobacterium*, *Eubacterium, Peptostreptococcus* and *Clostridium* (Qin et al., 2010).

The large bowel is an anaerobic environment and the pH is higher than in areas of the stomach and small intestine. As pH is a selection criteria for microbes, it also influences the microbial diversity of the large bowel. Numerous studies have been done to ascertain the pH values across the GI tract. Evans et al. (1988) found that gastric pH was highly acidic, ranging from 1.0-2.5 in subjects tested. The proximal small bowel had a mean pH of 6.6, which increased slowly into the distal small bowel and terminal ileum which averaged pH 7.5. From ileum to caecum, there was a sharp fall in pH to an average of 6.4. The pH then rises slowly from the right to the left colon to a final average of 7.0.

In the large bowel, facultative anaerobes such as *E. coli* are outnumbered by anaerobes such as *Bacteroides*, anaerobic *Streptococci* and *Clostridia* by 10^3. Recent improvement in genetic sequencing techniques have highlighted the variety of species that are present in the normal adult gut. There are currently a number of high profile projects sequencing large sample sizes to create libraries of sequencing data of individuals. These studies include the human microbiome project, the American gut project, the British and Canadian gut project, and

International Human Microbiome Consortium (IHMC). The IHMC is coordinating the microbiome initiatives around the world including those in the EU, China, Japan, Singapore, Australia and Canada.

2.5 Functions of the gut microbiome

The functions and mechanistic effects of the gut microbiota are an emerging field in human microbiology, and many of the roles that our microbial partners play in overall human digestion, regulation and homeostasis, are not well understood (Pflughoeft and Versalovic, 2012). Emerging research suggests that we are yet to discover all the benefits these co-inhabitants have on human systems and all the negative outcomes associated with an alteration or dysbiosis in this system (Gerritsen et al., 2011; Maruya et al., 2013; Galley and Bailey, 2014; Ha et al., 2014). Since the human microbiome assists human functioning via various critical roles, any alteration in their structure or function can impact human physiology and function, and ultimately overall health (HMPC, 2012; Sommer and Bäckhed, 2013).

Microorganisms perform a host of useful functions, such as fermenting unused energy substrates, training the immune system, preventing growth of pathogenic bacteria, regulating the development of the gut, and producing vitamins for the host (such as biotin and vitamin K) (Fujimura et al., 2010; Panda et al., 2014; D'Argenio and Salvatore 2015). These functions have been growing in description since the introduction of new molecular DNA sequencing techniques and it is thought that there are many more functions than currently recognised.

Gut microbiota are considered to have three broad functions in the human host – metabolic (supporting digestion), protective (supporting host immunity and defenses), and trophic (involved in cross-talk with the immune system and influencing cell growth and differentiation) (Montalto et al., 2009; Aziz et al., 2013; Rowland et al., 2018). In terms of metabolic functions, they aid in the digestion and utilization of energy. Protective functions include the development of immunity to harmful organisms. In the trophic functions, they promote cellular growth, differentiation and survival (Aziz et al., 2013).

2.5.1 Metabolic functions

The enteric microbiota have a collective function of metabolic activity that is equivalent to a virtual organ within the gastrointestinal tract. The gut microbes are essential for the normal process of digestion to access the molecular energy stored in the food intake (Cummings and MacFarlane, 1997; Rowland et al., 2018).

The gut microbiome have an important role in the process of digestion, influencing many aspects of the process and inhabiting many areas throughout the digestive tract. Gut microbes are actively involved in nutrient digestion helping in the synthesis of chemical energy such as adenosine triphosphate (ATP) from potential fuel sources including carbohydrates, fats, and proteins in diets. Anaerobic bacteria play an important role in the fermentation of dietary fibres creating short chain fatty acids that are used in metabolism (den Besten et al., 2013).

Gut microbes contain genes that code for proteins that help converting complex carbohydrates into simpler short chain fatty acids (SCFAs) (Rowland et al., 2018). These account for between 5 and 15 percent of our energy requirements. Other genes help with the breakdown of cellulose and complex sugars such as pectin which are found in fruit and vegetables, and without these gut microbial genes we would not be able to adequately utilise energy from these food sources (Clayton et al., 2009).

Dietary carbohydrates are substrates for fermentation by microbes in the proximal large intestine. This process converts them into SCFAs i.e. acetate, propionate and butyrate. Metabolites including lactate, pyruvate, ethanol, succinate as well as gases H_2, CO_2, CH_4, and H_2S are formed in this process (Flint et al., 2012a; Ibrahim and Anishetty, 2012; Chassard and Lacroix, 2013).

The SCFAs cause the pH of the lumen to decrease which thereby inhibits the growth of pathogenic species. These SCFAs also favour the absorption of ions (Ca, Mg and Fe) in the caecum and contribute towards the energy requirements (den Besten et al., 2013; Sun and O'Riordan, 2013). Butyrate is the major energy substrate for colonocytes (Ahmad et al., 2000; Bourassa et al., 2016). Propionate is taken up by the liver and acetate is metabolised by peripheral tissues after entering circulation (Wong et al., 2006; Louis et al., 2014). They stimulate colonic sodium and fluid absorption, acetate increases colonic blood flow and improves ileal motility.

Proximally, the colon is a saccharolytic environment (Wong et al., 2006) with the majority of the gut fermentation of carbohydrates occurring in this region. Carbohydrate abundance decreases in the distal colon and proteins derived from desquamated epithelium are increasingly used by bacteria as an energy source (Williams et al., 2017). Consequently, increased protein fermentation in the distal colon has been linked with disease states including colon cancer and chronic ulcerative colitis (Zeng et al., 2014; Makki et al., 2018). Hence increased emphasis towards shifting the gut fermentation towards saccharolytic activity by increasing dietary non-digestible carbohydrates is favourable (Senghor et al., 2018).

Starch and fibre poor diets result in a low production of SCFAs in the colon, and may contribute to the higher rates of colonic disorders in western countries (den Besten et al., 2013). The fermentation process results in the production of SCFAs, which effect gene expression and apoptosis signalling pathways in colonocytes, reduces the pH of the colon, which influences the host mineral absorption ability, and affect the satiety mediating hormones in the gut (Sun and O'Riordan, 2013).

The symbiotic microbes also help the human host by producing beneficial chemicals, including anti-inflammatory products, antibacterial molecules, and vitamins such as vitamin K which are synthesised by gut bacteria and would otherwise be difficult for humans to attain (O'Hara and Shanahan, 2006). Human systems are unable to intake or synthesise enough vitamin B or any vitamin K without the gut microbes. Substances formed as a result of bacteria activity include vitamin K, vitamin B12, thiamin and riboflavin (Shenkin, 2008; LeBlanc et al., 2011; Patel et al., 2013; de Angelis et al., 2014; Magnúsdóttir et al., 2015).

2.5.2 Protective functions

The adaptive immune system is essential in preventing an exaggerated inflammatory response to the microbiota and their translocation outside the gut associated lymphoid tissue (Hooper et al., 2012). Two way communication between the bacteria and the immune system with the production of immunoglobulin A (IgA) in response to the microbiota is an essential control pathway for this symbiotic relationship (Zimmerman et al., 2012; Belkaid and Hand, 2014; Kim et al., 2017).

Gut microbiota are actively involved in 'training' the immune response and defending against pathogens (Round and Mazmanian, 2009; Belkaid and Hand, 2014). There is a strong interaction between microbiome and the host which is influenced by environmental factors (Kers et al., 2018). Young (2012) proposed three paradigms to model the influence that environmental factors have on host microbe interactions and the microbiomes response to these factors. Firstly the microbiome can directly influence the host immune response. Using the murine model, Young (2012) showed that gut microbes regulate the TH-17 autoimmune response suggesting that environmental factors that affect the gut microbiota makeup may compromise the host's immune response. Secondly, there is evidence that shows microbes can change a host's immune response to prevent other microbes from residing in the gut. For example, Littman and Pamer (2011) showed that some gram negative microbes can use host signalling mechanisms to cause the production of antimicrobial peptides that work against gram positive microbes, such as *enterococcus* bacteria. This suggests that one organism can

trigger the host to produce something that can interfere with another organisms attempts to colonize the gut. Thirdly, the microbiome can alter the host's physiology and hence influence how xenobiotics are metabolised. For example, research by Nicholson et al (2005) showed that when gut microbe-free mice acquire microbiota, their expression of cytochrome P450 (CYP; enzymes involved in metabolism of xenobiotics) and nuclear receptors (proteins involved in regulation of specific gene expression) increased. These mice were also found to have an increased ability to metabolise bile salts (also an indication of CYP metabolic function).

These paradigms discussed by Young (2012) highlight the relationship between the microbiome and host immunity, alterations of the host microbiome by environmental factors, and the negative effect of host immunity through the interference of the human microbiome. There is mounting evidence to suggest that some autoimmune diseases may be associated with a changed microbial host interaction system (Belkaid and Hand, 2014; Li et al., 2018). Young (2012) speculated that antibiotic use and increased hygiene in recent years may have affected the host microbiome relationship. This example demonstrates the importance of taking the microbiome into consideration in drug and environmental interactions and also highlights how differences in the microbiome can lead to phenotypic variability amongst individuals.

2.5.3 Trophic functions

Gut microbiota can also have trophic functions – modulating and influencing gut epithelial cell differentiation and proliferation, affecting neuroendocrine pathways, and impacting on homeostatic regulation of the immune system (Aziz et al., 2013). The mammalian intestinal epithelial stem cell (IESC) niche is comprised of diverse epithelial, immune, and stromal cells, which together respond to environmental changes within the lumen and exert coordinated regulation of IESC behaviour. There is growing evidence for the role of the gut microbiota in modulating intestinal proliferation and differentiation, as well as other aspects of intestinal physiology (Peck et al., 2017). Gut microbiota help in the growth of intestinal epithelial cells and also control their proliferation and differentiation (Gordon et al., 1997; Guarner and Malagelada, 2003). Bacterial cells also alter intestinal growth by changing the expression of cell surface proteins such as sodium/glucose transporters (Drozdowski and Thomson, 2006; Chang and Leung, 2014). They may also cause lymphoid tissue near the gut to grow. Cell alterations by microbiota may prevent injury to the gut mucosa (Gori et al., 2008; Hill et al., 2009).

The gastro-intestinal (GI) mucosa is in continuous contact with the gut microbes, and it has been demonstrated that microbes present in the lumen of the gut affect GI health and functions including the regulation of the GI mucosal growth (Johnson, 1988; Rao and Wang, 2010). The epithelial cells lining the intestine function to keep bacteria from invading the body, but they also have mutually beneficial relationship with intestinal microbiota. Probiotics consisting of living microorganisms have been shown to provide health benefits to the host. They attach to epithelial cells and colonize in the intestine (Marteau et al., 1997; Dunne, 2001). Several studies also have attempted to identify specific positive health benefits of probiotics using different bacterial strains (Goldin, 1998; Gorbach, 2000) and the health promoting properties of probiotics are known to be strain-dependent (Chapman et al., 2011). For example, soluble proteins produced by probiotic bacteria *Lactobacillus rhamnosus* GG (LGG) have been shown to activate Protein kinase B (PKB or Akt), inhibit cytokine-induced epithelial cell apoptosis, and promote cell growth in human and mouse colon epithelial cells and in cultured mouse colon explants (Yan et al., 2007).

2.6 Factors effecting gut microbes

The microbial 'ecosystem' of a human throughout life is influenced by a sequence of complex and dynamic interactions, including dietary exposure, life-style choices, medical and health complications and antibiotic exposure (HMPC, 2012; Yatsunenko et al., 2012; Breton et al., 2013a; Subramanian et al., 2015; Jeffery et al., 2016). A number of extrinsic factors such as antibiotic use, diet, stress and disease constantly influence the functioning and diversity of the gut microbiota (Hooper et al., 2002).

Various factors affect the composition and function of gut microbes (Lozupone et al., 2012). As mentioned previously, the gut microbes in humans change over a period of time with age. The seeding birth gut microbiome of babies rapidly change due to selection pressures. These early changes are thought to be due to aging, diet, genetics, metabolism, geography, gender, stress and external environmental factors such as antibiotic exposure and exposure to environmental contaminants (Vemuri et al., 2018).

Even though intra-individual fluctuations in the composition of the gut microbes can be dramatic, the microbial ecosystem tends to return to their typical composition pattern with most strains being resident in an individual for decades - a phenomenon called microbiome resilience (Manichanh et al., 2012; Relman, 2012; Shade et al., 2012).

2.6.1 Diet

Diet is a key process to the symbiotic relationship between the gut microbes and the human host (De Filippo et al., 2010). Microbes contribute to human health while humans provide an adequate environment and nutrition for the gut microbes. Food provides a variety of substrates for microbial functioning and it is a key driving force in influencing the structure, composition and hence function of the microbial community (Shade et al., 2012). The best developmental evidence for this is shown by the greatest shift in the infant gut microbiota occurring at the point of introduction to solid foods (Marques et al., 2010; Rodriguez et al., 2015).

After delivery, the initial microbial community that colonises the infant gut is influenced by the birth modality as mentioned in Section 2.3.1. After the primary exposure to early colonisers, infants interact with microbes from the environment. This is in the form of physical contact with other individuals, the environment and through dietary exposure. This causes a rapid increase in bacterial diversity, with the greatest microbial shift in infant intestinal microbiota occurring with the introduction of solid foods (Marques et al., 2010; Rodriguez et al., 2015).

Breastfeeding is another early paradigm of environmental influence on the gut microbes. Human breast milk contains non-digestible oligosaccharides as the third largest component. These glycans remain whole as they travel to the colon where they promote the selective growth by nourishment of *Bifidobacterium* genus microbes. Studies have shown an increased proportion of bifidobacteria in breastfed infants compared with formula-fed infant (Stark and Lee, 1982; Balmer and Wharton, 1989; Fanaro et al., 2003; Knol et al., 2005).

Dietary interventions can lead to rapid alterations in the profile of the intestinal microbiota, although these changes are minor compared to inter person variability. Extreme changes in diet, for example a rapid shift to a diet with no complex carbohydrates (fibres) have been shown to have a significant effect on the human microbiota (Karasov et al., 2011). For example, David et al. (2014) observed that an uptake in animal-based fat exposure in the diet with a reduction in fibre is correlated with the increase in bile-tolerant microorganisms (*Alistipes, Bilophila* and *Bacteroides*) with a decrease in the levels of Firmicutes which metabolize dietary plant polysaccharides (*Roseburia, Eubacterium rectale* and *Ruminococcus bromii*). Likewise, increased consumption of dietary fibre from plant material is correlated with alterations in the gut microbiota.

Diet experiments with participants consuming resistant starch or non-starch polysaccharide caused shifts to specific bacteria taxonomic groups *Ruminococcus bromii* and

Eubacterium rectale. These groups have been shown to selectively metabolise specific insoluble carbohydrate substrates based on *in vitro* analyses of human faecal samples (Graf et al., 2015).

Different population groups have differing diets and this has been found to help shape the taxonomy of their gut microbiota. Traditional western diets rich in animal proteins, fats, carbohydrate sugars and low in fibre are associated and cultivate a different gut microbial profile than the high fibre diets of children in West Africa. De Filippo et al. (2010) showed that European children had lower amounts of *Prevotella* genus, higher amounts of *Bacteroides* genus, and lower microbial richness and produced lower levels of SCFAs than the microbiota of children from Burkino Faso. They speculated that the agrarian diet of children in Burkino Faso that is rich in non-animal protein, fibre and carbohydrate content contrasted to the high animal protein, sugar, starch, fat and low in fibre western diet had a predominant role in the difference between microbial profiles. This inverse relationship between *Prevotella* and *Bacteroides* genus has been shown in studies equating agrarian societies' intestinal microbiota to western industrialised societies (Kelsen and Wu, 2012).

There are however associations with the groups of organisms. *Prevotella* has been associated with an agrarian culture and diet, whereas profiles that are higher in proportion of *Bacteroides* are associated with more industrialised regions. Bacterial taxonomy has been seen to be affected by diet in the proportions of *Prevotella* versus *Bacteroides* in the US population (Yatsunenko et al., 2012). Hence there has been a link between the stable gut microbial communities and long-term dietary patterns.

Studies have associated the diversity of the gut microbiota at the taxonomic and gene level with diets higher in fruits, vegetables and fibre. In a study by Wu et al. (2011) that focused on linking long-term dietary patterns of 98 subjects with gut microbial enterotypes, there was a strong association with diet and enteric microbes. Long-term diets higher in protein and animal fat were associated with gut microbes from the *Bacteroides* genus. Alternatively, carbohydrate high diets were associated with *Prevotella* genus. Changes in the composition of the microbiome were detected within 24 hours of starting a controlled diet of either low-fat/high-fibre or high-fat/low-fibre indicating diet plays an integral and immediate role in the make-up of the gut microbiota.

Studies have also shown that certain microbes can be protective against weight gain from dietary factors. For example, Goodrich et al. (2014) noticed that the *Christensenallaceae* family microbes were more abundant in lean study participants than in those who were obese. They tested whether these microbes might protect against weight gain by introducing

microbes from obese participants into gut microbe free mice. To some of the mice, they also introduced bacteria from one species of *Christensenallaceae* and at the end of 3 weeks, they observed that the gut microbe free mice with obese donor's gut microbes were leaner in the presence of *Christensenallaceae* family microbes compared to those with only the obese donor's microbes. This shows the significance of this family of microbes towards weight gain and human metabolism. However, the presence or absence of Christensenallaceae microbes depends on the host microbiome and associated genes (Jacobs and Braun, 2014).

2.6.2 Genetics

Genetic factors influence many aspects of human health and have been found to influence the makeup of the gut microbiota (Ley et al., 2006; Goodrich et al., 2014). Twin studies have shown that monozygotic twins have a more similar microbiota than dizygotic twins implying a heritability aspect to gut microbiota (Turnbaugh et al., 2009). From the results of a mice study, they concluded that although the human gut microbiome is shared among family members, there is a difference in specific bacterial lineages among individual microbes, even between monozygotic and dizygotic twin pairs. The specific bacterial lineage has been proposed to alter the genetic make-up of host genetics.

It is well known that host genetics and the gut microbiome can influence metabolic phenotypes. This was further studied by Goodrich et al. (2014) to clarify if interaction of gut microbiome affected host phenotype, using more than 1000 faecal samples collected from 416 twin pairs in United Kingdom. While they found that the host genetics influenced microbial taxa abundance, the most heritable taxon was found to be from the family *Christensenellaceae*. The most striking phenotype was identified to be Christensenellacease enriched individuals with low BMI, where *Christensenella minuta* was responsible for limiting weight gain in mice. The genetics of the human gut microbiome can also be altered during dysbiosis as affected by antibiotics and drug intake.

Recently, Rothschild et al. (2018) examined genotype and microbiome data from 1,046 healthy individuals with several distinct ancestral origins who share a relatively common environment. Their results demonstrate that the gut microbiome is not significantly associated with genetic ancestry, and that host genetics have a minor role in determining microbiome composition. The results also indicate that over 20% of the inter-person microbiome variability is associated with factors related to diet, drugs and anthropometric measurements. Their results suggest that microbiome alterations aimed at improving clinical outcomes can be carried out across diverse genetic backgrounds.

2.6.3 Antibiotics intake

Gut microbes are impacted by antibiotics intake, through the imbalance it could cause to the microbial population in gut (Dethlefsen et al., 2008; von Rosenvinge et al., 2013a). Microbiome composition can be rapidly altered by exposure to antibiotics, potentially leading to the selection of resistant opportunistic pathogens that can cause acute disease. As discussed above, the mutualistic gut microbes interact with many physiological processes, and participate in the regulation of immune and metabolic homeostasis. Therefore, antibiotic exposure can alter many basic physiological equilibria, promoting long-term disease. In addition, excessive antibiotic use fosters bacterial resistance, and the overly exposed human microbiome has become a significant reservoir of resistance genes, contributing to the increasing difficulty in controlling bacterial infections.

The interaction between human intestinal cells and the gut microbes are highly co-evolved and any disruptions in the population of the microbial community can affect the important functions such as nutrition, development, metabolism, pathogen resistance, and regulation of immune responses (Dethlefsen et al., 2008). The researches found that the use of ciprofloxacin antibiotic affected the presence of one third of bacterial taxa in the human gut, thereby limiting the diversity and distribution of the community. Dethlefsen et al. (2008) also noticed that the taxonomic composition of the gut microbiome failed to recover even after six months of treatment with ciprofloxacin, supporting their hypothesis of functional redundancy of human gut microbiota. Another ten month study by Dethlefsen and Relman (2011) on three individuals treated with ciprofloxacin in two different courses, they found that the communities began to return to their initial state within a week of antibiotic treatment, but incompletely. This shows that antibiotic intake can result in long-term irreversible effects on human gut microbiome.

Other possible reasons for dysbiosis of the human gut microbiome include the exposure of gut to environmental pollutants including heavy metal(loid)s and toxic substances. The intake of such contaminants can be through food, air, water and some complementary medicines (Naidu et al., 2008; Wijayawardena et al., 2016).

2.7 Sources of heavy metal(loid) intake

Heavy metal(loid)s are elements with an atomic density greater than 6g/cm^3 and include both metabolically essential and non-essential elements. Essential elements such as copper (Cu),

zinc (Zn) and iron (Cu) contribute to certain physiological functions in human systems (Adriano, 2001). Although essential, these metal(loid)s are toxic in high concentrations. Non-essential heavy metal(loid)s such as cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg) have been associated with human health risks. Through various exposure pathways, these heavy metal(loid)s can lead to metal(loid) toxicity and poisoning (Naidu et al., 2008).

Metal(loid) mining and smelting industries, human activities, and indiscriminate disposal of agricultural and industrial wastes have resulted in the pollution of terrestrial and aquatic environments with heavy metal(loid)s (Senesi et al., 1999; Adriano, 2001; Nicholson et al., 2003; Naidu et al., 2008; Tchounwou et al., 2012; Wuana and Okieimen, 2011; Zhang et al., 2012; Xia et al., 2014). Health authorities in many parts of the world are becoming increasingly concerned about the effects of heavy metal(loid)s on environmental and human health (Järup, 2003; Naidu et al., 2008; Edwards and Prozialeck, 2009; Rehman et al., 2017). More recently high concentrations of heavy metal(loid)s, such as, Cd, Pb, Hg, Cu and Zn reaching aquatic and terrestrial environments have often been reported in number of countries. Arsenic, Cd, Pb and Hg are some of the most common metal(loid)s which readily reach human food chain causing toxicity (ATSDR, 2007 a,b; Navas-Acien et al., 2007; Naidu et al., 2008; Hughes et al., 2011; Tchounwou et al., 2012; Rehman et al., 2017). In this study, the bioavailability of selected heavy metal(loid)s including As, Cd, Pb and Hg as impacted by gut microbes is examined.

Arsenic reaches human food chain through As rich drinking water and As-enriched food commodities including rice (Mahimairaja et al., 2005; ATSDR, 2007a). Arsenic exists in two forms – arsenate (AsV) and arsenite (AsIII), which vary in their adsorption and toxicity characteristics. Arsenic is classified as a class one carcinogen by the World Health Organisation's International Agency for Research on Cancer (IARC, 2004). High levels of As intake can raise the risk of developing lung, bladder and skin cancer, cardiovascular disease, diabetes mellitus, skin lesions, gastrointestinal illness, and other serious health problems, eventually leading to death (WHO, 2009; Zhang et al., 2016).

Mining activities, industrial processes and tobacco smoking deposit Cd in the atmosphere, and application of phosphate fertilisers rich in Cd result in soil contamination (Naidu et al., 1994; Loganathan et al., 2003; Bolan et al., 2014). These activities increase human uptake of Cd through ingestion of Cd contaminated produce grown under such affected environment (Page et al., 1986; Thornton, 1992; Loganathan et al., 2003; Pinot et al., 2011; Bolan et al., 2014; Jaishankar et al., 2014). Cadmium accumulation in humans can cause renal complications through the impairment of Vitamin D metabolism and also potentially

carcinogenic due to oxidative stress and DNA damage, thereby resulting in the inhibition of apoptosis (Johri et al., 2010; Bishak et al., 2015).

Lead (Pb) is a common contaminant in industrialized regions globally, with major sources being shooting ranges and base-metal(loid) tailings (Lafond et al., 2004; Kumpiene et al., 2008; Sanderson et al., 2012). Oral ingestion of contaminated soil is an important pathway of Pb toxicity, especially in young children (Hou et al., 2013; Cao et al., 2014). Lead is one of the most common heavy metal(loid)s added as a therapeutic ingredient in complementary medicines for the treatment of diabetes mellitus, spleen enlargement, diarrhoea and various skin diseases (Nagarajan et al., 2014; Bolan et al., 2016b). The developing fetus and children are more sensitive to high levels of Pb exposure than adults due to neurological effects of Pb exposure, which may cause irreversible learning disabilities, attention deficit disorders, lowered IQ, and behavioral difficulties (ATSDR, 2007b; Park et al., 2011).

Mercury (Hg) is abundantly found in industry contaminated sites and can accumulate over time thereby polluting the environment (Rice et al., 2014). Fish consumption is a major source of Hg intake by humans (Bushkin-Bedient and Carpenter, 2010; Silbernagel et al., 2011). Long-term Hg exposure through the above sources can lead to health issues to humans and the environment. Mercury is added a therapeutic agent in some complimentary medicines including Ayurvedic medicines and regular use of these medicines (Saper et al, 2008; Bolan et al., 2017a) may result in severe abdominal cramps, peptic ulceration and as a result bloody diarrhoea (Lehman-McKeeman, 2003). Acute cases of Hg exposure may also lead to renal failure because of the continuous Hg load to the kidney.

The toxicity of ingested contaminants including heavy metal(loid)s is determined ultimately by the extent to which they are solubilised in the gut (bioaccessibility), their permeability through intestinal epithelial cells and subsequent circulation in the blood (bioavailability), and their assimilation and metabolic action in any tissues that subsequently absorb them (bioactivity). The bioaccessibility-bioavailability-bioactivity continuum (Figure 2.3) play a critical role in the toxicity of heavy metal(loid)s to biota (Naidu et al., 2008; Jaishankar et al., 2014; Kim et al., 2015; Bolan et al., 2017a). Bioaccessibility is usually evaluated *in vitro* by physiologically based extraction tests and gastrointestinal digestion procedures. Thus, for example, bioaccessibility can be used as a conservative estimate for bioavailability, as bioaccessibility is a theoretical maximum possible bioavailability (Laparra et al., 2003, 2007; Versantvoort et al., 2016). Bioavailability, which expresses the fraction of the bioaccessible compound that enters the blood circulation, refers to the rate and extent to which the compound permeates through the intestinal epithelial cells (Jaishankar et al., 2014; Kim et al., 2015).

Bioactivity refers to the physiological and metabolic interactions between the compound and the human tissue or organ, which disturb homeostasis (Rehman et al., 2018). Heavy metal(loid) toxicity can be mitigated by reducing their permeability in the intestine, thereby reducing the amount of metal(loid) entering the systemic circulation (Jaishankar et al., 2014; Egorova and Ananikov, 2017).

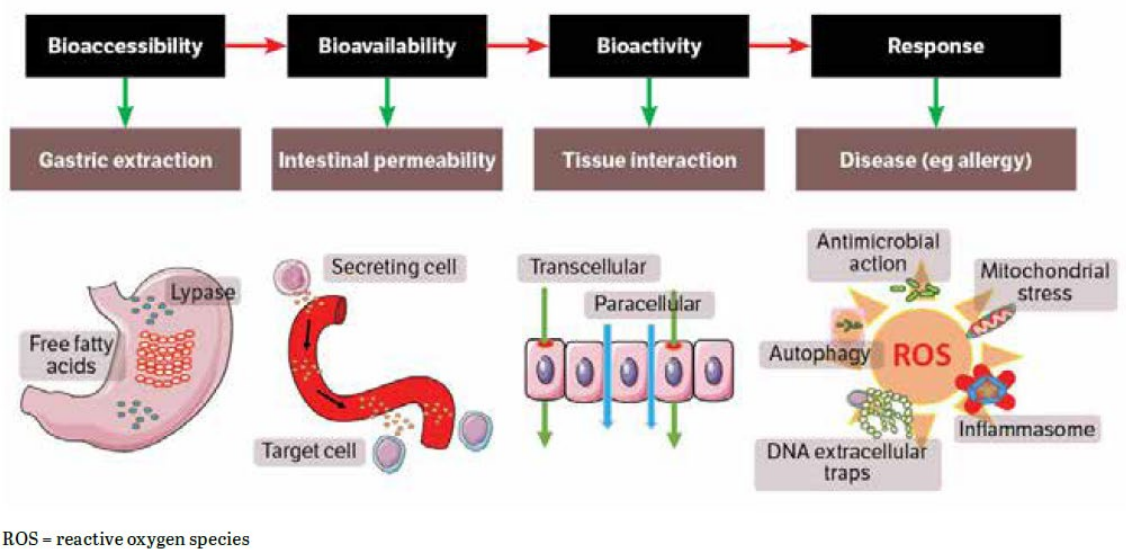


Figure 2.3 The bioaccessibility–bioavailability–bioactivity continuum of contaminants including heavy metal(loid)s in relation to toxicity response

The amount of metal(loid) absorption into systemic circulation (the bioavailable fraction) depends on the nature and solubility of metal(loid) source (i.e., bioaccessibility) and the properties of the ingested compound (Naidu et al., 2008; Deshommes et al., 2012; Ruby et al., 2016). Bioaccessible metal(loid) concentrations are critical for health- and environmental-risk assessment, and hence bioaccessibility measurements are necessary for quantifying human intake of heavy metal(loid)s from various sources for use in risk assessments to establish safe metal(loid)s threshold values (Naidu et al., 2008). Studies on metal(loid) bioaccessibility are often conducted using *in vitro* digestive techniques aiming to simulate the human digestive system (Van de Wiele et al., 2010; Laird et al., 2007, 2013; Wijayawardena et al., 2015; Juhasz et al., 2016). These tests, however, do not take into account the role of the human gut microbiome and its interactions with environmental contaminants via the transformation of environmental contaminants.

2.8 Interactions of heavy metal(loid)s and gut microbes

Environmental factors influence the human gut microbiome right from birth, with the environment of the delivery area - hospital delivery vs home delivery - effecting the makeup of the initial gut microbiota (Fanaro et al., 2003; Dogra et al., 2015). These environmental factors play a role in shaping the microbiome ecology in the human gut (De Filippo et al. 2010; Collado et al., 2012). Therefore, environmental disturbances including heavy metal(loid)s can play a role in the composition of the human gut microbiota.

The gut microbes not only can be affected by environmental contaminants but they themselves can alter the speciation and bioavailability of these contaminants (Monachese et al., 2012; Williams et al., 2015). This creates a two way interaction between the microbiome and contaminants, gut microbial diversity and function being impacted by contaminants, and the contaminants being changed or impacted by gut microbes. These interactions can have both positive and negative consequences for the host.

A number of studies have demonstrated that environmental contaminants including heavy metal(loid) intake alters the genomic and functional diversity of gut microbes, which they attributed mainly to the toxicity of heavy metal(loid)s to gut microbes (Fazeli et al., 2011; Monachese et al., 2012; Liu et a., 2014; Claus, 2016; Rosenfeld, 2017). In a classical study, Breton et al. (2013b) examined the impact of up to 8 weeks of oral Pb and Cd ingestion on the composition of the murine intestinal microbiome. Pyrosequencing of 16S RNA sequences revealed specific changes in bacterial commensal communities (at both family and genus levels) following oral exposure to the heavy metals, with notably low numbers of *Lachnospiraceae* and high numbers levels of *Lactobacillaceae* and *Erysipelotrichaceacae* (mainly due to changes in *Turicibacter spp*), relative to control animals. Non-absorbed heavy metals have a direct impact on the gut microbiota, which in turn, may impact the alimentary tract and overall gut homeostasis.

Gut microbes can affect the way toxic compounds including heavy metal(loid)s react with the human host by altering the solubility and bioavailability of heavy metal(loid)s. Experiments done with the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), a system that mimics digestive processes in the gut showed that microbes can affect the bioavailability of toxic compounds (Williams et al., 2015). The population and diversity of microorganisms present in each organ in the gastrointestinal tract depends on the pH gradient of the entire human digestive system (Flint et al., 2012b; Figure 2.4).

Van de Wiele et al. (2010) have shown that the bioavailability of As ingested through As contaminated rice from China is likely to be altered as it moves through the digestive system. The ability of the human microbiome to methylate and demethylate arsenic is important due to

the implications for the toxicity profile for As and the chronic health outcomes related to As exposure. New evidence is showing that the ability of the human microbiome to methylate inorganic As and demethylate arsenic is challenging our current thinking about the exposure assessments about As (Laird et al., 2007, 2013). Historically, regulations have focused mainly on human exposure to organic methylarsenic, but since our gut microbes may create a greater exposure risk to inorganic As which has a higher toxicity profile than methylarsenic in relation to hurting immune function, this now has implications surrounding the current regulations.

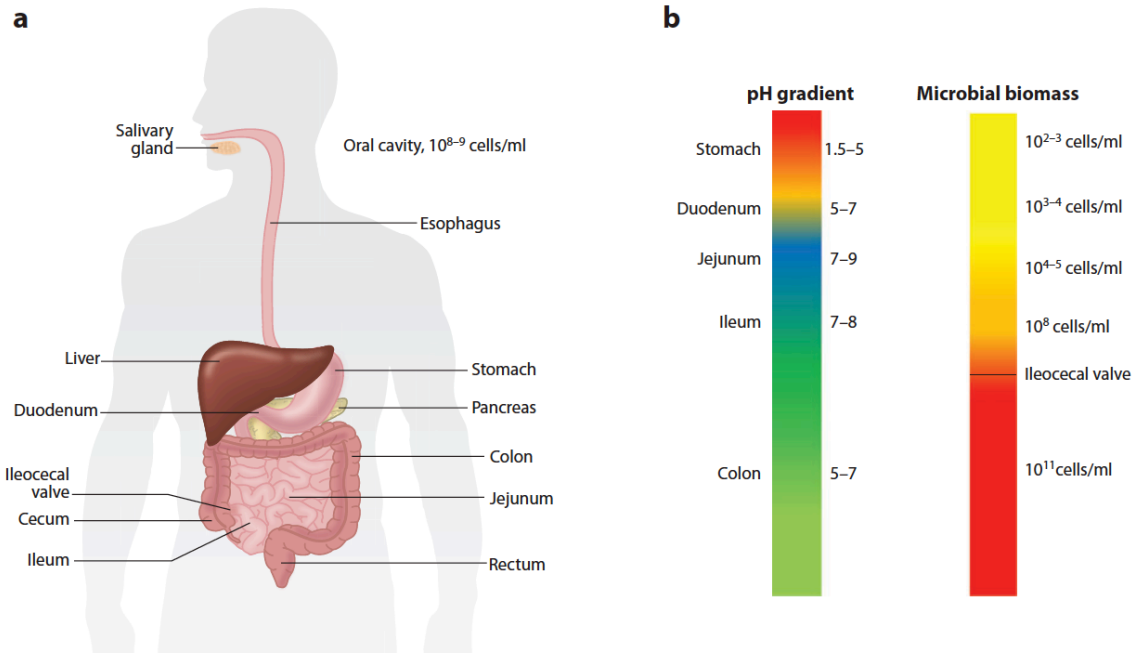


Figure 2.4 pH gradients in various organs of a human digestive system with the corresponding microbial biomass distribution.

This new research area opens up interest in how other metal(loid)s that are known to be bioavailable may be affecting our microbiomes. For example, some fresh food items such as salads incorporate silver nanomaterials in the packaging for preservation. Bacteria have developed silver resistance genes and these can then cause problems with treatments such as those used in burns units where antibiotics and silver are used together for management. These metal(loid)s when used in small scale maybe effective in hospital settings but when used in other products where exposure to healthy individuals occurs may lead to dysbiosis (Knetsch and Koole, 2011).

Heavy metal(loid)s are not the only environmental contaminants that may alter and deter the gut microbiome to then interact with humans. Van de Wiele et al. (2010) showed how

polycyclic aromatic hydrocarbons (PAHs) – including naphthalene, phenanthrene, pyrene and benzo[*a*]pyrene interacted with gut microbes to produce compounds that are attracted to estrogen receptors where the precursor compounds had no estrogenic effects.

The effects of cigarette smoke which also contains PAHs, such as benzo[*a*]pyrene have been studied to see the effects on the lung and gut microbiome. Studies have shown small changes to the gut microbiome and a change in immunity against entero-pathogens as a result of exposure to cigarette smoke (Verschuere et al., 2011; Berk et al., 2013; Shanahan et al., 2018)

Van de Wiele et al. (2010) provided an example of how ignoring the importance of the human microbiome and environmental factors interactions can have severe unintended consequences. Ignoring the importance the microbiome plays in modulating foreign chemicals can lead to negative outcomes. For example, Japanese physicians found case studies of patients who had died due to the interactions of drugs with the microbiome (Sato et al., 2014). Tegafur (5-fluorouracil (5-FU)) a chemotherapy agent used to treat colorectal cancer is metabolised into 5-fluorouracil, which is toxic to cancer cells. Normally a liver enzyme detoxifies the excess 5-fluorouracil and it is cleared from the body. However when Tegafur was given in conjunction with sorivudine, a common antiviral agent given to cancer patients, eighteen patients died. Further studies showed that the gut microbes of some patients converted sorivudine into a compound that blocked the liver enzyme that detoxified 5-fluroouracil (Li et al., 2016).

The modulation of bioaccessibility of metal(loid)s by gut microbes could be attributed to the (im)mobilization through adsorption, complexation, and precipitation reactions (Unz and Shuttleworth, 1996; Halttunen et al., 2007a,b,c; Monachese et al., 2012; Jarosławiecka and Piotrowska-Seget, 2014; Zoghi et al., 2014). The microbial cell wall is a natural barrier for metal(loid)s, since the functional groups of several macromolecules are involved in the immobilization of metal(loid)s. In Gram-negative bacteria, lipopolysaccharide, a major component of the outer membrane, is effective in the immobilization of metal(loid) ions. In Gram-positive bacteria, peptidoglycan along with teichoic and teichuronic acids are involved in metal(loid) binding (Beveridge and Graham, 1991). For example, Cabuk et al. (2006) demonstrated that hydroxyl and carboxyl groups, along with nitrogen-based bio-ligands such as amide and sulfonamide, are responsible in the complexing Pb^{2+} by *Bacillus* sp.

Most microorganisms excrete extracellular polymeric substances (ESPs) that bind toxic metal(loid) ions, thereby protecting metal(loid)-sensitive biochemical components (Gupta and Diwan, 2017). The components of ESPs including proteins, polysaccharides, and nucleic acids,

involve in the chelation of metal(loid)s (Guibaud et al., 2003; Pal and Paul, 2008). Binding of Pb^{2+}, Cd^{2+} and Hg^{2+} and other metal(loid)s by ESPs has been observed for bacteria (Perez et al., 2008; Chakravarty and Banerjee, 2012). Additionally, metal(loid) cations such as Pb^{2+}, Cd^{2+} and Hg^{2+} forms strong soluble and insoluble complexes with organic compounds including tryptone, cysteine, neopeptone, casamino acid, and succinic acid (Tan et al., 1994; Nigam et al., 2000; Mayer and Godwin, 2006; Gadd, 2010; Hajdu and Slaveykova, 2012; Ndu et al., 2012). Microorganisms release a number of organic compounds including short chain fatty acids and carboxylic acids (e.g., lactic acid) that are involved in nutrient absorption and energy regulation (Krajmalnik-Brown et al., 2012). These organic compounds form complexes with metal(loid)s, resulting in the removal of these metal(loid)s from solution. Metal(loid) cations such as Cd^{2+} and Pb^{2+} also react with inorganic anions such as chlorides, carbonates, phosphates, and hydroxyl ions to form precipitates (Kumpiene et al., 2008; Gao et al., 2017). The precipitation of Pb^{2+} is employed by microorganisms to lower the free Pb^{2+} ion concentration by sequestering it in the form of both extracellular and intracellular phosphate salts.

2.9 Conclusions

Gut microbiome refers to the entire collection of microorganisms, their complete genetic makeup (genomes), and the interactions of these in gut environment. The influence of the gut environment is very important for the toxicity exerted by heavy metal(loid)s, and therefore, further progress in their understanding is crucial to characterize the mode of action of these toxic elements and to modulate their toxicity. Although the main route of exposure of the toxic elements is oral, the toxic effects is exerted mostly through their passage in the gastrointestinal tract where absorption of heavy metal(loid)s takes place. However, we have little knowledge on the events that occur during the passage of these elements, in most cases. Some of the influencing factors will be diet, water, pH changes in the gastrointestinal tract and microbial-induced heavy metal(loid) transformations in the gut.

Therefore, this study on heavy metal(loid) – gut microbe interactions, will help in understanding the effect of selected bacterial species on heavy metal(loid) transformation in the gastrointestinal tract and their influence on human health. Given that our gut microbiota influence human development, susceptibility to disease, and even the outcomes of drug treatment, perhaps microbiota biobanking for future generations facing unknown biological and infectious disease challenges will be an innovative approach (Bolan et al., 2016a).

Several studies have aimed at classifying the organisms that make up the gut microbiota and the interactions between these and environmental factors (Spor et al., 2011; Clemente et al., 2012). However only limited studies have been done on the interactions between heavy metal(loid) intake and these microbes (Breton et al., 2013a). The major research gaps are as follows:

- A number of studies have examined the toxicity of heavy metal(loid)s on microbes in environmental substrates such as soil and water. However, there is only limited work on the effect of heavy metal(loid)s on microbes from various parts of the GI tract.
- The effect of the gut microbiome on the transformation and bioavailability of heavy metal(loid)s hasn't been examined in detail. For example, *in vitro* bioaccessibility tests do not include microbes.
- A number of studies have examined the diversity of the gut microbiome. However, the effect of long-term exposure of heavy metal(loid)s on the genomic and functional diversity of the gut microbiome hasn't been examined in detail.

Hence, this research aims to demonstrate the impact of heavy metal(loid) exposure on the toxicity to gut microbes, and the effect of gut microbes on the bioaccessibility and subsequent bioavailability of these heavy metal(loid)s.

Chapter 3

HEAVY METAL(LOID) TOXICITY TO GUT MICROBES

3.1 Introduction

Health authorities in many parts of the world are becoming increasingly concerned about the effects of heavy metal(loid)s on environmental and human health (Järup, 2003; Naidu et al., 2008; Edwards and Prozialeck, 2009; Rehman et al., 2017). More recently high concentrations of heavy metal(loid)s, such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), copper (Cu) and zinc (Zn) reaching aquatic and terrestrial environments have often been reported in a number of countries. Arsenic, Cd, Pb and Hg are some of the most common metal(loid)s which readily reach human food chain causing toxicity (ATSDR, 2007a,b; Navas-Acien et al., 2007; Naidu et al., 2008; Hughes et al., 2011; Tchounwou et al., 2012; Rehman et al., 2017).

In this chapter, the toxicity of As, Cd, Hg and Pb metal(loid)s on selected gut bacteria is reported. The sources and toxicities of these four metal(loid)s are presented in Section 2.7 (Chapter 2). Briefly, As exists in two forms – arsenate (AsV) and arsenite (AsIII), and reaches human food chain through As rich drinking water and As-enriched food commodities including rice (Mahimairaja et al., 2005; ATSDR, 2007a). Arsenic is classified as a class one carcinogen and high levels of As intake can raise the risk of cancer and cardiovascular diseases. Fertilizer application is a major source of Cd input to soil, and Cd accumulation in humans can cause renal complications. Lead (Pb) is a common contaminant in industrialized regions globally, and oral ingestion of contaminated soil is an important pathway of Pb toxicity, especially in young children (Hou et al., 2013; Cao et al., 2014). Young children are more sensitive to high levels of Pb exposure than adults, which may cause irreversible learning disabilities and attention deficit disorders. Fish consumption is a major source of Hg intake by humans (Bushkin-Bedient and Carpenter, 2010; Silbernagel et al., 2011). Long-term Hg exposure can lead to health issues to humans including renal failure.

The above mentioned heavy metal(loid)s that include As, Cd, Pb and Hg have been shown to impact microorganisms, thereby affecting the functional and genomic diversity of microbiomes both in the environment and human (Gadd, 1990; Giller et al., 1998; Betts, 2011). The microorganisms found in the human digestive tract are often collectively referred to as the

'gut microbiome' (HMPC, 2012). Although gut microbiota play a critical role in maintaining normal physiology and energy production in a human body, they are sensitive to changes in the intestinal environment in the presence of contaminants including heavy metal(loid)s (Carding et al., 2015; Lu et al., 2015; Claus et al., 2016; Jin et al., 2017; Tasmin et al., 2017).

There have been a large number of studies examining the toxicity of various heavy metal(loid)s on microorganisms in the terrestrial and aquatic ecosystems (Gadd, 1990; Chander et al., 1995; Giller et al., 1998; Kahru et al., 2005; Baby et al., 2010; Hodson, 2012). These studies have demonstrated that heavy metal(loid) toxicity to microorganisms varies with both the metal(loid) species and the microbial species (Gadd, 1990; Giller et al., 1998; Naidu et al., 2006). There have been only limited research work on the interactions between heavy metal(loid)s and gut microbes (Monachese, 2012). For example, As has been shown to cause deleterious effects in the human intestine by altering the gut microbiome and thereby affecting overall health of an individual (Van de Wiele et al., 2010). However, As and Pb resistance in *Lactobacillus* species, Hg resistance and Cd efflux in *Bacillus* species and As efflux in *E. coli* were also observed (Carlin et al., 1995; Monachese, 2012). Gut microbes including bacteria play an important role in the transformation of metal(loid)s including As and Hg in the intestinal ecosystem (Monachese et al., 2012; Claus et al., 2016). While heavy metal(loid)s are toxic to gut microbiome, the human bioaccessibility and bioavailability of these metal(loid)s are affected by the activity of gut microbes (Claus et al., 2016).

The present study reported in this thesis examines the interactions between heavy metal(loid)s and gut microbes in relation to heavy metal(loid) toxicity in gut microbes (Chapter 3), and the effects of these gut microbes on the bioaccessibility of heavy metal(loid)s in the gastro-intestinal tract (Chapters 4 - 6) and the subsequent intestinal bioavailability of these heavy metal(loid)s as measured by intestinal permeability (Chapter 7).

3.2 Objectives

The overall aim of this chapter was to compare the toxicity of As, Cd, Pb and Hg on selected gut microbes such as *Escherichia coli, Lactobacillus rhamnosus* and *Lactobacillus acidophilus*. The specific objectives of this study were to:

(i) Determine the growth of selected gut bacterial species in the presence of heavy metal(loid)s.
(ii) Measure heavy metal(loid) toxicity to gut microbes as impacted by pH.

(iii) Examine the effect of pH and bacterial species on variable toxicity of As species.
(iv) Monitor the speciation of metal(loid)s in the bacterial growing media.

3.3 Hypothesis

Some of the common heavy metal(loid)s that are toxic to biota include As, Cd, Pb and Hg. This study hypothesized that:

(i) The growth of gut bacteria decreases with increasing concentration of heavy metal(loid)s.
(ii) The metal(loid) toxicity is dependent of metal(loid) species and pH conditions of the growing media.
(iii) Arsenite [As(III)] is more toxic to bacteria than does arsenate [As(V)], and the difference in toxicity depends on the pH conditions of the growing media.

3.4 Experiments

The major experiments conducted to test the hypothesis and the treatments used in this chapter are listed in Table 3.1.

Table 3.1 The major experiments and components used in Chapter 3

No.	Experimental sections	Components and treatments
1.	Growth curve study	Selected bacterial species (*Escherichia coli, Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) and relevant bacterial growth media (Luria Bertani (LB) broth and de Man Rogosa Sharpe (MRS))
2.	Toxicity study	Selected bacterial species (*Escherichia coli, Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) and relevant bacterial growth media (Luria Bertani (LB) broth and de Man Rogosa Sharpe (MRS)), and selected heavy metal(loid)s (Arsenic (As), Cadmium (Cd), Lead (Pb) and Mercury (Hg)) at defined concentrations
3	pH effect on toxicity	Selected bacterial species (*Escherichia coli, Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) and relevant bacterial growth media (Luria Bertani (LB) broth and de Man Rogosa Sharpe (MRS)), and selected heavy metal(loid)s (Arsenic (As), Cadmium (Cd), Lead (Pb) and Mercury (Hg)) at various concentrations and pH conditions
4	Metal speciation	Selected bacterial species (*Escherichia coli, Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) and relevant bacterial

		growth media (Luria Bertani (LB) broth and de Man Rogosa Sharpe (MRS)), and selected heavy metal(loid)s (Arsenic (As), Cadmium (Cd), Lead (Pb) and Mercury (Hg)) at a defined concentration and various pH conditions

3.5 Materials and methods

3.5.1 Bacterial culture and media preparation

The toxicity of As, Cd, Pb and Hg on selected gut microbes that include *Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) was examined in this study (Table 3.2). It is important to recognise that the human gut microbiota are a composite structure of a large number of distinct bacterial species that reside in the human digestive tract. In this study, these three bacterial species were used based on their predominance in the gut, differences in their pH optimum in the gut and their location in various parts of the human gut. One of the limitations in this study is the use of this narrow range of bacterial species which is explained in conclusion chapter (Chapter 8).

Subcultures of *Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) were inoculated from their respective mother cultures purchased from American Type Culture Collection (ATCC, Melbourne; https://www.atcc.org/). The media used for the subculturing of the bacterial species and their preparation protocols employed for this study are given below (de Man et al., 1960; Bertani, 2004):

- Luria Bertani (LB) broth (Bertani, 2004) powder was used to prepare the medium for *E. coli*. About 25g LB broth powder was added to 1000 mL Milli Q water in an autoclavable container and swirled to mix. The mixture was autoclaved at 121°C for 20 minutes and cooled to room temperature. Care was taken to maintain the sterility of the medium after autoclaving.
- For both *Lactobacillus* species, de Man Rogosa Sharpe (MRS) medium (de Man et al., 1960) was prepared using 46 g of MRS broth powder following the above procedure. In addition, 40 mL of amylase extract was also added to the media before sterilising the medium contents.

Table 3.2 Bacterial species used in Chapter 3

Bacteria	Family	Primary location	Optimum pH	Cell wall
Lactobacillus acidophilus	Lactobacillaceae	Mouth and stomach	3 - 5	Gram positive
Lactobacillus rhamnosus	Lactobacillaceae	Large intestine	5 - 6	Gram positive
Escherichia coli	Enterobacteriaceae	Large intestine (lower)	6 - 7.5	Gram negative

3.5.2 Subculturing

The mother cultures of the three bacterial species were stored using ultralow freezer (Forma™ 88000 Series Upright Ultra Low Temperature Freezer) maintained at around -86°C temperature, thereby allowing the cells to remain viable for several years. The low temperature generated by ultralow freezers substantially reduces chemical reactions within the culture. However, molecular motion still occurs in frozen cells and thus the viability of the culture is likely to decline with time (Burg et al., 2007; Kiehl et al., 2011).

Bacterial liquid cultures were prepared as explained in the protocol given in Appendix 3.1. The bacterial species used in this experiment were maintained by subculturing every week for the experimental duration. The bacterial species were taken from their previous cultures and inoculated before incubating in suitable incubators. For *E. coli,* the subcultures were incubated in shaking incubator at 37°C, and for *Lactobacillus* species, CO_2 incubator was used for maintaining anaerobic conditions at 37°C. The growth of bacterial cultures was monitored by measuring optical density (@600 nm) over time in a microplate reader (BMG LABTECH FLUOstar OPTIMA Fluorescence Microplate Reader, Germany) (Andrews, 2001; Stevenson et al., 2016).

3.5.3 Preparation of metal(loid) stock solutions

The stock solution (1000 mg/L) of the heavy metal(loid)s were prepared using appropriate salts listed in Table 3.3 and autoclaved sterile MQ water. After preparation of the stock solutions, they were stored in fridge for further experimental use. Before the bacterial growth experiment, the stock solution was diluted to appropriate concentrations using autoclaved sterile water. Care was taken to maintain the sterile condition during the gut bacterial growth experiments.

Table 3.3 Metal(loid)s used for gut bacterial toxicity experiments in Chapter 3

Heavy metal(loid)	Chemical compound*	Stock solution (mg/L)	Secondary stock (mg/L)	Concentration range used for toxicity test**	
				MCOE value (ug/L)	LD_{50} value (mg/L)
Arsenate	Sodium arsenate	1000	100	0-50	0-100
Arsenite	Arsenic oxide	1000	50	0-20	0-50
Cadmium	Cadmium acetate	1000	50	0-100	0-50
Lead	Lead acetate	1000	100	0-100	0-100
Mercury	Mercuric chloride	1000	50	0-100	0-50

**These metal(loid) chemical compounds are selected based on their use as a reference compound in bioaccessibility tests.*
***MCOE = Minimum metal(loid) concentration at which there is an observable effect on bacterial growth; LD_{50} = Lethal dose refers to metal(loid) concentration at which the bacterial growth is reduced by 50% maximum growth for the control treatment without metal(loid) addition*

3.5.4 Growth response to heavy metal(loid)s

The growth of the three selected bacterial species was examined in the presence of various metal(loid) concentrations. Two concentration ranges were used in this experiment: 0 - 100µg/L (ppb) to calculate minimum metal(loid) concentration at which there is an observable effect on bacterial growth (MCOE); 0 - 100 mg/L (ppm) to calculate lethal dose for 50% growth reduction (LD_{50}) (Monachese, 2012). The bacterial species were inoculated in the metal(loid) containing media and monitored over a period of 24 hours in a 96-well round bottom microplate (Costar 3799, Corning Incorporated, USA) under sterile anaerobic conditions at 37°C. The bacterial growth was monitored by measuring optical density (@600 nm) over time in a microplate reader (BMG LABTECH FLUOstar OPTIMA Fluorescence Microplate Reader, Germany) (Koch, 1970; Stevenson et al., 2016). The experiments were carried out in triplicate and the medium without bacterial inoculation served as blank, and the bacterium inoculated media without metal(loid) served as control.

3.5.5 Effect of pH on the growth and heavy metal(loid) toxicity

The effect of pH on the growth and heavy metal(loid)-induced toxicity was determined by monitoring bacterial growth at various pH values - 1.5 (acidic gastric pH), 5.8 (acidic intestinal pH) and 7.0 (neutral pH) and 9.0 (alkaline pH). The pH values 1.5 (gastric pH) and 5.8 (intestinal pH) were selected because these are the two pH values at which the gastric (pH

1.5) and intestinal (pH 5.8) bioaccessibility of heavy metal(loid)s as impacted by gut bacteria was tested (Chapters 4 – 6). The pH of the bacterial growth media was emended using autoclaved dilute (0.1mM) HCl or NaOH solutions (Basu et al., 2015).

3.5.6 Distribution of metal(loid)s

The distribution of free and complexed metal(loid)s in the gastric and intestinal extracts was measured using chelate/ion-exchange disk/cartridge (Empore, iminodiacetate functionalized poly(styrene divinylbenzene) - 234877 Aldrich) (Pu and Fukushi, 2013). Five mL of 3.0M nitric acid and 5mL of Milli-Q water were sequentially passed through the cartridge. Then, 3mL of the metal(loid) containing bacterial growing medium was passed through the cartridge, and 5mL of Milli-Q water was passed through to rinse the cartridge. The 8mL of leachate was collected and determined for metal(loid)s using ICP MS. Free ionic forms of metal(loid)s are retained in the ion-exchange resin cartridge. The metal(loid) concentration in the leachate solution is considered to be stable complexed metal(loid)s, and the difference between total concentration and complexed metal(loid) concentration measured in the filtrate gives the free metal(loid) concentration. The distribution of As(V) and As(III) species was measured using HPLC-ICP-MS hyphenated set-up (Alava et al., 2013). A system of liquid chromatography hyphenated to an inductively coupled plasma mass spectrometer (HPLC-ICP-MS) from Perkin Elmer (Sunnyvale, CA, USA) was used, consisting of a P680 HPLC pump, an ASI-1 00 automated sample injector and an Elan DRC-e ICP-MS detector (Perkin Elmer, Sunnyvale, CA, USA).

3.5.7 Data analysis

Bacterial growth responses to pH and heavy metal(loid)s were carried out in triplicate. The data for bacterial growth was described using Eq. 3.1 (Juška et al., 2006; Peleg and Corradini, 2011).

$$Y = Y_m\,(1\text{-}exp^{-rx}) \qquad (3.1)$$

where Y = bacterial growth as measured by optical density (OD), Y_m is the maximum growth, r = rate constant, and x = growth period (minutes). The maximum growth response at various metal(loid) concentrations was calculated relative to the maximum growth in the absence of metal(loid) input. The relationship between maximum growth and metal(loid) concentration was described by log-logistic dose-response curve (Eq. 3.2) (Gardner, 2002; Rozman and Doull, 2000; Murado and Vázquez, 2002; Focke et al., 2017).

$$y = (D - C)/\{1 + \exp[B(\log(z) - \log(LD_{50})]\} \quad (3.2)$$

where, y = bacterial growth, z = metal(loid) concentration, D = upper limit of growth (i.e., growth in the control treatment), C = lower limit of growth, LD_{50} value = metal(loid) concentration at which the maximum growth of gut bacteria decreases to 50% of maximum growth achieved for the control treatment in the absence of metal(loid) input (i.e., 50% between the upper and lower limits); B = the proportional slope of the curve around LD_{50} (the point of inflexion). Metal(loid) toxicity was expressed as lethal dose for 50% growth reduction (LD_{50}) using Eq. 3.2 (Murado and Vázquez, 2002).

To test for significant differences in various treatments, statistical comparisons were made using analysis of variance (ANOVA) in Predictive Analytics SoftWare (PASW) statistics, release version 18.0.0 (SPSS, Inc., 2009, Chicago, IL). Duncan's multiple range test was used to compare the means of the treatments; variability in the data was expressed as the standard deviation and a $p < 0.05$ was considered to be statistically significant.

3.6 Results and discussion

3.6.1 Bacterial growth as impacted by pH

Growth of all the three bacterial strains followed the typical growth response curve (Eq. 3.1) with the maximum growth reaching within 12 hours of incubation (Figure 3.1 a, b and c). Maximum growth at various pH values and metal(loid) concentrations was estimated using Eq 3.1 (Figure 3.2). While the maximum growth of *Lactobacillus* species decreased at the higher pH tested, there was no significant effect of pH on the growth of *E. coli* (Figure 3.2). This indicates that *Lactobacillus* species are adapted to acidic pH conditions (Hood and Zoitola, 1988; Wijtzes et al., 1995; O'May et al., 2005). Gastric acid is considered to provide an effective barrier to microbial colonization at pH values of < 4 (Gianella et al., 1972; von Rosenvinge et al., 2013b). However, O'May et al. (2005) noticed that gastric acidity did not affect the microbial population recovered from intestinal aspirates but influenced microbiota composition. While *Lactobacillus* species are aciduric, a significant number of both *E. coli* and Lactobacillus species were noticed even at pH 3. However, O'May et al. (2005) also observed that the recovery of *E. coli* decreased as pH was reduced.

A number of studies have examined the effect of pH on probiotic bacterial species including *Lactobacillus* species in relation to their potential suitability as gastric probiotic

cultures (Fayol-Messaoudi et al., 2005; Sanhueza et al., 2015). For example, Saarela et al. (2008) have indicated that the potential value of *L. rhamnosus* as a gastric probiotics depends on the initial pH conditions under which the bacterial species is cultured. Fermentation at low pH may ensure a better performance of *L. rhamnosus* cells during the subsequent acid-stress conditions. Horáčková et al. (2011) compared the stability of the selected *Lactobacillus* species (*L. acidophilus* CCDM 151; *L. casei* CCDM 198; *L. rhamnosus* CCDM 150, and *L. fermentum* ST 68) with the commercial probiotic strain *L. casei* LAFTI L-26. All *Lactobacillus* species had the ability to adapt in the environment of bile salt including various pH conditions. Similarly, although a number of studies have indicated that *E. coli* growth decreased with increasing acidity (decreasing pH) of the growing medium, this bacterial species tend to develop resistance to extreme pH conditions (Lin et al., 1995; Presser et al., 1997; Li et al., 2012). Gut-resident *E. coli* strains deploy a complex set of responses to counter the impact of the low pH they experience as they travel through the gut passage (Hingorani and Gierasch, 2013). Some of their responses, such as the amino acid decarboxylases, act to keep the cytoplasmic pH above a dangerous level (Cotter and Hill, 2003; Hingorani and Gierasch, 2013).

Figure 3.1a. Escherichia coli

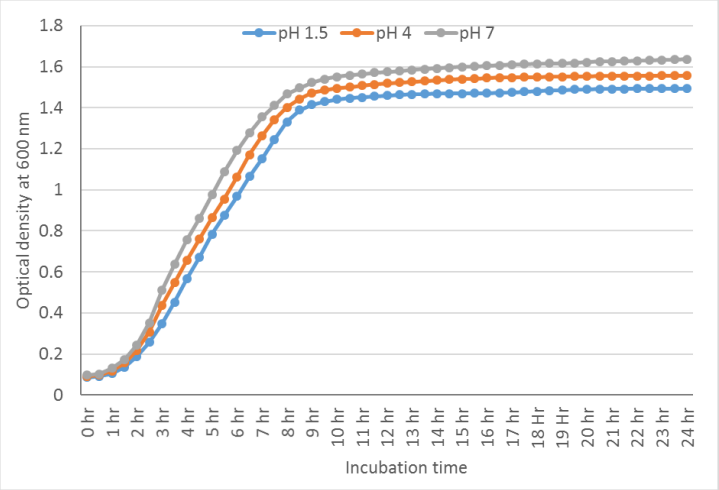


Figure 3.1b. Lactobacillus rhamnosus

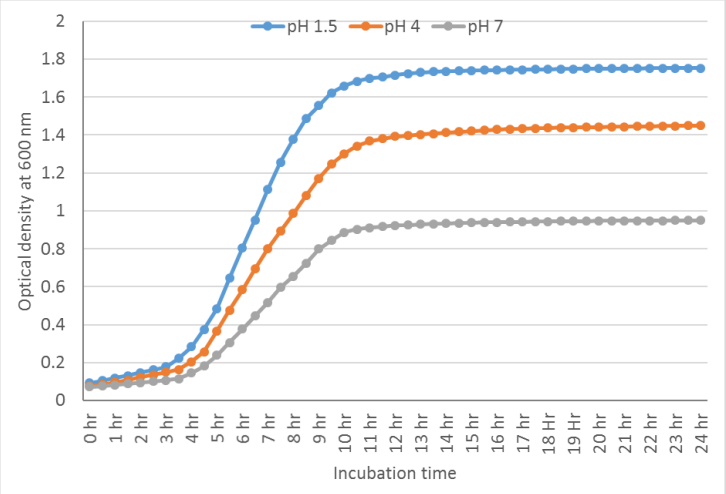


Figure 3.1c. Lactobacillus acidophilus

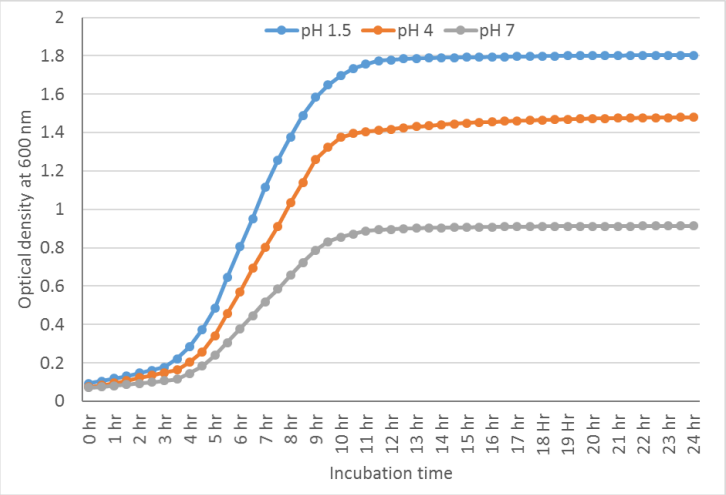


Figure 3.1 Growth response of *Escherichia coli* (a), *Lactobacillus rhamnosus* (b) and *Lactobacillus acidophilus* (c) to pH

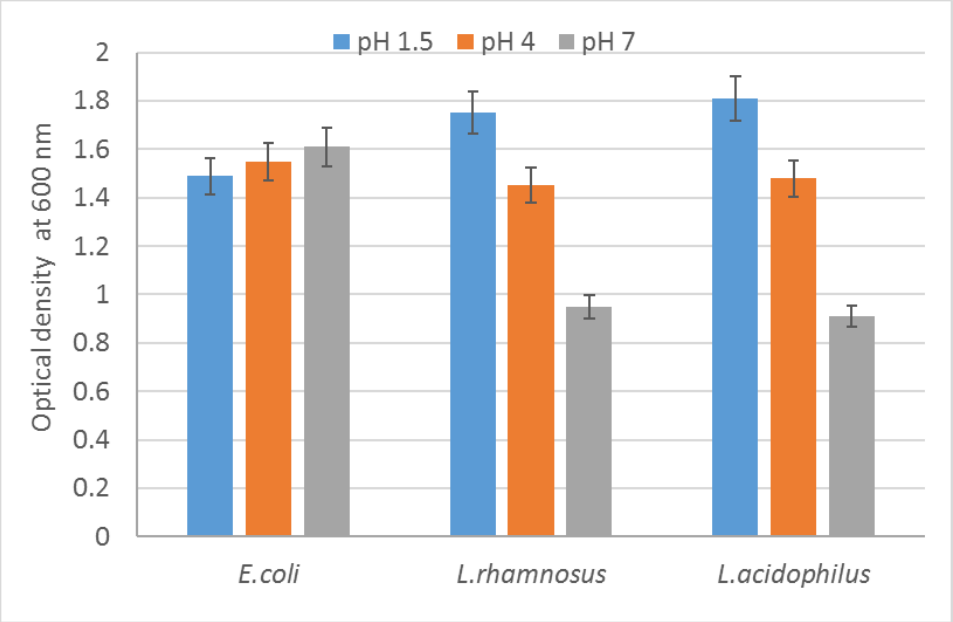


Figure 3.2 Maximum growth of gut bacteria as a function of pH

3.6.2 Metal(loid) toxicity

The growth response of three gut bacteria to four metal(loid)s at various concentrations is presented in Appendix (Appendix Figures 3.2 – 3.7). There was a slight decrease in bacterial growth when the metal(loid) concentrations were tested at the µg/L (ppb) level. The bacterial growth data at these concentrations was used to calculate minimum metal(loid) concentration at which there is an observable effect on bacterial growth (MCOE) (Table 3.4). The results indicated that MCOE values varied between the metal(loid) and bacterial species. As evident from MCOE values, As and Hg were more toxic to all three gut bacteria than Cd and Pb. The MCOE values in general are less for *E. coli* than for the *Lactobacillus* species indicating that the latter species are more tolerant to these metal(loid)s. There was no difference in MCOE values between the two *Lactobacillus* species.

Metal(loid) toxicity to microorganisms including bacteria is also often expressed as lethal dose (concentration) (LD_{50}), effective concentration (EC_{50}) or minimum inhibitory concentration (MIC). The LD_{50} and EC_{50} values represent the metal(loid) concentration at which the bacterial growth is reduced by 50% of the growth obtained for the control treatment in the absence of metal(loid) input. The MIC value represents the minimum metal(loid) concentration at which the bacterial growth is completely inhibited. In the present study, since the bacterial growth

was decreased only by a maximum of 10% at the highest concentration (ppb level) used to measure MCOE value, the LD_{50} values were estimated using higher metal(loid) input concentrations. It was not possible to calculate the MIC values because bacterial growth was observed even at the highest metal(loid) concentration tested in this study. Generally, for most bacteria, these toxicity values for metal(loid)s follow: MCOE > LD_{50} (EC_{50}) > MIC (Harrison et al., 2005; Rathnayake et al., 2013).

At the higher (i.e., ppm) concentration range, the maximum growth of all three gut bacteria decreased with increasing concentrations of As(III), As(V), Cd, Pb and Hg in the growing medium (Appendix Figures 3.2 – 3.7). The decrease in maximum growth with increasing metal(loid) input varied between both the gut bacteria and metal(loid)s. Metal(loid) input resulted in a lag period in the growth of gut bacteria, and the extent of lag period varied between both the gut bacteria and metal(loid)s. The relationship between maximum growth and metal(loid) concentration followed a log-logistic curve (Eq. 3.2) (Figure 3.3). The toxicity of metal(loid)s to gut bacteria as measured by LD_{50} values using Eq. 3.2 (Table 3.4) is discussed below.

Table 3.4 Minimum toxic metal(loid) concentration (MCOE) values at pH 7.0 and lethal dose (LD_{50}) values for As(V), As(III), Cd, Pb and Hg at various pH values for three gut bacteria

Gut bacteria	**Metal(loid)**	**MCOE value (µg/L)***	**LD_{50} value (mg/L)****			
			pH level			
			1.5	**5.8**	**7.0**	**9.0**
Escherichia coli	As(III)	5.0	13.4	13.2	14.5	15.3
	As(V)	10.0	106.3	89.5	71.3	32.3
	Cd	25.0	14.5	32.3	49.2	64.4
	Pb	35.0	24.4	37.5	56.5	98.3
	Hg	15.0	12.7	23.3	39.1	57.6
Lactobacillus acidophilus	As(III)	15.0	19.3	17.5	15.6	14.6
	As(V)	30.0	115.6	102.3	65.7	48.6
	Cd	55.0	16.2	17.4	24.4	32.4
	Pb	75.0	26.2	31.2	37.1	46.6
	Hg	25.0	12.4	12.7	18.3	26.3
Lactobacillus rhamnosus	As(III)	15.0	17.5	15.3	13.1	16.0
	As(V)	30.0	112.2	99.3	70.1	53.7
	Cd	55.0	17.3	18.4	22.2	28.7
	Pb	75.0	27.6	33.5	35.2	43.7

	Hg	25.0	13.1	13.4	17.5	23.8

**MCOE value refers to minimum metal(loid) concentration at which there is an observable effect on bacterial growth*

***Lethal dose (LD_{50}) refers to metal(loid) concentration at which the bacterial growth is reduced by 50% maximum growth for the control treatment without metal(loid) addition. LD_{50} value was calculated using the following equation:*

$$y = (D - C)/\{1 + \exp[B(\log(z) - \log(LD_{50})]\} \quad (3.2)$$

where, y = bacterial growth, z = metal(loid) concentration, D = upper limit of growth (i.e., growth in the control treatment), C = lower limit of growth, LD_{50} value = metal(loid) concentration required to achieve 50% of maximum growth (i.e., 50% between the upper and lower limits); B = the proportional slope of the curve around LD_{50} (the point of inflexion).

E. coli and the *two Lactobacillus* species exhibited slightly different patterns in terms of metal(loid) toxicity. In *E. coli*, the toxicity pattern followed As(III) > Hg > Cd > Pb > As(V), and the toxicity pattern of two *Lactobacillus* species followed Hg > As(III) > Cd > Pb > As(V). Mariscal et al. (1995) studied the toxicity effects of Cd, Hg and Pb in *E. coli* and found a similar toxicity pattern. Although, the *E. coli* MG1655 strain used in this study is considered to be resistant to As (Carlin et al., 1995; Chrysostomou et al., 2015), there was no significant difference in As toxicity between *E. coli* and two *Lactobacillus* species. All bacterial species were found to be more tolerant to cationic metal(loid)s including Cd, Pb and Hg compared to As(III). This is attributed to the abundance of exopolysaccharides and anionic groups present on the cell wall of bacteria, which are effective in the adsorption and complexation of cationic metal(loid)s, thereby rendering them less toxic to bacteria (Nwodo et al., 2012; Vijaydeep and Sastry, 2014).

The LD_{50} values for As(V) for *E. coli* and *Lactobacillus* species were much higher than that of As(III), indicating that the latter As species is more toxic than the former (Figure 3.3 a and b). However, all bacterial species showed similar trends in terms of the LD_{50} values for both As(V) and As(III) species. A number of studies demonstrated that As(III) is more toxic than As(V) to biota including bacteria (Ehrlich, 2002; Ordóñez et al., 2005). Two reasons have been attributed to the difference in microbial toxicity between As species. Firstly, As(V) is strongly adsorbed compared to As(III), resulting in the less bioaccessibility of the former species. Secondly, there is a difference in the uptake of As species within the cells. Arsenate enters the cell through phosphate transporters. With high serum concentrations of P (~ 34,000 µg/L,) As(V) is likely to have very little cellular uptake due to competition of phosphate. Arsenite enters the cell via cell diffusion or through aquaporin transporters. Since As(III) can gain entry into the cell by diffusion, this species potentially is much more toxic than As(V) (Baastrup et al., 2008; Straif et al., 2009; Hughes et al., 2011; Singh et al., 2011).

Figure 3.3a: As(V)

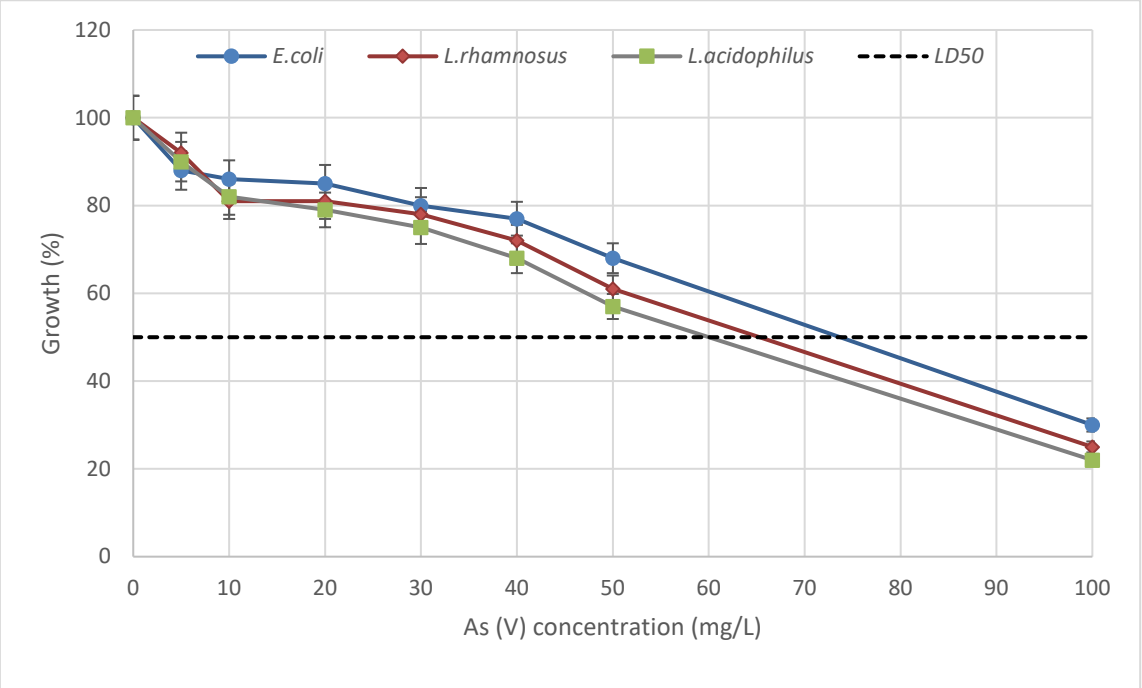


Figure 3.3b. As(III)

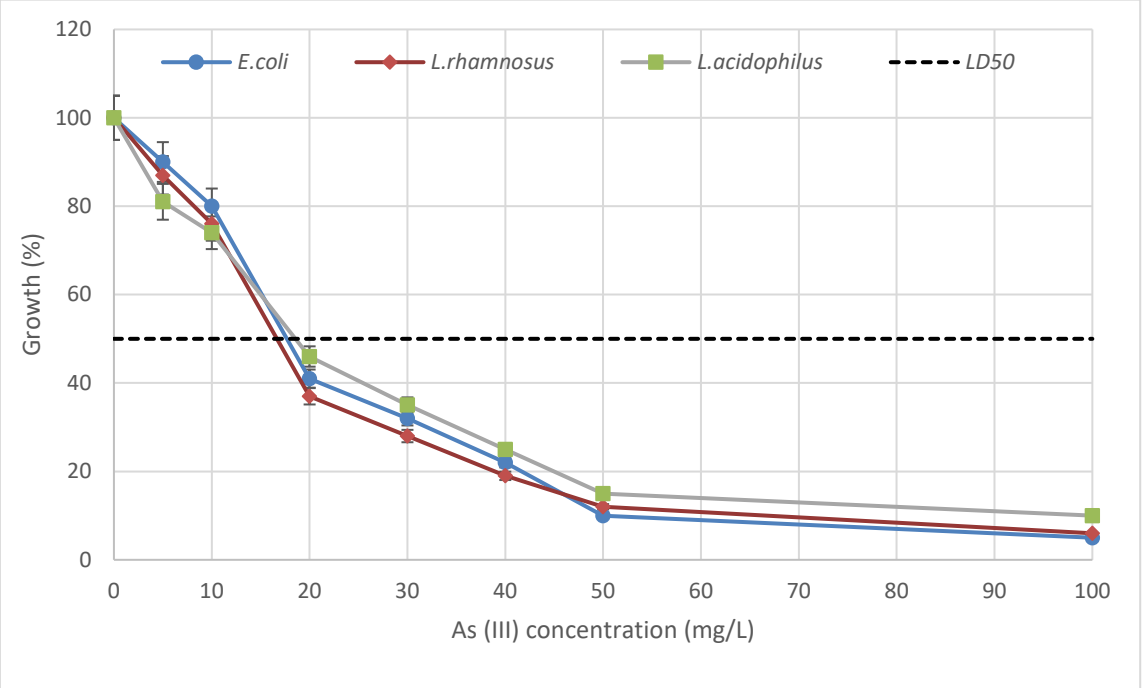


Figure 3.3c. Cd

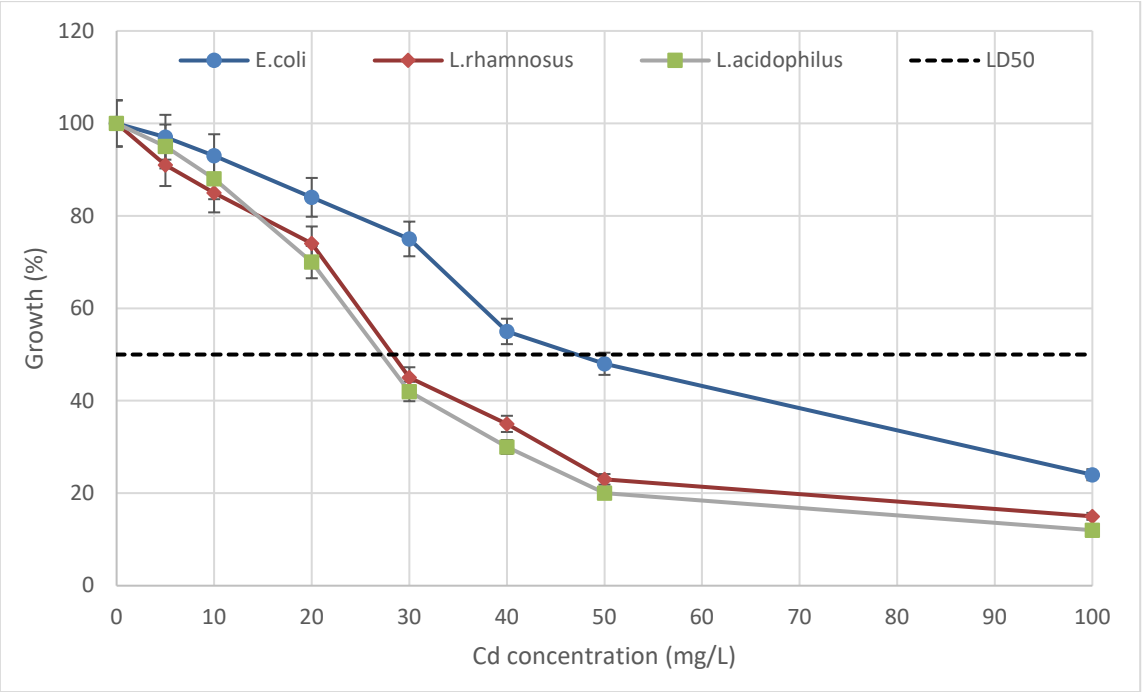


Figure 3.3d. Pb

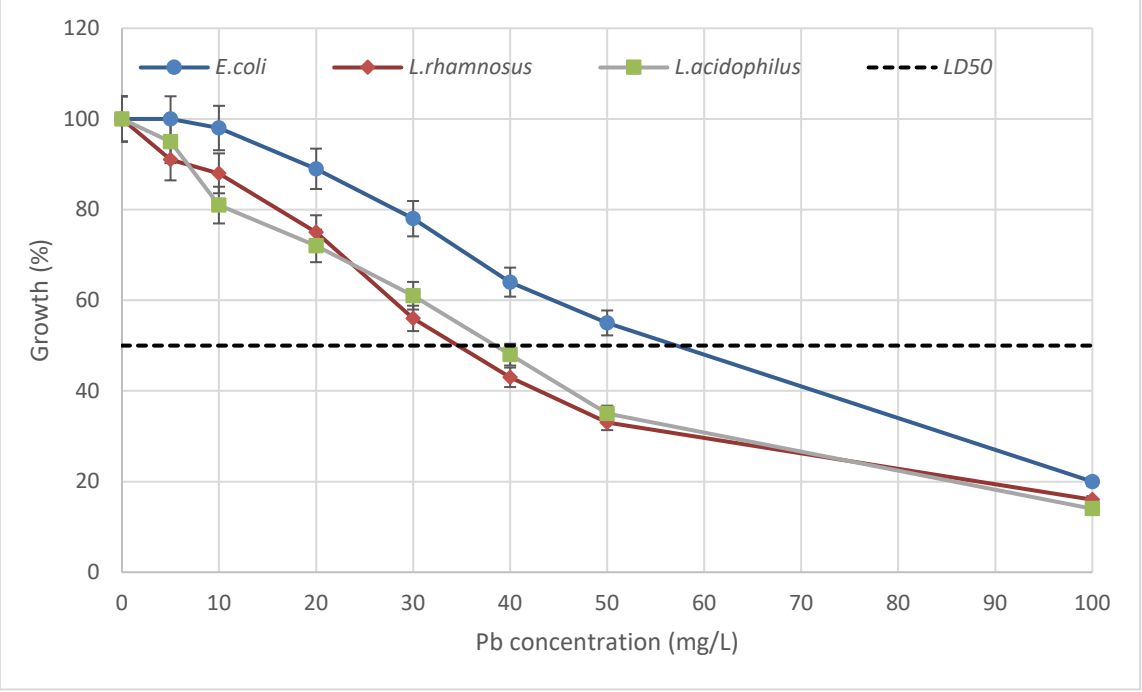


Figure 3.3e. Hg

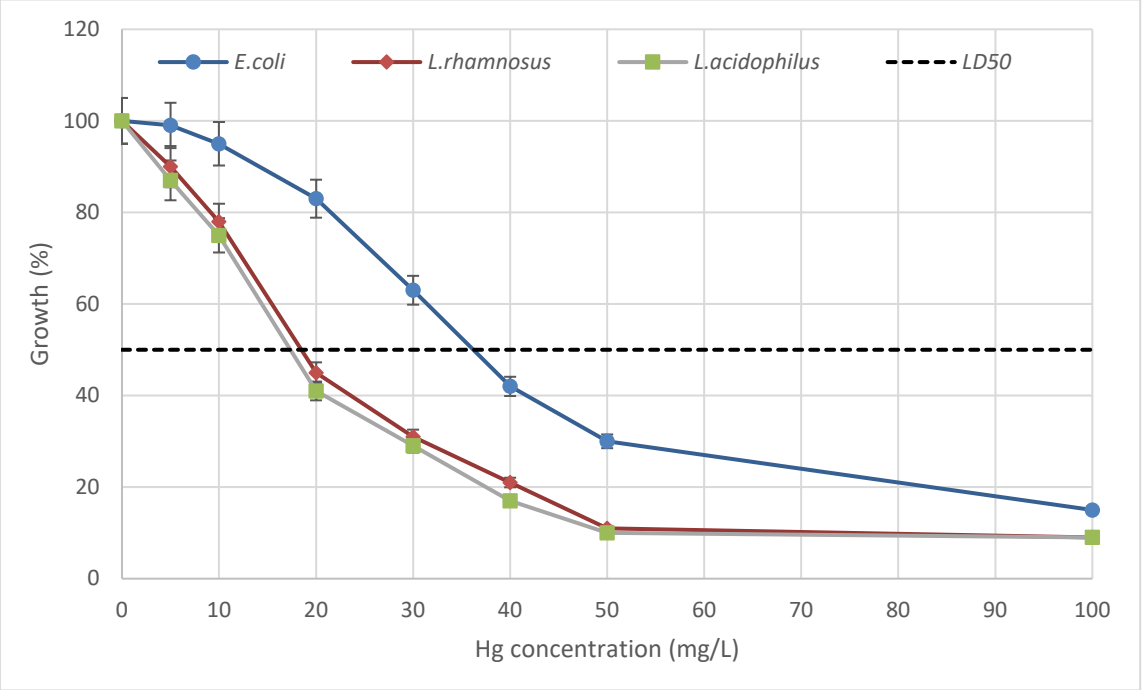


Figure 3.3 Bacterial growths response to increasing As(V) (a) and As(III) (b), Cd (c), Pb (d) and Hg (e) concentrations

In this study, the LD_{50} values for both Cd and Pb were higher for *E. coli* compared to *Lactobacillus* species, indicating that the former species is more resistant to these two metals (Figure 3.3). Heavy metal(loid) toxicity to microbes depends on a number of factors including the nature of metal(loid) and microbial species, and pH the composition of the growing media. Generally, gram positive bacteria including *lactobacillus* species are more resistant to cationic metals such as Cd and Hg compared to gram negative bacteria like *E. coli* (Vijaydeep and Sastry, 2014). The large amount of exopolysaccharides present in the cell wall of gram positive bacteria with a number of anionic groups (carboxyl, hydroxyl and phosphate) favour cationic metal binding (Nwodo et al., 2002). Most of the research indicated that Cd increased the lag phase of lactic acid bacterial growth (Arauz et al., 2007; Fazeli et al., 2011; Jama et al., 2012). The physiological reasons for the higher resistance of *E. coli* compared to *Lactobacillus* species to Cd and Pb toxicity are not clear. The main reasons for the toxic effects on bacterial growth include oxidative stress and the resultant modification of their metabolic processes (Birbin et al., 2012; Bhattacharyya et al., 2014; Rahal et al., 2014).

Although a large number of studies have examined Pb toxicity to environmental microorganisms including *E. coli* in soil, composts and biosolids (Giller et al., 1998; Roane et

al., 2000), and *Lactobacillus* species in aquatic system (Upreti et al., 2004; Halttunen et al., 2007a), there are only very few studies covering gut bacteria. For example, a recent work by Gao et al. (2017) using 16S rRNA sequencing demonstrated that Pb exposure not only altered the gut microbiome community structures and diversity but also greatly affected metabolic functions, leading to gut microbiome toxicity. de Boever et al. (2000) showed that 11 strains (out of 53 studied) of lactic acid bacteria including *L. rhamnosus* and *L. acidophilus* are tolerant to Pb and Cd. The toxicity of Pb is relatively low compared to other metal(loid) cations including Cd because of its lower bioavailability as a result of its tendency to form the insoluble lead phosphate and precipitate in bacterial cells (Levinson et al., 1996; Nies, 1999). Monachese (2012) also found relatively less toxicity of Pb to gut bacteria, *Lactobacillus* species compared to that of Cd.

In this experiment, Hg exhibited higher toxicity in all the three bacterial species, compared to Cd and Pb. As evident from the LD_{50} values, Hg toxicity was higher for *Lactobacillus* species than *E. coli* (Figure 3.3 e). Jardim et al. (1993) studied acute toxicity of Hg in *E. coli* and found that 50 µg/L can result in toxicity. They suggested that the glucose in the LB media can reduce the Hg ions into elemental Hg thereby rendering less toxicity. However, in the current study, glucose was not added to the media to avoid the conversion of Hg ions into elemental Hg. In terms of Hg toxicity, the two *Lactobacillus* species showed lower LD_{50} values compared to that of *E. coli*. Vijaydeep and Sastry (2014) observed that gram negative bacteria like *E. coli* was more resistant to Hg compared to the gram positive ones. There is limited information of Hg binding to gut bacteria. While Hg is cationic and analogous to Cd and Pb, their binding to bacterial cell wall is different from the latter two metal(loid)s (Monachese et al., 2012).

3.6.3 Effect of pH on metal(loid) toxicity

The effect of pH on metal(loid) toxicity, as measured by LD_{50} value, is presented in Table 3.4 and Figure 3.4. The LD_{50} values for the cationic metal(loid)s (Cd, Pb and Hg) increased with increasing pH and the effect was more pronounced in *Lactobacillus* species compared to *E. coli* (Figure 3.4). This indicates that the toxicity of these cationic metal(loid)s to gut bacteria decreased with increasing pH. Moreover, cationic metal(loid)s were less toxic to *E. coli* than to *Lactobacillus* species, especially at high pH conditions.

Both Cd and Pb showed similar trends in LD_{50} values throughout the pH changes, while Hg showed no significant changes in LD_{50} values at lower pH levels (Figure 3.4). Research on lactic acid bacteria showed the abundance of negatively charged carboxyl and phosphoryl groups (Sengupta et al., 2013). Hynönen and Palva (2013) conducted electrophoretic studies on *Lactobacillus* species and found that the net surface charges on the bacterial cell wall becomes negative at neutral pH thereby the bacteria can accommodate more cations. Halttunen (2007) showed that low pH facilitates the competition between the metal(loid) cationic and hydrogen ions (H^+) towards the negative charge, thereby decreasing the adsorption of metal(loid) cations. Zoghi et al. (2014) monitored metal(loid) removal from solution at various pHs by probiotic gut bacteria and found that the maximum removal occurred at pH 6, where the concentrations of free ionic species of metal(loid)s were found to be maximum. However, Esbaugh et al. (2011) suggested that alkaline pH can affect the competition between Ca and metal(loid) ions for the ligand binding, thereby decreasing metal(loid) toxicity.

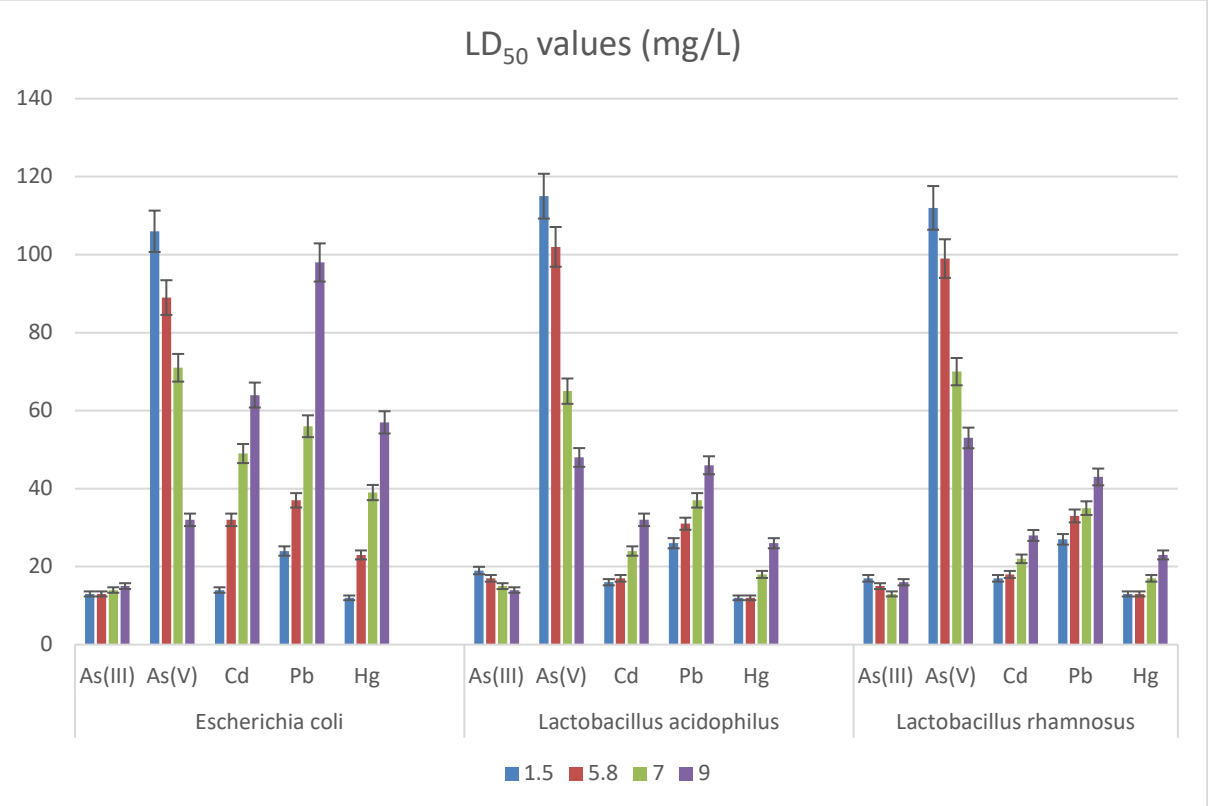


Figure 3.4 Effect of pH on As(III) (a), As(V) (b), Cd (c), Pb (d) and Hg (e) toxicity to gut bacteria as measured by LD_{50} values

The LD_{50} values for anionic metal(loid) As varied between the two As species, and As(III) was found to be more toxic than As(V) at all pH values tested in this study. However, while the LD_{50} value for As(V) decreased with increasing pH, there was no significant effect of pH on the LD_{50} value for As(III) species (Figure 3.4). This indicates that while the effect of pH on As(III) toxicity on bacterial growth was not significant, As(V) toxicity on bacterial growth increased with increasing pH (Table 3.4). The increased As(V) toxicity with increasing pH may be due to reduction of As(V) to the more toxic As(III). For example, Fulladosa et al. (2004) examined the effect of pH on As toxicity in the gram-negative *Vibrio fischeri* and noticed As(V) toxicity increased with increasing pH.

The present study using *E. coli*, which comes under the same phylum (Proteobacteria) showed results similar to that of Fulladosa et al. (2004), where As(III) was found to be more toxic than As(V), independent of the pH value. The difference in toxicity between these two As species decreased with increasing pH as a consequence of the strong influence of pH on the reduction of As(V) to As(III). In an *in vitro* intestinal experiment, Calatayud et al. (2010) observed that acidification of the growth medium (pH 5.5) resulted in a marked increase in As(V) permeability in intestine, which may be attributed to the reduction of As(V) to As(III) species which is readily absorbed by intestinal epithelial cells (Styblo et al., 2000; Calatayud et al., 2010; Dopp et al., 2010; Calatayud et al., 2012a). The intestinal permeability of these two As species is reported in Chapter 7. However, the reduction of As(V) to As(III) in the acidic intestinal environment was less compared to higher pH regions of large intestine and colon predominantly harbouring *E. coli* (Rowland and Davies, 1981; Alava et al., 2013).

3.6.4 Distribution of metal(loid) species in the bacterial growth medium

The data on the distribution of metal(loid) species in the bacterial growth medium are presented in Figure 3.5. The data indicated that As(V) was reduced to As(III) both in the presence and absence of bacteria which increased with increasing pH (Figure 3.5 a and b). The extent of As(V) reduction in the presence of bacteria was slightly higher than that occurred in the absence of bacteria especially at high pH values, indicating that both the growing medium and bacteria facilitated the As(V) reduction process. Fulladosa et al. (2004) argued that As speciation and oxidation state influence their biological effects and the subsequent toxicity. They noticed that within a 5.0-8.0 pH range, LD_{50} values for As(V) were found to decrease as

pH became basic, reflecting an increase in toxicity; whereas in the case of As(III), LD_{50} values were almost unchanged within a 6.0-8.0 pH range and lowered only at pH 9.0. While some bacterial species are resistant to As toxicity, gram-negative strains obtain their energy from As(V) during the reduction process to As(III) by arsenate reductase enzyme, which is an energy-dependent efflux (Mukhopadhyay et al., 2002; Tseng, 2007). It has often been found that microbial reduction of As(V) to As(III) species increases with pH (Masscheleyn et al., 1991; Valles-Aragón et al., 2013) which is attributed to ready supply of electrons, thereby facilitating reduction reaction (Mahimairaja et al., 2005).

Figure 3.5a: As(V)

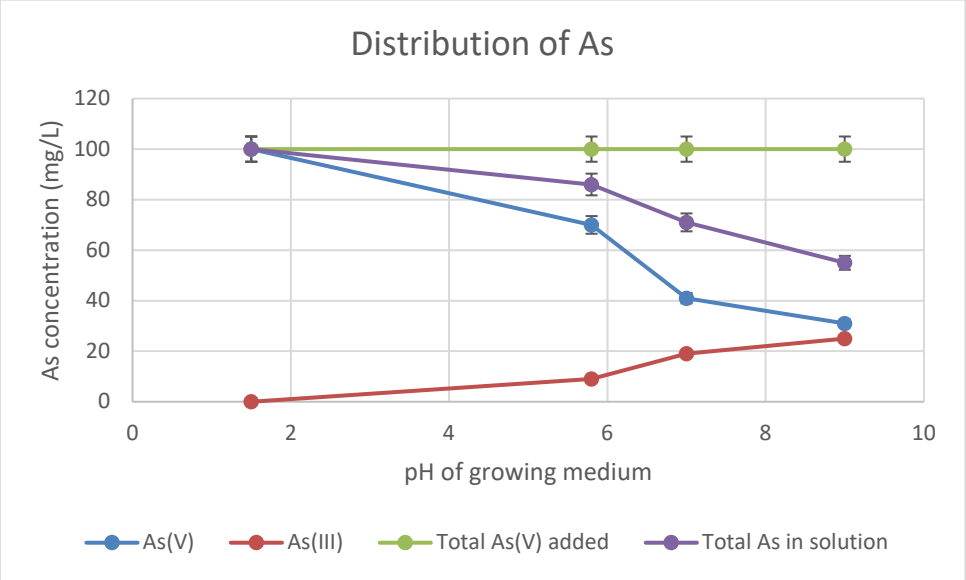


Figure 3.5b: Cd

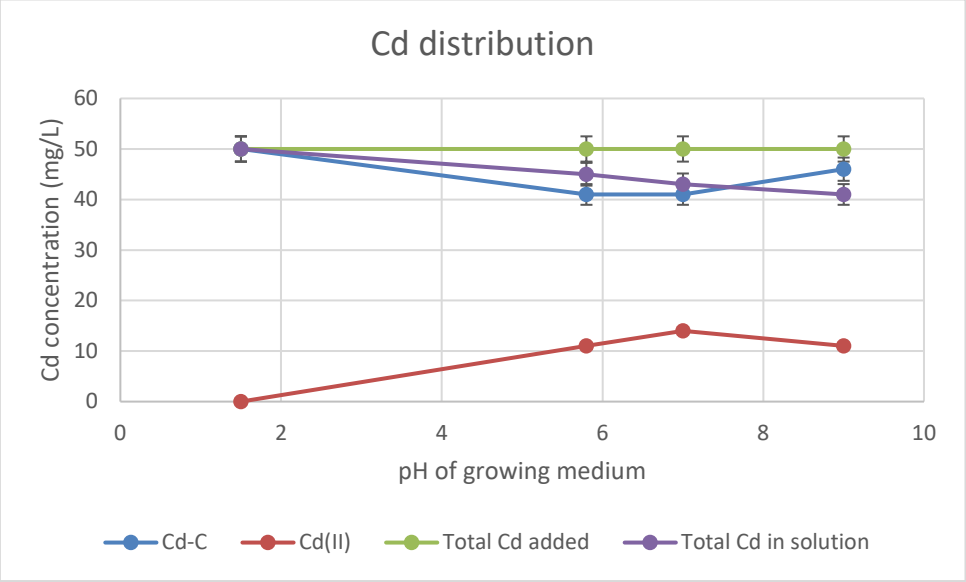

Cd distribution
Cd concentration (mg/L)
60
50
40
30
20
10
0
0
2
4
6
8
10
pH of growing medium
Cd-C
Cd(II)
Total Cd added
Total Cd in solution

Figure 3.5c: Pb

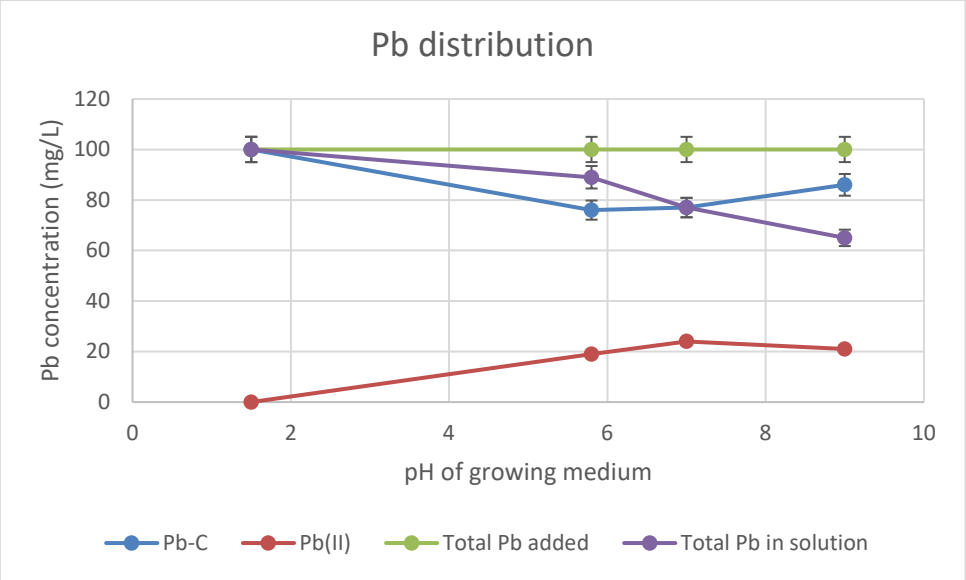


Figure 3.5d: Hg

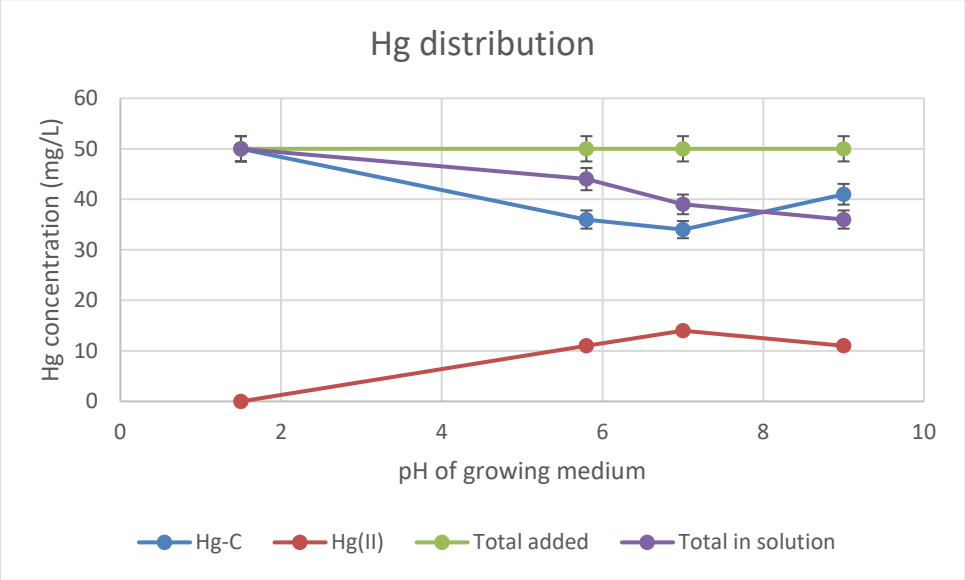


Figure 3.5 Speciation of As(V) (a), Cd (b), Pb (c) and Hg (d) in the bacterial growth medium

In the absence of bacteria, all the cationic metal(loid)s (Cd, Pb and Hg) remain as free ions at neutral pH but were complexed at acidic and alkaline pHs (Figure 3.5 c, e and g). The concentration of free metal(loid) ions in the absence of bacteria was high for Cd and Hg compared to Pb. Several researchers demonstrated that free metal(loid) ion concentration can

affect the bioavailability of metal(loid) ions to bacteria and thereby contribute to the toxicity of the metal(loid)s (Babich et al., 1983; Archibald and Duang, 1984; Schalk et al., 2011). They claimed that pH of the bacterial medium along with other factors (e.g. metal(loid) concentration, competing ions, chelating agents and ligands) can affect the transformation of free metal(loid) ions.

In this study, presence of bacteria decreased the free cationic metal(loid) ion concentrations in the medium, thereby reducing the toxicity of the metal(loid) ions (Figure 3.5 d, f and h). The extent of free metal(loid) ion formation was high for Cd and Hg in the medium but in the presence of bacteria, the free metal(loid) ion formation was low. This can be attributed to the adsorption and/or uptake of the metal(loid)s by the bacteria as free ions. Kirillova et al. (2017) showed that metal(loid) uptake is mainly through physical adsorption which can be affected by pH and presence of other cations. For instance, acidic conditions can reduce the uptake of cationic metal(loid)s due to the competition by protons (Olaniran et al., 2013; Ayangbenro and Babalola, 2017). Other than ionic competition, precipitation and complex formation are important factors that could influence toxicity to bacteria. Some studies indicate that bacteria can release OH^- to increase the pH and thereby precipitate cationic metal(loid)s (Rayner and Sadler, 1990; Yoshida et al., 1999).

3.7 Conclusions

All the cationic metal(loid)s (Cd, Pb and Hg) showed similar trend in terms of pH-toxicity relationship, while the anionic As species (As(V) and As(III)) exhibited an opposite effect. The order of metal(loid) toxicity was As(III) > Hg > Cd > Pb > As(V) for *E. coli* and Hg > As(III) > Cd > Pb > As(V) for the two *Lactobacillus* species In the case of cationic metal(loid)s such as Cd, Pb and Hg, toxicity decreased with increasing pH because of the increased adsorption of these metal(loid) cations by the bacterial species. Arsenite (AsIII) is more toxic than arsenate (AsV) to both gram-positive and gram-negative gut bacteria that include *L. rhamnosus, L. acidophilus* and *E. coli*. The toxicity of As(V) species increased with increasing pH. This is attributed to the reduction of less toxic As(V) to more toxic As(III) with increasing pH. Also, different As species have been shown to alter the composition and/or the metabolic activity of the gastrointestinal bacteria including *E. coli*, which may be an important factor contributing to the formation of an individual's microbiotype. The physiological consequences of these alterations have not been studied in detail but As-induced alterations of the gut bacteria are likely to contribute to their toxicity. There are other factors including the methylation of inorganic As(V) and As(III) by gut

bacteria. Hence, more *in vitro* and *in vivo* studies are required to deduce the entire As metabolism pathway in bacterial and human systems to assess the As toxicity for better human risk assessment.

While As is an anion, other three metal(loid)s are cations and hence the binding capacity of these heavy metal(loid)s to the bacterial cell wall varied based on the charge dependent functional groups. The toxicity of these metal(loid)s to the bacteria also depends on their bioavailability and bioaccessibility, which will be discussed in the forthcoming chapters.

Chapter 4

BIOACCESSIBILITY OF ORALLY INGESTED HEAVY METAL(LOID)S

4.1 Introduction

Metal mining and smelting industries, human activities, and indiscriminate disposal of agricultural and industrial wastes have resulted in the pollution of terrestrial and aquatic environments with heavy metal(loid)s (Senesi et al., 1999; Adriano, 2001; Nicholson et al., 2003; Naidu et al., 2008; Tchounwou et al., 2012; Wuana and Okieimen, 2011; Zhang et al., 2012; Xia et al., 2014). For instance, shooting ranges and base-metal tailings are a major source of heavy metal(loid)s that include arsenic (As), cadmium (Cd) copper (Cu), lead (Pb), antimony (Sb), mercury (Hg) and zinc (Zn) (Kumpiene et al., 2008; Sanderson et al., 2012).

In this chapter, the bioaccessibility of As, Cd, Hg and Pb from various sources is reported. Arsenic, Cd, Hg and Pb are the most important toxic metal(loid)s which reach human biological systems through oral ingestion (Jaishankar et al., 2014). For example, incidental ingestion of soil and dust by young children is an important pathway for Pb exposure (Hou et al., 2013; Cao et al., 2014). Lead is also included as a therapeutic compound in *Ayurvedic* medicines for treating diabetes mellitus, diarrhoea, and skin diseases (Nagarajan et al., 2014; Bolan et al., 2017a). Lead has no known natural biological function and, in humans, the developing fetus and children are more sensitive to high levels of Pb exposure than are adults due to neurological effects of Pb exposure; these include irreversible learning disabilities, attention deficit disorders, and behavioral difficulties (ATSDR, 2007b; Park et al., 2011).

Arsenic (As), which occurs naturally in a number of forms (or species), is classified as a class one carcinogen by the World Health Organisation's International Agency for Research on Cancer (IARC, 2004). High levels of As intake can raise the risk of developing lung, bladder and skin cancer, cardiovascular disease, diabetes mellitus, skin lesions, gastrointestinal illness and other serious health problems, eventually leading to death (WHO, 2009; Zhang et al., 2016). Significant detrimental impacts of As on human health have been reported in some endemic areas of Bangladesh, India, Chile, and China, and millions of people are potentially at risk from As poisoning (Mahimairaja et al., 2005; Naidu and Bhattacharya, 2009; Bhattacharya

et al., 2012; Hojsak et al., 2015). In these countries, As reaches food chain mainly through the use of potable water and plant uptake resulting from the irrigation of As-rich water. Regular rice consumption has been considered as a major source of As intake in humans in many countries including Bangladesh and Vietnam where As-rich groundwater is used to irrigate rice crop (Williams et al., 2006; Rahman and Hasegawa, 2011; Meharg and Zhao, 2012). Arsenic is also included as a therapeutic compound in *Ayurvedic* medicines for treating cancer, skin diseases, anti-inflammation, malaria, and antidote to venoms (Khandpur et al., 2008; Bolan et al., 2017a,b).

Cadmium (Cd) has been identified as one of the major heavy metals reaching the food chain through various activities (Naidu et al., 1994; Bolan et al., 2013; Loganathan et al., 2012). For example, in New Zealand and Australia, Cd has been identified as the most common heavy metal reaching the food chain mainly through animal transfer in pastoral agriculture (McLaughlin et al., 1996; Loganathan et al., 2012). Similarly, in many East and South Asian countries including Japan, Bangladesh, Indonesia, and Korea, Cd accumulation in rice ecosystems and its subsequent transfer to the human food chain is a major environmental issue (Kawada and Suzuki, 1998; Simmons et al., 2008; Bolan et al., 2013). In Australia (Williams and David, 1973; McLaughlin et al., 1996; Mann et al., 2002) and New Zealand (Longhurst et al., 2004), most of the Cd that has accumulated in the topsoil has been derived from impurities in phosphate (P) fertilizers added during normal farming practice. The paddy soils in many countries have been affected by Cd derived not only from fertilizer application, but also mine tailings and refining plants (Zarcinas et al., 2004; Bolan et al., 2013).

Mercury (Hg) occurs naturally in the earth's crust. It is released into the environment from volcanic activity, weathering of rocks and as a result of human activity, particularly coal-fired power stations, residential coal burning for heating and cooking, industrial processes, waste incinerators and as a result of mining for mercury, gold and other metals (Stylo et al., 2016). Mercury exists in various forms: elemental (or metallic), inorganic and organic, and these forms of mercury differ in their degree of toxicity and in their effects on the nervous, digestive and immune systems, and on lungs, kidneys, skin and eyes (Rice et al., 2014). Once in the environment, Hg can be transformed by bacteria into methylmercury, and then bioaccumulates in fish and shellfish. Mercury exposure mainly occurs through consumption of fish and shellfish contaminated with methylmercury (Bushkin-Bedient and Carpenter, 2010; Silbernagel et al., 2011; Dong et al., 2015). Mercury is also included as a therapeutic compound in some *Ayurvedic* medicines for treating insomnia, throat and skin infections and intestinal parasites (Kew et al., 1993; Liu et al., 2008; Bolan et al., 2017a). Neurological and behavioural disorders

may be observed after inhalation, ingestion or dermal exposure of different mercury compounds. Symptoms include tremors, insomnia, memory loss, neuromuscular effects, headaches and cognitive and motor dysfunction (Carocci et al., 2014).

The toxicity of ingested contaminants including heavy metal(loid)s is determined ultimately by the extent to which they are solubilised in the gut (bioaccessibility), their permeability through intestinal epithelial cells and subsequent circulation in the blood (bioavailability), and their assimilation and metabolic action in any tissues that subsequently absorb them (bioactivity). The bioaccessibility-bioavailability-bioactivity continuum (Figure 2.1; Section 2.7 in Chapter 2) play a critical role in the toxicity of heavy metal(loid)s to biota (Naidu et al., 2008; Jaishankar et al., 2014; Kim et al., 2015; Bolan et al., 2017a). Bioaccessibility is usually evaluated *in vitro* by physiologically based extraction tests and gastrointestinal digestion procedures. Bioavailability, which expresses the fraction of the bioaccessible compound that enters the blood circulation, refers to the rate and extent to which the compound permeates through the intestinal epithelial cells (Jaishankar et al., 2014; Kim et al., 2015). Bioactivity refers to the physiological and metabolic interactions between the compound and the human tissue or organ, which disturb homeostasis (the body's usual healthy equilibrium) (Rehman et al., 2018).

The amount of metal(loid) absorption into systemic circulation (the bioavailable fraction) depends on the nature and solubility of metal(loid) source (i.e., bioaccessibility) and the properties of the ingested compound (Naidu et al., 2008; Deshommes et al., 2012; Ruby et al., 2016). Studies on metal(loid) bioaccessibility are often conducted using *in vitro* digestive techniques aiming to simulate the human digestive system (Van de Wiele et al., 2010; Laird et al., 2007, 2013; Wijayawardena et al., 2015; Juhasz et al., 2016). These tests, however, do not take into account the role of the human gut microbiome and its interactions with environmental contaminants. In this chapter (Chapter 4), the bioaccessibility of selected metal(loid)s including As, Cd, Pb and Hg from various common orally ingested sources will be reported. In the subsequent chapters, the effects of gut microbes (Chapter 5) and chelating agents (Chapter 6) on the bioaccessibility will be reported.

4.2 Objectives

The overall objective of this work was to scrutinize the bioaccessibility of orally ingested As, Cd, Hg and Pb as impacted by gut microbes. The specific objectives in this chapter were to:

(i) Monitor the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb in various sources

(ii) Monitor the distribution of various species of As, Cd, Hg and Pb in gastric and intestinal extractions

4.3 Hypothesis

(i) Bioaccessibility depends on the nature of metal(loid)s and its sources.

(ii) Bioaccessibility of metal(loid)s varies between gastric and intestinal extractions

(iii) Gut bacteria modulate bioaccessibility of metal(loid)s through their effects on the solubilisation of metal sources and subsequent interactions with metals via adsorption and speciation processes.

Hypotheses (i) and (ii) will be tested in this chapter (Chapter 4) by comparing the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb in various sources. Hypothesis (iii) will be tested in Chapter 5 by comparing the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb in various sources as impacted by three gut bacteria.

4.4 Experiments

The major experiments and analyses conducted to test the hypothesis, and the treatments used in this chapter are listed in Table 4.1.

Table 4.1 The major experiments and treatments used in Chapter 4

No.	Title	Treatments
1	Sequential fractionation of metal(loid)s	4 oral metal(loid) sources (Table 4.4); sequential extraction tests (Table 4.5)
2	Bioaccessibility of metal(loid)s	4 metal(loid) sources (4 reference metal(loid)s and 4 oral metal(loid) sources (Table 4.3 & 4.4); Gastric and intestinal bioaccessibility tests
3	Speciation of metal(loid)s	4 metal(loid) sources (4 reference metal(loid)s and 4 oral metal(loid) sources (Table 4.3 & 4.4); Gastric and intestinal bioaccessibility extractions

4.5 Materials and methods

4.5.1 Metal(loid) sources

The metal(loid) sources included in this study are the common orally ingested sources, and are based on their heavy metal(loid) content derived from literature data (Table 4.2). The metal(loid) sources include offal pet food (cadmium), fish meal (mercury), complementary medicine (lead) and rice grain (arsenic) (Table 4.3 & 4.4). The pet food is derived from sheep and cattle offal including kidney and liver which are rich in Cd (Zhang et al., 2012). The fish meal is derived from fish wastes which are rich in Hg (Bushkin-Bedient and Carpenter, 2010; Silbernagel et al., 2011). Lead is added as a therapeutic agent in some of the complementary medicines including ayurvedic medicines (Saper et al., 2004; Nagarajan et al., 2014; Bolan et al., 2016b, 2017a). Rice grains harvested from paddy fields irrigated with As rich groundwater tend to contain high levels of As (Rahman and Hasegawa, 2011; Bolan et al., 2017b). Arsenic oxide, Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3).

Table 4.2 Orally ingested sources of arsenic (As), cadmium (cd), lead (Pb) and mercury (Hg)

Metal(loid)	Source	Concentration (mg/kg)	References
Arsenic (As)	Rice	<0.0022 - 1.095	Duxbury et al. (2003); Williams et al. (2005); Williams et al., 2007; He and Zheng (2010); Hu et al. (2011); Kuramata et al. (2011); Rahman and Hasegawa (2011); Bhattacharya et al. (2012); Halder et al. (2012); He et al. (2012); Meharg and Zhao (2012); Sun et al. (2012); Azad et al. (2013a); Hu et al. (2013b); Bolan et al. (2017a,b); Taylor et al. (2017)
Cadmium (Cd)	Animal offal	<0.012 – 54.6	Tahvonen (1996); D'Ilio et al. (2008); Loganathan et al. (2012); Lazarus et al. (2014); Magwedere et la. (2013); Skaljaca et al. (2015); Bazargani-Gilani et al. (2016); Lazarus et al. (2014); Tyokumbur (2016); Tomović et al. (2017)
Lead (Pb)	Complimentary medicine	340 - 36000	Saper et al. (2004, 2008); Cooper et al. (2007); Koch et al. (2011); Ihedioha and Okoye (2012); Nagarajan et al. (2014); Bolan et al. (2016b; 2017a)
Mercury (Hg)	Fish	0.005 – 32.6	Cabanero et al. (2004); Burger et al. (2005); Dórea (2006); Berntssen et al (2014); Kuras et al. (2017)

Table 4.3 Standard reference chemical compounds and characteristics of heavy metal(loid) sources used in this study

Heavy metal(loid)	Chemical compound	Oral ingestion metal(loid) sources
Arsenic	Arsenic oxide (As_2O_3; Molar mass = 197.841 g/mol)	Rice grain
Cadmium	Cadmium acetate ($Cd(CH_3COO)_2$; Molar mass = 230.5 g/mol)	Offal pet food
Lead	Lead acetate ($Pb(C_2H_3O_2)_2$; Molar mass = 325.29 g/mol)	Complementary medicine
Mercury	Mercuric chloride ($HgCl_2$; Molar mass = 271.52 g/mol)	Fish meal

Table 4.4 Characteristics of heavy metal(loid)s sources used in this study (values are mean ± standard deviation of triplicate)

Property	Heavy metal(loid)s tested in this study			
	Cadmium	Mercury	Lead	Arsenic
Source	Offal pet food	Fish meal	Complementary medicine	Rice grain
Total metal (mg/kg)	35.67± 0.56	26.78± 0.23	5724± 11.4	1.095 ± 0.03
pH	6.23±0.11	7.12± 0.13	5.87± 0.21	6.01± 0.05
EC (µS/cm)	586±13.2	345±11.3	1267± 23.4	213± 13.1
Metal compounds	Organic bound	Methyl mercury	lead carbonate; lead sulphide	Inorganic As

4.5.2 Total and extractable metal(loid)s

To determine the total heavy metal(loid) content, 0.50 g of air-dried metal(loid) source samples (< 250 µm) were digested with 5 mL of *aqua regia* in Teflon microwave oven as outlined in Method 3051H (USEPA, 1997). For quality assurance, the appropriate number of blank and standard reference material samples [certified reference material (CRM) - Montana Soil (SRM 271)] were included in the digestion procedure and sample analysis. For the measurement of extractable metal(loid) concentration, 5.0 g of metal(loid) source was extracted with 12.5 mL of 1 M NH_4NO_3 solution (DIN 19730 – Sigma-Aldrich) for 2 h in an end-over-end shaker (Naidu

and Harter, 1998; Krishnamurti et al., 2000). All digested (total) and extracted solutions were diluted with MQ water and selected samples were spiked with an internal standard solution. The metal(loid) concentration was measured using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS, Agilent) using a combination of internal standardisation and external calibration.

4.5.3 Forms and distribution of heavy metal(loid)s

Distribution of metal(loid)s in environmental and food samples has been monitored using selective chemical fractionation techniques (Arpadjan et al. 2006; Bolan et al., 2017a). The distribution of metal(loid)s among various forms depends on the chemical properties of both the individual metal(loid) and the metal(loid) source substrates.

There is currently no standard fractionation scheme that is specific to heavy metal(loid) species in some of the metal(loid) matrices used in this study (for example, complementary medicines). In this study, a simple sequential fraction technique (Tessier et al. 1979) was used to determine the distribution of heavy metal(loid)s in various sources (Table 4.5; Figure 4.1). This method divides metal(loid)s into five chemical fractions: soluble, carbonate-bound, oxide-bound, organic-bound and residual. The metal(loid) concentrations in the sequential extraction were analysed by ICP-MS (Agilent).

Table 4.5 Sequential fractionation scheme for heavy metal(loid)s in source materials (Tessier et al., 1979)

Step	Fraction	Extractant	Solid (g) : solution (mL) ratio	Conditions
1	Soluble fraction	1M $MgCl_2.6H_2O$	1:8	Shaken 1 hr
2	Carbonate-bound	1 M NaOAc	1:8	Shaken 5 hrs
3	Oxide-bound	0.04 M $NH_2OH.HCl$	1:20	Boiling in water bath 6 hrs
4	Organic-bound	0.02 M HNO_3 30 % H_2O_2	1:8	Boiling in water bath 2 hrs
5	Residual	Concentrated HNO_3 and HCl	1:5	Digested at 160 ºC

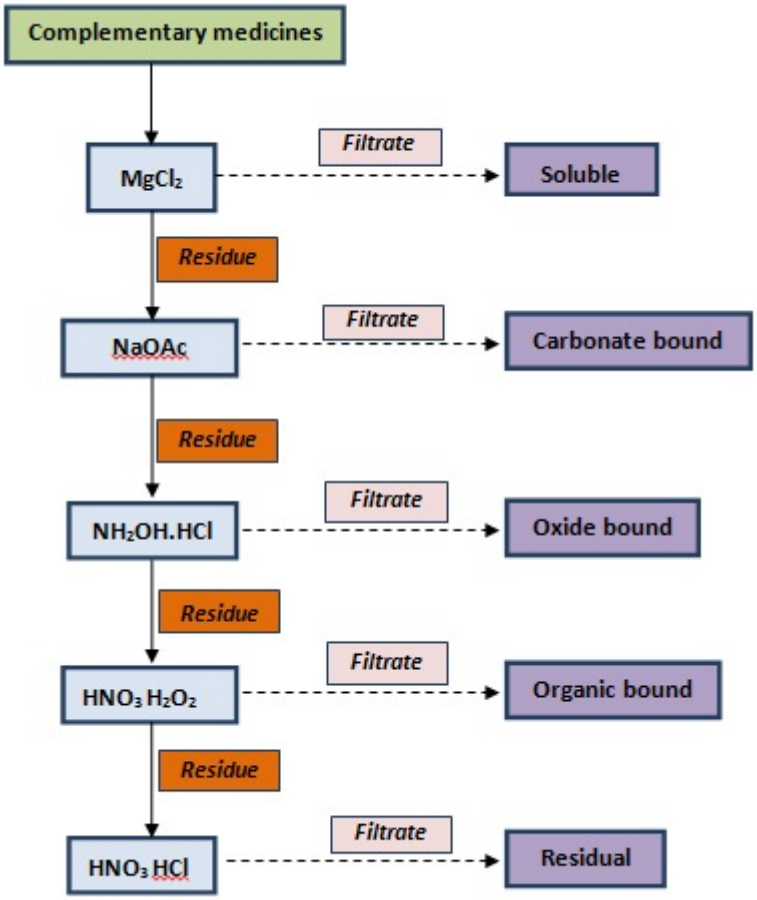


Figure 4.1 Sequential fractionation scheme for heavy metal(loid)s in source materials (Tessier et al., 1979)

4.5.4 *In vitro* gastrointestinal bioaccessibility tests

Bioaccessible metal(loid) contents in various metal(loid) sources were measured following the *in vitro* gastrointestinal (IVG) method by Rodriguez et al. (1999) which involves a two-step sequential extraction: a gastric followed by an intestinal extraction. Arsenic oxide, Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3). These compounds have been used as reference standard materials for the bioaccessibility test because these are readily soluble and have often been used for *in vivo* metal(loid) bioaccessibility assessment (Bannon et al., 2009; Juhasz et al., 2016), and also in toxicity studies in the Integrated Risk Information System (IRIS, 2004). The *in vitro* gastrointestinal bioaccessibility of these reference compounds were used to estimate the relative bioaccessibility of the metal(loid) sources.

Firstly, the standard compounds or the metal(loid) sources were combined with gastric phase solution at a solid:solution ratio of 1:100 in polyethylene screw cap tubes. The gastric phase solution was 0.15 M NaCl and 1% porcine pepsin (pH 1.8) (Sigma Chemical Company, Australia) (Rodriguez et al., 1999)). The tubes with the extracting solution were bubbled with argon gas to maintain anaerobic condition, and the tubes were incubated at 37°C on a Ratek suspension mixer with a shaking speed of 40 rpm for 1 h. Following gastric phase dissolution, the solution was modified to the intestinal phase by adjusting the pH to 5.8 with NaOH and with the addition of bovine bile (1750 mg L^{-1}) and porcine pancreatin (500 mg L^{-1}) (Rodriguez et al., 1999). After 4 h, intestinal phase samples were collected. Both gastric and intestinal phase samples were filtered through 0.2 *μ*m syringe filters for metal(loid) analysis by ICP-MS.

4.5.5 Distribution of free and complexed metal(loid)s

The distribution of free and complexed metal(loid)s in the gastric and intestinal extracts was measured using cation/anion-exchange resin cartridge (Empore, iminodiacetate functionalized poly(styrene divinylbenzene) - 234877 Aldrich) (Pu and Fukushi, 2013). The method is described in Section 3.5.6 (Chapter 3).

4.5.6 Data analysis

Both absolute (AB) and relative (RB) metal(loid) bioaccessibility values for gastric and intestinal phases were calculated using Eqs. 4.1 – 4.4.

$$Gastric\ AB\ (\%) = [Gastric\ in\ vitro\ metal(loid)/Total\ metal(oid))]\ x\ 100 \quad (4.1)$$

where, gastric AB = gastric absolute bioaccessibility, gastric *in vitro* metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by gastric phase solution, total metal(loid) = total metal(loid) content in the source material or reference metal(loid) sample added to the *in vitro* assay.

$$Gastric\ RB\ (\%) = (Gastric\ AB\ source/Gastric\ AB\ reference)\ x\ 100 \quad (4.2)$$

where, gastric RB = gastric relative bioaccessibility, Gastric AB source = absolute bioaccessibility for metal(loid) source in gastric phase, and Gastric AB reference = absolute bioaccessibility for reference metal(loid) compound in gastric phase.

$$Intestinal\ AB\ (\%) = [Intestinal\ in\ vitro\ metal(loid)/Total\ metal(oid))]\ x\ 100 \quad (4.3)$$

where, intestinal AB = intestinal absolute bioaccessibility, intestinal *in vitro* metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by intestinal

phase solution, total metal(loid) = total metal(loid) content in the source material or reference metal(loid) sample added to the *in vitro* assay.

$$Intestinal\ RB\ (\%) = (Intestinal\ AB\ source / Intestinal\ AB\ reference)\ x\ 100 \quad (4.4)$$

where, intestinal RB = intestinal relative bioaccessibility, Intestinal AB source = absolute bioaccessibility for metal(loid) source in intestinal phase; and intestinal AB reference = absolute bioaccessibility for reference metal(loid) compound in intestinal phase.

All experimental analyses were carried out using three replications. Statistical comparisons were made using analysis of variance (ANOVA) in Predictive Analytics SoftWare (PASW) statistics (version 18.0.0; SPSS, Inc., 2009, Chicago, IL) in order to examine the significant differences in various treatments. Duncan's multiple range test was also employed to compare the means of various treatments; variability in the data was presented as the standard deviation and a $p < 0.05$ was considered statistically significant.

4.6 Results and discussion

4.6.1 Total and extractable metal(loid) content

Montana Soil (SRM 271) was used as a reference material for total heavy metal(loid) analysis. The total and extractable concentrations of heavy metal(loid)s in the reference material, metal(loid) source materials and the blank solutions are presented in Table 4.6. The data indicate that the *aqua regia* solution used for total heavy metal(loid) analysis gave a good recovery of metal(loid)s (94.3 – 98.6% recovery) (Table 4.6). All blank solutions used for various metal(loid) analyses contained very low concentrations of these metal(loid)s (Table 4.6). The total metal(loid) contents of source materials were 1.104 mg/kg for As in rice grain, 37.5mg/kg for Hg in fish meal, 112.4mg/kg for Cd in pet food and 1023mg/kg for Pb in complementary medicine. These values were within the range reported in literature (Table 4.2).

Table 4.6 Total and 1M NH4NO3-exctracble metal(loid) concentrations in Montana Soil (Reference material; SRM 271), standard metal(loid) samples used for bioaccessibility tests, test samples and blank solutions used for total, speciation and bioaccessibility measurements. The values within brackets give 1M NH_4NO_3-exctracble metal concentration.

Samples	Arsenic (As)	Cadmium (Cd)	Lead (Pb)	Mercury (Hg)
Reference sample (mg/kg)				
Montana soil (SRM 271)*	103 (3.12)	51.2 (4.17)	1370 (8.26)	7.01 (0.24)
Standard samples (%)**				
Arsenic oxide	76.9 (74.2)	-	-	-
Cadmium acetate	-	42.2 (41.5)	-	-
Lead acetate	-	-	61.5 (59.5)	-
Mercuric chloride	-	-	-	89.5 (82.3)
Test samples (ppm (mg/kg))				
Rice grains (As)	1.095 (0.005)	0.001	0.001	0.001
Offal (Cd)	0.004	35.67 (1.36)	0.002	0.001
Complementary medicine (Pb)	0.010	0.023	5724 (2.34)	0.024
Fish meal (Hg)	0.001	0.012	0.001	26.78 (1.23)
Blank solutions (µg/L (ppb))				
Aqua regia (for total)	<0.5	<0.5	<0.5	<0.5
NH4NO3 (for extractable metal)	<0.5	<0.5	<0.5	<0.5
1M $MgCl_2.6H_2O$ (for speciation)	<0.5	<0.5	<0.5	<0.5
1 M NaOAc (for speciation)	<0.5	<0.5	<0.5	<0.5
0.04 M $NH_2OH.HCl$ (for speciation)	<0.5	<0.5	<0.5	<0.5
0.02 M HNO_3 30 % H_2O_2 (for speciation)	<0.5	<0.5	<0.5	<0.5
Gastric extract (for bioaccessibility)	<0.5	<0.5	<0.5	<0.5
Intestinal (for bioaccessibility)	<0.5	<0.5	<0.5	<0.5

**Certified values for Montana Soil (SRM 271) (mg/kg): Arsenic (As) = 107; Cadmium (Cd) = 54.1; Mercury (Hg) = 7.42; Lead (Pb) = 1,400.*

***Standard metal(loid) samples (%):* Arsenic oxide - *Arsenic (As) = 77.73;* Cadmium acetate - *Cadmium (Cd) = 48.76;* Mercuric chloride - *Mercury (Hg) = 92.91;* Lead acetate - *Lead (Pb) = 63.69;*

BD = below detection limit - ICPMS: As, Cd, Hg, Pb, Zn, Cu – 0.5 µg/L (ppb)

The NH_4NO_3 extractable metal(loid) contents are presented in Table 4.6. NH_4NO_3 extraction is often used to measure bioaccessible metal(loid) concentration in soil (Krishnamurti et al., 2000). The data in this study indicated that while almost 100% of total metal(loid) content in the standard reference samples were extracted by NH_4NO_3, only a small fraction (< 10%) of total metal(loid) content in the source materials were extracted. This indicates the low bioaccessibility of the tested metal(loid)s in these source materials.

4.6.2 Speciation of metal(loid)s

The speciation of heavy metal(loid)s in various sources was examined using a sequential fractionation technique (Tessier et al., 1979). This sequential fractionation technique divides heavy metal(loid)s into five 'operationally defined' chemical fractions that include: soluble, carbonate-bound, oxide-bound, organic-bound and residual fractions (Figure 4.1).

The recovery of total heavy metal(loid) content by these fractions was examined by comparing the independently measured total heavy metal(loid) content using *aqua regia* (Table 4.6) with the summation of the five fractions measured using the sequential fractionation technique (Table 4.5). The data indicate that the recovery of total metal(loid) content by the fractionation technique for all metal(loid)s tested in this study ranged from 85.6 to 104.7%. The recovery of total metal(loid) content by the fractionation technique for Hg was generally less (85.6%) than that for other metal(loid)s. The range in the recovery of total metal(loid)s by sequential fractionation may be attributed to various factors including the nature of metal(loid) species, sample substrate (i.e., organic and inorganic) and analytical error resulting from loss of samples during filtration of sequential extracts. Nevertheless, the high percentage recovery indicates that sequential fractionation technique used in this study was effective in quantifying various species of heavy metal(loid)s in these source materials.

The amounts of various species of metal(loid)s and their distribution as a percentage of total content are presented in Figure 4.2. The soluble fraction of metal(loid) contributed only 5.14%, 6.71%, 2.34% and 3.25% for As, Cd, Pb and Hg, respectively of the total metal(loid) contents in the respective sources. The remaining was contributed by insoluble fractions that include carbonate-bound, oxide-bound, organic-bound, and residual fractions. Within the insoluble fractions, organic bound fractions dominated in the case of As in rice grains, Cd in offal and Hg in fish meal, while inorganic fractions dominated in Pb in complementary medicines. While only a small portion of As, Cd and Hg (<5.25%) was present as the residual fraction, a significant amount of Pb in complementary medicine (23.4%) was present in residual fraction. The difference in metal(loid) fractions between various sources may be attributed to the difference in original source of metal(loid) input in these sources (Nagarajan et al. 2014; Juhasz et al., 2016).

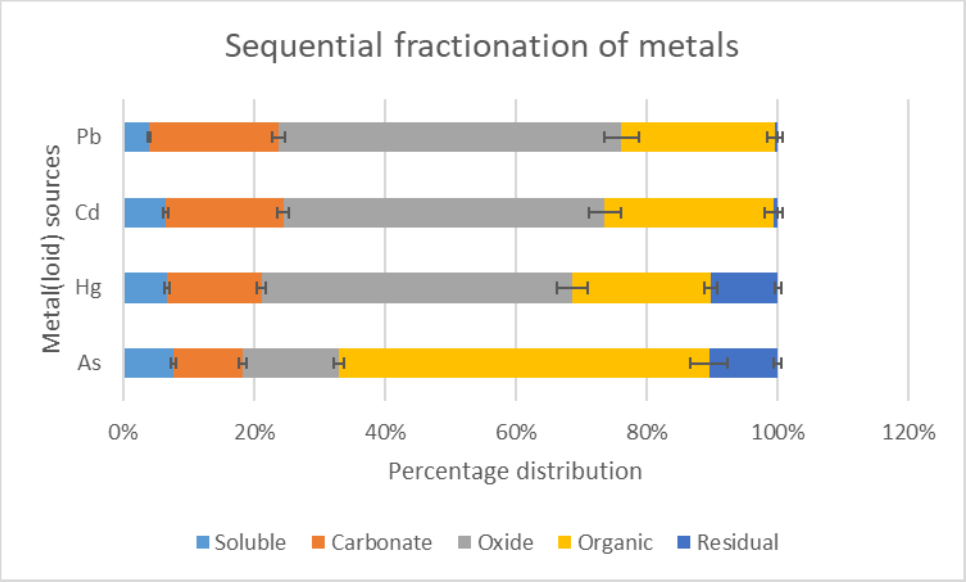


Figure 4.2 Speciation of metal(loid)s in various sources used in this study as measured by sequential fractionation

4.6.3 Bioaccessibiity of metal(loid)s

Generally, the metal(loid) extracted by the gastric solution was greater than that extracted by the intestinal solution for both reference compounds and the respective metal(loid) sources (Table 4.7; Figure 4.3). In the gastric phase (pH 1.5), the solubility of all the standard reference samples was close to 100%. pH is the dominant factor which controls the solubility of most metal(loid)s in aqueous solutions. In the metal(loid) source samples tested, the absolute bioaccessibility of As, Cd, Pb and Hg was 43.2, 53.2%, 61.7% and 72.3%, respectively following gastric phase dissolution (Table 4.7).

Table 4.7 Absolute and relative bioaccessibility of metal(loid)s in Montana Soil (Reference material; SRM 271), standard metal(loid) samples, and test samples. (values are mean ± standard deviation of triplicate)

Metal sources	Absolute bioaccessibility*		Relative bioaccessibility*	
	Gastric	Intestinal	Gastric	Intestinal
Reference soil				
Montana soil (SRM 271) - As	21.7 ± 1.03	11.4 ± 0.87	21.42 ± 1.78	12.08 ± 1.53
Montana soil (SRM 271) - Cd	18.6 ± 2.15	4.13 ± 0.34	18.36 ± 1.34	5.33 ± 0.78
Montana soil (SRM 271) - Pb	25.1 ± 2.36	3.36 ± 0.21	25.51 ± 1.98	4.34 ± 0.68
Montana soil (SRM 271) - Hg	17.5 ± 1.76	5.15 ± 0.52	17.00 ± 1.56	7.69 ± 0.75
Test samples				
Rice grain (As)	43.2 ± 3.76	34.6 ± 2.13	43.87 ± 2.31	37.36 ± 2.87
Offal (Cd)	53.2± 4.32	8.14 ± 0.24	54.08 ± 2.38	10.66 ± 1.32
Fish (Hg)	72.3 ± 4.23	14.6 ± 1.57	73.46 ± 3.45	20.28 ± 1.89
Complementary medicine (Pb)	61.7 ± 5.31	6.13 ± 0.54	61.00 ± 4.16	9.23 ± 1.23
Standard samples				
Arsenic oxide	98.4 ± 3.45	91.4 ± 3.45	NA	NA
Cadmium acetate	98.3 ± 4.54	75.6 ± 4.12	NA	NA
Mercuric chloride	98.7 ± 4.35	69.3 ± 3.43	NA	NA
Lead acetate	100.2 ± 0.03	65.4 ± 3.56	NA	NA

**Absolute bioaccessibility (%) = (in vitro metal(loid)/ total metal(loid)) × 100*
where, in vitro metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by gastric phase or intestinal phase solutions, total metal(loid) = total metal(loid) content in the source material or reference metal sample added to the in vitro assay.

Relative metal(loid) bioaccessibility (%) = ($AB_{metal(loid)\ source}$ /$AB_{meta(loid)l\ ref}$) x 100
where, $AB_{metal(loid)\ source}$ = absolute bioaccessibility for metal(loid) source and $AB_{metal(loid)\ ref}$ = absolute bioaccessibility for reference metal(loid) compound.

When the *in vitro* solution was changed to the intestinal phase (pH 5.8), the solubility of As, Cd, Pb and Hg in the respective reference compounds decreased to 91.4%, 75.6%, 69.3% and 65.4% (Table 4.7). A similar significant decrease in soluble metal(loid) concentration was noticed for the metal(loid) sources tested. Correspondingly, absolute bioaccessibility in the intestinal phase decreased to 34.6%, 8.14%, 6.13% and 14.6% for As, Cd, Pb and Hg, respectively (Table 4.7).

Numerous *in vitro* assays used to assess metal(loid) bioaccessibility indicate gastric bioaccessibility of metal(loid)s in various sources including As in rice grains, Cd in offal, Pb in complimentary medicines and soil, and Hg in fish meal ranges from 21.3% to 56.7%, from 13.6% to 67.5%, from 7.18% to 72.5% and from 32.5% to 87.9%, respectively (Ruby et al., 1996; Juhasz et al., 2006; He and Zheng, 2010; Satarug et al., 2010; Torres-Escribano et al., 2010; Chunhabundit et al., 2011; Koch et al., 2011; Ouédraogo and Amyot, 2011; He et al., 2012; Signes-Pastor et al., 2012; Fu and Cui, 2013; Wang et al., 2013; Tyokumbur, 2016; Bolan et al., 2017a; Bradley et al., 2017). As metal(loid) dissolution in these samples is influenced by composition and mineralogy, metal(loid) bioaccessibility is likely to be influenced by the source of metal(loid) contamination. For example, Juhasz et al. (2016) have indicated that the dissolution of Pb carbonate and sulphide minerals such as galena and anglesite are likely to be low compared to the soluble Pb oxides such as cerussite and manganese-Pb oxides. Lead mineralogy in the shooting range field soil used in their study indicates the presence of both discrete Pb minerals such as cerussite, hydrocerussite, galena, and anglesite, and Pb sorbed by iron oxy-hydroxides. Razić et al. (2008), Jayawardene et al. (2010), Koch et al. (2011), and Bolan et al. (2017a) have measured a wide range in the bioaccessibility of As, Cd, Hg and Pb in Ayurvedic and Chinese traditional medicines, which they attributed to the nature of therapeutic compounds such as added to these medicines. Similarly, Wang et al. (2013) have shown that *in vitro* total Hg bioaccessibility ranged from 21% to 52%, and MeHg bioaccessibility ranged from 20% to 59% from raw seafood in Hong Kong. *In vitro* total Hg bioaccessibility from 16 raw seafood species including swordfish in Spain ranged from 35% to 106% (Calatayud et al., 2012b). *In vitro* Hg bioaccessibility from 10 raw seafood species in Montreal ranged from 50% to 100% (Siedlikowski et al., 2016). These results document substantial variability in Hg bioavailability from seafood.

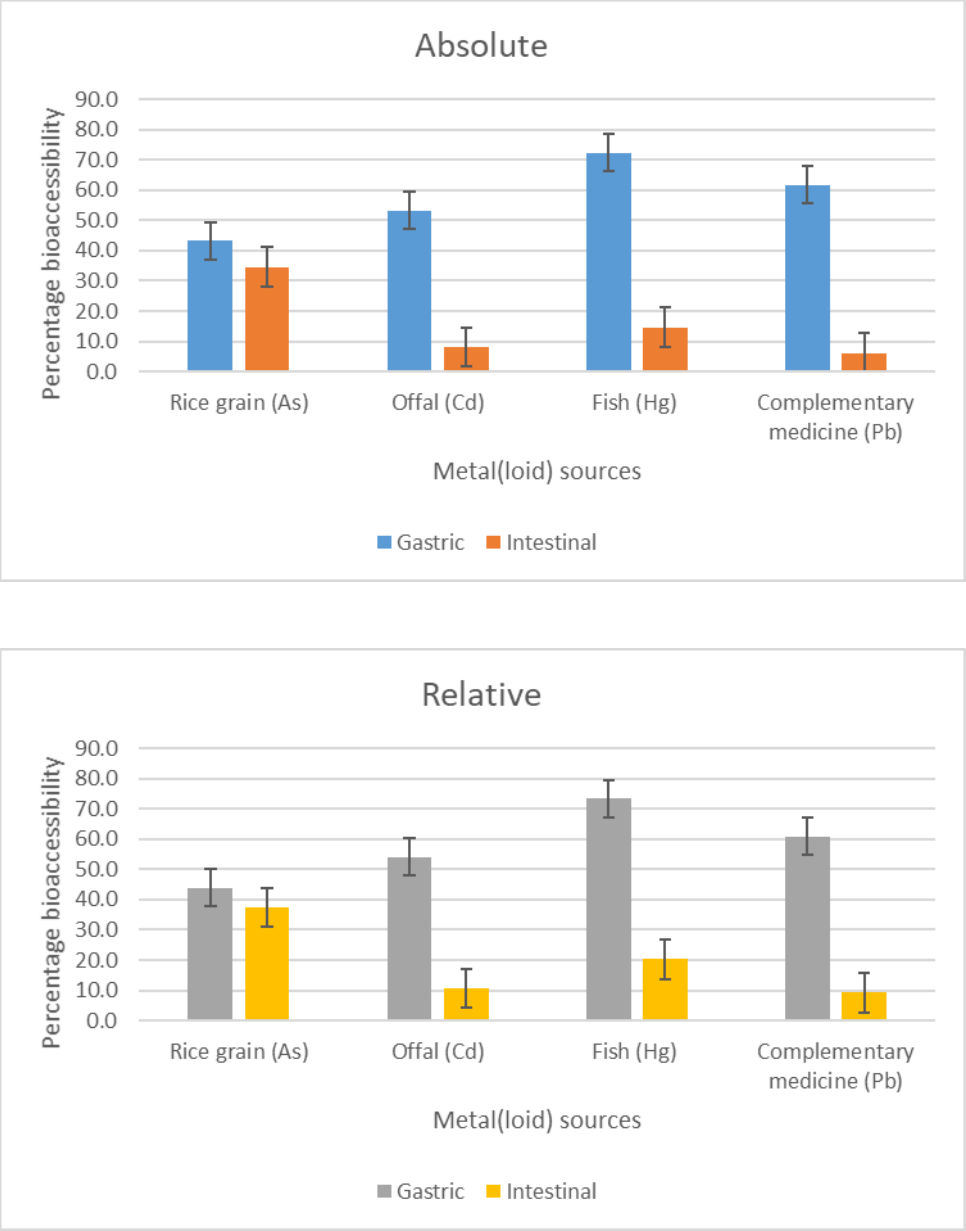


Figure 4.3 Absolute and relative bioaccessibility of various metal(loid) sources in gastric and intestinal extractions

The reduction of measured metal(loid) bioaccessibility between gastric and intestinal phases can be related to the decreased solubility of metal(loid)s such as Pb, Cd and Hg in the higher pH of the intestinal solution compared with the gastric phase (pH 5.8 vs. 1.8). For example, it has been observed that a major proportion of solubilized Pb in the gastric phase is readsorbed onto soil components or precipitated at the neutral intestinal phase pH, thereby reducing the bioaccessibility in the intestinal phase (Jayawardene et al., 2010; Juhasz et al.,

2016; Boros et al., 2017). In fact, Pb intestinal bioaccessibility values have been observed to be 2- to 45-times lower than gastric Pb bioaccessibility values (Juhasz et al., 2016).

In the gastric phase, the relative bioaccessibility was comparable to absolute bioaccessibility (Table 4.7). However, in the intestinal phase, the relative bioaccessibility was slightly higher than the absolute bioaccessibility, which is attributed to the decrease in the solubility of reference standard metal(loid) compounds at the higher pH (pH 5.8) in the intestinal phase. Juhasz et al. (2016) observed that the relative Pb bioaccessibility values for soils in the intestinal phase (pH 6.5) estimated from the solubility of Pb acetate as a reference compound were approximately 10-fold greater than the corresponding absolute bioaccessibility values.

4.6.4 Distribution of metal(loid)s in the gastric and intestinal extracts

The distribution of solution metal(loid)s as free ions and complexes in the gastric and intestinal extracts is presented in Table 4.8 and Figure 4.4. The data indicate that the percentage of free metal(loid) ions is less in both gastric and intestinal extracts when compared to water extracts (data not shown). The gastric and intestinal extracts contain pepsin and bovine bile and porcine pancreatin. These compounds have been found to be very effective in complexing metal(loid)s (Ding et al., 2015). Metal(loid)s are well known to complex enzyme proteins, thereby inhibiting their functions (Kilpin and Dyson, 2013). For example, metallothioneins are a family of cysteine-rich, low molecular weight (MW ranging from 500 to 14000 Da) metelloproteins (Klaassen et al., 1999, 2009; Waalkes and Liu, 2009; Babula et al., 2012; Freisinger and Vašák, 2013). These protein compounds have the capacity to bind both metabolically essential (such as zinc, copper, selenium) and xenobiotic (such as cadmium, mercury, lead, arsenic) heavy metal(loid)s through the thiol group of its cysteine residues, which represent nearly 30% of its constituent amino acid residues (Rooney, 2007; Babula et al., 2012). The relative distribution of free and complexed metal(loid)s in gastrointestinal extracts may impact the bioavailability of metal(loid)s as measured by intestinal permeability test, which will be discussed in Chapter 7. The effects of gut microes and chelating agents on the relative distribution of free and complexed metal(loid)s in gastrointestinal extracts will be discussed in Chapter 5 and 6, respectively.

Table 4.8 Percentage of total solution concentration of metals as free metal ionic species in bioaccessibility tests in Montana Soil (Reference material; SRM 271), standard metal(loid) samples, and test samples. (values are mean ± standard deviation of triplicate)

Metal sources	Gastric		Intestinal	
	Free ionic	**Complexed**	**Free ionic**	**Complexed**
Reference soil				
Montana soil (SRM 271) - As	51.4± 3.45	48.6± 3.45	37.7± 6.52	62.3± 6.52
Montana soil (SRM 271) - Cd	21.3± 4.52	78.7± 4.52	17.8± 3.23	82.2± 3.23
Montana soil (SRM 271) - Pb	26.7± 4.12	73.3± 4.12	25.6± 3.56	74.4± 3.56
Montana soil (SRM 271) - Hg	32.8± 4.56	67.2± 4.56	35.4± 5.63	64.6± 5.63
Test samples				
Rice grain (As)	57.3± 7.61	42.7± 7.61	18.91± 6.23	81.09± 6.23
Offal (Cd)	47.6± 5.43	52.4± 5.43	6.72± 5.12	93.28± 5.12
Fish (Hg)	61.2± 4.59	38.8± 4.59	11.5± 6.73	88.5± 6.73
Complementary medicine (Pb)	34.3± 4.63	65.7± 4.63	8.19± 5.98	91.81± 5.98
Standard samples				
Arsenic oxide	73.4± 5.87	26.6± 5.87	45.6± 7.12	54.4± 7.12
Cadmium acetate	67.3± 5.67	32.7± 5.67	23.6± 4.78	76.4± 4.78
Mercuric chloride	72.1± 5.62	27.9± 5.62	27.6± 6.23	72.4± 6.23
Lead acetate	43.7± 7.12	56.3± 7.12	19.8± 4.23	80.2± 4.23

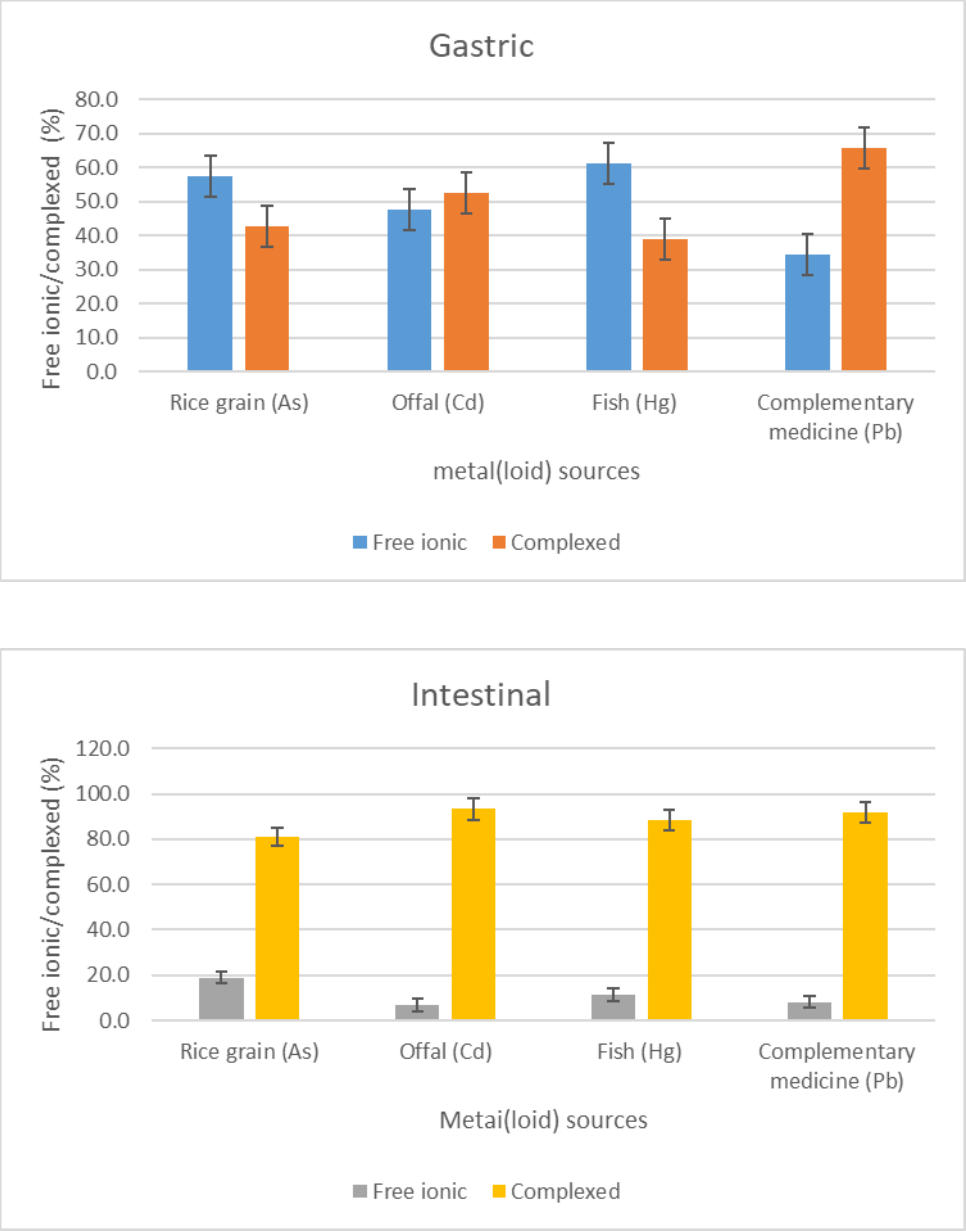


Figure 4.4 Percentage distribution of free ionic and complexed forms of metal(loid)s in various sources in gastric and intestinal extractions

A number of schemes have been presented to describe the types of interactions that can occur between metal(loid)s (M), enzyme proteins (E) and substrate (S) resulting in metal(loid) complex formation (Kilpin and Dyson, 2013). The first of these represents an interaction between the substrate and the metal(loid) ion to form a complex that acts as the true substrate. Substrate-metal(loid) complexation can occur prior or subsequent to the formation of the

enzyme-substrate complex (M + S = MS; E + MS = EMS). This type of behaviour is typically observed with metal(loid)-activated enzymes. The second scheme indicates that the metal(loid) first binds to the protein and then serves as a site of interaction with substrate (E + M = EM; EM + S = EMS). In this instance, the metal(loid) can function either as a binding site, as a component of the catalytic apparatus of the enzyme or both. A third scheme would have the metal(loid) acting at a site on the enzyme remote from the active site (E + M = ME; ME + S = MES). In such instances, the metal(loid) could either serve to maintain protein structure and only influence catalytic activity indirectly or else it could regulate activity by stabilizing more or less active conformations of the protein. The latter situation would more likely pertain for metal(loid)-activated enzymes where the metal(loid)-protein interaction is more readily controlled by manipulation of the ambient metal(loid) ion concentration.

4.7 Conclusions

Bioaccessibility of contaminants, including heavy metal(loid)s, underpins their bioavailability and toxicity to biota including microorganisms and human being (Figure 2.3). The intestinal bioaccessibility of As, Cd, Pb and Hg was less than that of gastric bioaccessibility, the difference was less pronounced when bioaccessibility was expressed as relative bioaccessibility than absolute bioaccessibility. Majority of the metal(loid)s extracted in gastric and intestinal extracts was present as metal(loid) complexes. The distribution of metal(loid)s in the gastric and intestinal extracts will have implications on their bioavailability as measured by intestinal permeability which will be discussed in Chapter 7.

Chapter 5

BIOACCESSIBILITY OF HEAVY METAL(LOID)S AS IMPACTED BY GUT MICROBES

5.1 Introduction

The human gut microbiota is a composite structure of more than 1,000 species-level phylotypes with distinct bacterial species that reside in the human digestive tract (Li et al., 2012; Pflughoeft and Versalovic 2012; Weinstock 2012; Thursby and Juge, 2017; Cani, 2018). Fungi, protozoa, and archaea are found in lesser numbers than bacteria and constitute the rest of the gut microbiota. The normal gastrointestinal microbiota aid and facilitate numerous functions within the gut and in exogenous sites, that include digestion, immune system modulation and barrier effects, and synthesis and modification of antibiotics and vitamins (HMPC, 2012; Pflughoeft and Versalovic, 2012; Thursby and Juge, 2017). Gut microbes also play a critical role in the transformation and bioavailability of contaminants including heavy metal(loid)s (Rowland et al., 1975, 1981; Carding et al., 2015; Claus et al., 2016; Jin et al., 2017; Tasmin et al., 2017). For example, Van de Wiele et al. (2010) have shown that the bioavailability of As ingested through As-contaminated rice is likely to be altered as it moves through the digestive system. The ability of the human microbiome to methylate and demethylate As is important due to the implications for the toxicity profile of As and the chronic health outcomes related to As exposure. Similarly, Laird et al. (2013) have demonstrated the ability of the human microbiome to methylate inorganic As, which also has implications surrounding the current regulations of As levels. Adsorption to the bacterial cell wall (extracellular polysaccharides) also controls the bioaccessibility and bioavailability of metal(loid)s including Pb, Cd, As and Hg (Jarosławiecka and Piotrowska-Seget, 2014). However, most of the bioaccessibility tests have been carried out in the absence of gut microbes (Naidu et al., 2008; Juhasz et al., 2016).

The amount of metal(loid) absorption into systemic circulation (the bioavailable fraction) depends on the nature and solubility of metal(loid) source (i.e., bioaccessibility) and the properties of the ingested compound (Naidu et al., 2008; Deshommes et al., 2012; Sun et al., 2012; Ruby et al., 2016). Bioaccessible metal(loid) concentrations are critical for health- and environmental-risk assessment, and hence bioaccessibility measurements are necessary for

quantifying human intake of heavy metal(loid)s from various sources for use in risk assessments to establish safe metal(loid)s threshold values (Naidu et al., 2008). Studies on metal(loid) bioaccessibility are often conducted using *in vitro* digestive techniques aiming to simulate the human digestive system (Van de Wiele et al., 2010; Laird et al., 2007, 2013; Wijayawardena et al., 2015; Juhasz et al., 2016). These tests, however, do not take into account the role of the human gut microbiome and its interactions with environmental contaminants (Naidu et al., 2008).

Gut microbes influence gastrointestinal bioaccessibility of heavy metal(loid) derived from oral ingestion by a number of ways. Firstly, gut microbes influence the solubilisation of heavy metal(loid) sources through secretion of organic acids. For example, *Lactobacillus* species have been shown to excrete lactic acid thereby increasing the solubilisation of metal(loid) sources (Monachese et al., 2012; Patel et al., 2018). Secondly, gut bacteria adsorb the metal(loid)s released from food sources, thereby reducing their concentration in gastric and intestinal solution. For example, a number of bacteria species have been shown to adsorb metal(loid)s including As, Cd, Hg and Pb (Monachese et al., 2012; Elsanhoty et al., 2016; Patel et al., 2018). Thirdly, gut microbes are involved in the transformation of metal(loid)s, thereby influencing the bioaccessibility and bioavailability of these metal(loid)s (Mayer and Godwin, 2006; Teemu et al., 2008; Gupta and Diwan, 2017).

5.2 Objectives

The overall objective of this work was to scrutinize the bioaccessibility of orally ingested As, Cd, Hg and Pb as impacted by gut microbes. The specific objectives of the study were to:

(i) Monitor the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb in various sources as impacted by gut microbes.

(ii) Monitor the distribution of various species of As, Cd, Hg and Pb in gastric and intestinal extractions.

(iii) Measure the adsorption of As, Cd, Hg and Pb by these microbes in relation to bioaccessibility.

5.3 Hypothesis

(i) Gut bacteria modulate bioaccessibility of metal(loid)s through their effects on the solubilisation of metal(loid) sources and subsequent interactions with metal(loid)s via adsorption and speciation.

5.4 Experiments

The major experiments and analyses conducted to test the hypothesis, and the treatments used in this chapter are listed in Table 5.1.

Table 5.1 The major experiments and treatments used in Chapter 5

No.	Title	Treatments
1	Bioaccessibility of metal(loid)s	3 gut bacteria (Table 5.2); 4 metal(loid) sources (4 reference metal(loid)s and 4 oral metal(loid) sources (Table 4.2 and 4.3); Gastric and intestinal bioaccessibility tests
2	Adsorption of metal(loid)s	3 gut bacteria (Table 5.2); 4 metal(loid)s sources (Table 4.3)
3	Speciation of metal(loid)s	3 gut bacteria (Table 5.2); 4 metal(loid) sources (4 reference metal(loid)s and 4 oral metal(loid) sources (Table 4.2 and 4.3); Gastric and intestinal bioaccessibility extractions
4	FTIR	3 gut bacteria (Table 5.2); 1 metal(loid) (Pb)
5	TEM	3 gut bacteria (Table 5.2); 1 metal(loid) (Pb)

5.5 Materials and Methods

5.5.1 Bacterial cultures and metal(loid) sources

The effect of gut bacteria on the bioaccessibility of As, Cd, Pb and Hg sources was examined using selected gut microbes that include *Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) (Table 5.2). In this study, these three bacterial species were used based on their predominance in the gut, differences in their pH optimum in the gut and their location in various parts of the human gut. Subcultures of *Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) were inoculated from their respective mother cultures purchased from American Type Culture Collection (ATCC, Melbourne; https://www.atcc.org/). The media used

for the subculturing of the bacterial species and their preparation protocols employed for this study are described in Section 3.5.1 (Chapter 3).

Table 5.2 Gut bacteria used in this study

Bacteria	Family	Primary location	Optimum pH	Cell wall
Lactobacillus acidophilus	Lactobacillaceae	Mouth and stomach	3-5	Gram positive
Lactobacillus rhamnosus	Lactobacillaceae	Large intestine	5-6	Gram positive
Escherichia coli	Enterobacteriaceae	Large intestine (lower)	6-7.5	Gram negative

The metal(loid) sources included in this study are the common orally ingested sources, and are based on their heavy metal(loid) content derived from literature data (Table 4.2). The metal(loid) sources include offal pet food (cadmium), fish meal (mercury), complementary medicine (lead) and rice grain (arsenic) (Table 4.3), which are described in Section 4.5.1 (Chapter 4). Arsenic oxide, Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3).

5.5.2 *In vitro* gastrointestinal bioaccessibility tests

Bioaccessible metal(loid) contents in various metal(loid) sources were measured following the *in vitro* gastrointestinal (IVG) method by Rodriguez et al. (1999) which involves a two-step sequential extraction: a gastric followed by an intestinal extraction. Arsenic oxide Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3). The *in vitro* gastrointestinal bioaccessibility of these reference compounds were used to estimate the relative bioaccessibility of the metal(loid) sources.

The gastrointestinal bioaccessibility test is described in Section 4.5.4 (Chapter 4). The gastrointestinal extraction was carried out with the addition of 1 mL of broth with and without (control) bacterium. A negative control without the addition of broth was also used to determine the effect of broth on bioaccessibility and speciation of metal(loid). Both gastric and intestinal phase samples were filtered through 0.2 μm syringe filters for metal(loid) analysis by ICP-MS.

5.5.3 Adsorption of metal(loid)s

The non-metabolic interaction (i.e., adsorption) between metal(loid)s and gut microbes was investigated using batch adsorption studies. Bacterial cells were cultured anaerobically at 37°C in LB broth and (*E. coli)* and MRS broth (*Lactobacillus* sp.) and incubated for 24 h in a shaking incubator at 120 rpm. After incubation, the bacteria were collected by centrifugation at 7000 rpm, and then rinsed 5 times with a sterile 0.I M $NaClO_4$ electrolyte solution. The extent of metal(loid) adsorption by bacteria was quantified using batch experiments where 100 mg of bacterial pellet was mixed with 10 mL of metal(loid) solution ranging in concentrations from 0 to 1000 mg/L. The ionic strength in all experiments was maintained using 0.1 M $NaClO_4$. The solution pH adjusted to a specified initial pH, ranging from pH values of 1.5 to 7.5 (covering the gastric and intestinal pH), using HCl or NaOH, and mixed thoroughly by end-over-end rotation at 24 rpm for two hours. The bacterial suspension was centrifuged, and the supernatant solution was filtered through a 0.45 µm nylon syringe filter. The filtered solution was stored at 4°C prior to analysis for total dissolved metal(loid) and free ionic metal(loid) species. The concentration of dissolved metal(loid) was determined using ICP-MS with matrix-matched standards. Bacteria-free controls were included using the same concentrations of background electrolyte and metal(loid). The controls exhibited virtually no metal(loid) removal indicating that any metal(loid) lost from solution during experiments was due solely to adsorption by the bacteria. Metal(loid) adsorption was calculated using Eq. 5.1.

$$X = (C_i - C_e) \times V/M \qquad (5.1)$$

where X = amount of metal(loid) adsorbed (mg metal(loid)/g bacteria), C_i = metal(loid) input concentration (mg/L), C_e = Metal(loid) equilibrium concentration (mg/L), M = mass of bacterial pellet (g) and V = volume of solution (L).

5.5.4 Distribution of free and complexed metal(loid)s

The distribution of free and complexed metal(loid)s in the gastric and intestinal extracts was measured using cation/anion-exchange resin cartridge (Empore, iminodiacetate functionalized poly(styrene divinylbenzene) - 234877 Aldrich) (Pu and Fukushi, 2013). The method for the measurement of distribution of free and complexed metal(loid)s in the gastric and intestinal extracts is described in Section 3.5.6 (Chapter 3).

5.5.5 FTIR

Since highest adsorption (X_m) values were observed for Pb adsorption by all gut bacteria species, the FTIR spectra and TEM observations of the bacterial biomass with and without Pb loading were analysed. Bacterial moist pellets were used to obtain ATR-FTIR spectra on an Agilent Cary 600 Series FTIR spectrometer instrument using a Germanium detector. The scan conditions were 2000 scans with a 4 cm^{-1} resolution; the scans were collected and averaged for each sample. The slit opening for the incident beam was set at 4 mm. The Ge-60 (refractive index = 4.0 and incident angle = 60°), ATR crystals used for collecting infrared spectra were cleaned with water, and the samples were spread to cover the entire crystal surface. The spectra for the bacterial growth medium were used to correct the spectral background.

5.5.6 Transmission and scanning electron microscopy (TEM)

The distribution of Pb on gut microbes after adsorption experiments was monitored using TEM (Dunham-Cheatham et al., 2011). Bacterial cultures after the adsorption experiments were spun at 3000 rpm for 10 mins. Supernatant was removed and 1 mL of modified Karnovsky's fixative was added to bacterial cultures and were mixed gently with a thin spatula. The cultures were left overnight at 4°C, and washed twice with 0.1 M cacodylate buffer. The cultures were then postfixed in 1% osmium tetroxide for 1 hour at room temperature, and then washed twice with deionised water. The samples were dehydrated by passing through a series of alcohol washings. The dehydrated pellet was suspended in a series of Spurs resin solutions, enabling infiltration of the bacteria by the resin. The infiltrated pellet was placed in the tip of a 1 mL BEEM capsule, and the capsules were filled with 100% resin and placed in a 70°C oven for 24 h. The sample blocks were removed from the capsules, sectioned by ultramicrotomy to a 110 nm thickness, and mounted onto 200 mesh copper grids. The grids were stained with uranyl acetate, and TEM images were collected using a Hitachi H-600 TEM operated at 75 kV acceleration voltage, as well as a JEOL 2100F TEM operated at 200 kV using various modes: bright field (BF), dark field (DF), and scanning TEM (STEM). Chemical maps were determined by an electron dispersive X-ray (EDX) detector using the K line for Pb using the JEOL 2100F TEM.

5.5.7 Data analysis

Both absolute (AB) and relative (RB) metal(loid) bioaccessibility values for gastric and intestinal phases were calculated using Eqs. 4.1 – 4.4 described in Section 4.5.6 (Chapter 4). Metal(loid) adsorption followed Langmuir adsorption isotherm (Eq. 5.2).

$$X = 1/[(1/X_m) + (1/C*X_m b)] \quad (5.2)$$

Where, 'X' is uptake of metal(loid) (mg/g); 'C' is equilibrium concentration of metal(loid) in solution (mg/L); 'X_m' is the maximum uptake (mg/g); and 'b' is binding constant (L/mg).

All experimental analyses were carried out using three replications. Statistical comparisons were made using analysis of variance (ANOVA) in Predictive Analytics SoftWare (PASW) statistics (version 18.0.0; SPSS, Inc., 2009, Chicago, IL) in order to examine the significant differences in various treatments. Duncan's multiple range test was also employed to compare the means of various treatments; variability in the data was presented as the standard deviation and a $p < 0.05$ was considered statistically significant.

5.6 Results and discussion

5.6.1 Bioaccessibility of metal(loid)s

The metal(loid) extracted by the gastric solution was greater than the metal(loid) extracted by the intestinal solution for the reference compounds such as arsenic oxide, cadmium acetate, lead acetate and mercuric chloride, and also for the respective metal(loid) sources both with and without gut bacteria (Table 5.3). As discussed in Chapter 4, the reduction of measured metal(loid) bioaccessibility between gastric and intestinal phases can be related to the decreased solubility of metal(loid)s in the higher pH of the intestinal solution compared with the gastric phase (pH 5.8 vs. 1.8). It has been observed that a major proportion of solubilized metal(loid) in the gastric phase is readsorbed onto bacterial growth media components or precipitated at the neutral intestinal phase pH, thereby reducing the bioaccessibility in the intestinal phase (Jayawardene et al., 2010; Juhasz et al., 2016; Boros et al., 2017).

Table 5.3 Absolute bioaccessibility of metal(loid)s (values are mean ± standard deviation of triplicate)

Metal(loid) sources	Gastric/ Intestinal phase	Absolute control (no bacteria/no broth)	Control (only broth – no bacteria)	With *L. acidophilus*	With *L. rhamnosus*	With *E. coli*
Arsenic oxide	Gastric	98.4±3.3	94.1±2.6	87.3±1.9	84.6±2.2	72.1±1.6
Rice grain		43.3±1.3	40.5±1.3	35.6±1.1	33.7±1.2	29.4±1.1
Arsenic oxide	Intestinal	91.2±3.2	87.2±2.5	81.1±2.3	78.4±2.1	66.3±1.7

Rice grain		34.5±1.1	32.3±1.3	28.4±0.9	26.2±1.1	21.7±0.8
Cadmium acetate	Gastric	98.3±3.5	94.7±3.2	72.6±1.7	75.8±1.9	86.1±2.1
Offal		53.3±2.6	49.3±2.8	33.4±1.9	36.1±2.1	45.5±2.5
Cadmium acetate	Intestinal	75.6±3.2	71.5±3.1	59.6±2.4	61.2±2.5	69.3±2.7
Offal		8.19±0.17	6.32±0.14	3.51±0.12	3.86±0.12	4.78±0.13
Lead acetate	Gastric	100.2±4.8	95.7±3.7	76.5±2.9	78.1±3.1	92.4±3.6
Complementary medicine		62.2±2.5	58.6±2.3	47.8±1.8	49.1±1.9	53.6 ±2.4
Lead acetate	Intestinal	65.3±2.6	61.4±2.3	51.2±2.1	52.5±1.8	56.1±2.2
Complementary medicine		6.14±0.15	4.53±0.13	2.27±0.08	2.41±0.09	3.63±1.11
Mercuric chloride	Gastric	98.8±4.3	94.3±4.1	75.1±3.5	77.3±3.6	90.4±4.2
Fish		72.6±3.6	68.1±3.9	56.3±2.5	57.8±2.3	62.6±3.7
Mercuric chloride	Intestinal	69.5±4.1	65.4±3.8	54.5±3.5	56.1±3.2	60.2±3.7
Fish		14.7±1.1	11.2±0.9	7.3±0.6	7.8±0.7	9.1±0.8

Absolute bioaccessibility (%) = (in vitro metal(loid)/ total metal(loid)) × 100
where, in vitro metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by gastric phase or intestinal phase solutions, total metal(loid) = total metal(loid) content in the source material or reference metal sample added to the in vitro assay.

In the present study, while the gastric bioaccessibility of the metal(loid)s decreased slightly with the addition of bacterial growing medium alone in the absence of gut bacteria, there was no significant effect of growing medium on the intestinal bioaccessibility (Table 5.3; Figure 5.1). However, there was a significant decrease in both gastric and intestinal bioaccessibility with the addition of bacterial growing medium in the presence of gut bacteria, and the effect was more pronounced for the gastric bioaccessibility. The effect of gut bacteria on the intestinal and gastric bioaccessibility varied between the bacterial and metal(loid) species. The bioaccessibility of metal(loid)s in the presence of bacteria, decreased in both gastric and intestinal phases in the following order: *L. acidophilus>L. rhamnosus>E. coli.* The bacterial-induced reduction in the bioaccessibility followed: Pb > Cd > Hg > As (Figure 5.1).

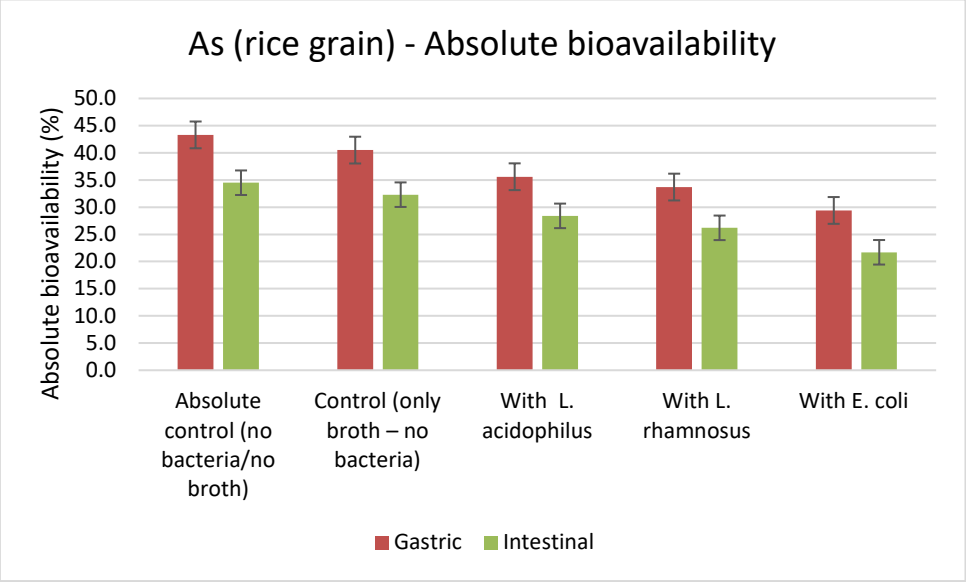
In the gastric phase, the relative bioaccessibility was comparable to absolute bioaccessibility both in the presence and absence of gut microbes (Table 5.4). However, in the intestinal phase, the relative bioaccessibility was slightly higher than the absolute bioaccessibility, which is attributed to the decrease in the solubility of the metal(loid) salts at the higher pH (pH 5.8) in the intestinal phase. For example, Juhasz et al. (2016) observed that

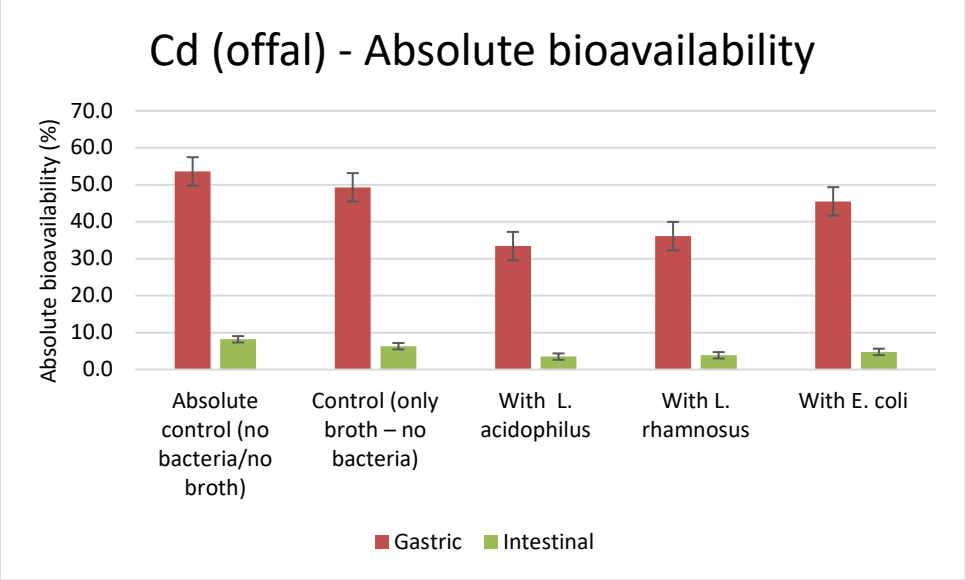
the relative Pb bioaccessibility values for soils in the intestinal phase (pH 6.5) estimated from the solubility of Pb acetate as a reference compound were approximately 10-fold greater than the corresponding absolute bioaccessibility values.

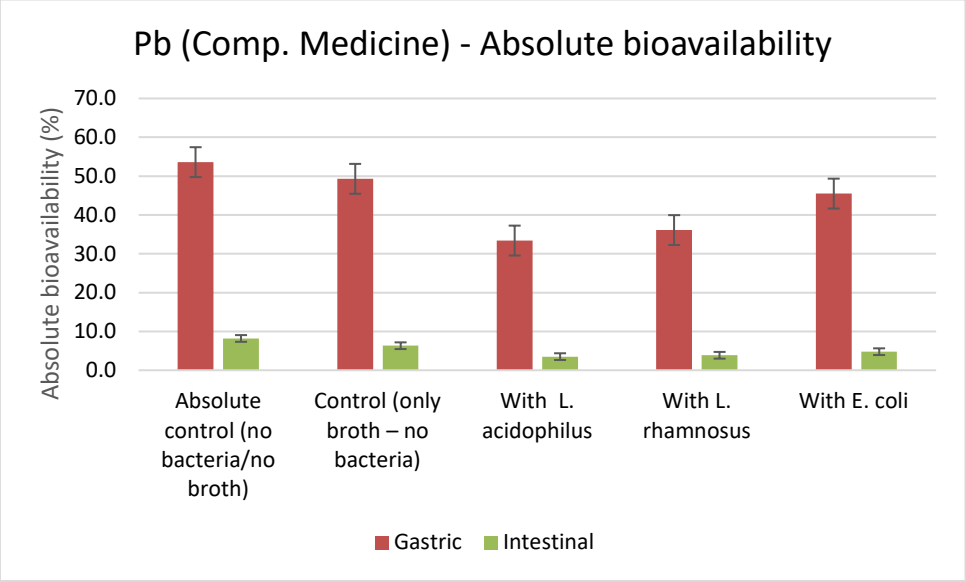
Table 5.4 Relative bioaccessibility of metal(loid)s (values are mean ± standard deviation of triplicate)

Metal(loid) source	**Gastric/ Intestinal phase**	**Absolute control (no bacteria/no broth)**	**Control (only broth – no bacteria)**	**With *L. acidophilus***	**With *L. rhamnosus***	**With *E. coli***
Arsenic	Gastric	44.0±2.6	43.0±2.5	40.8±2.2	39.8±2.3	40.8±2.1
	Intestinal	37.8±2.1	37.0±1.9	35.0±1.9	33.4±1.6	32.7±1.4
Cadmium	Gastric	54.2±3.1	52.1±2.9	46.0±2.7	47.6±2.8	52.8±3.3
	Intestinal	10.8±0.14	8.84±0.11	5.89±0.09	6.31±0.11	6.90±0.11
Lead	Gastric	62.1±3.7	61.2±3.6	62.5±3.6	62.9±3.3	58.0±3.4
	Intestinal	9.40±0.16	7.38±0.14	4.43±0.08	4.59±0.08	6.47±0.09
Mercury	Gastric	73.5±4.3	72.2±4.1	75.0±4.4	74.8±4.4	69.2±4.1
	Intestinal	21.2±1.6	17.1±1.4	13.4±1.6	13.9±1.3	15.1±1.6

Relative metal(loid) bioaccessibility (%) = ($AB_{metal(loid)\ source}$ /$AB_{meta(loid)l\ ref}$) x 100
where, $AB_{metal(loid)\ source}$ = absolute bioaccessibility for metal(loid) source and $AB_{metal(loid)\ ref}$ = absolute bioaccessibility for reference metal(loid) compound.


As (rice grain) - Absolute bioavailability
Absolute bioavailability (%)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Absolute control (no bacteria/no broth)
Control (only broth – no bacteria)
With L. acidophilus
With L. rhamnosus
With E. coli
Gastric
Intestinal


Cd (offal) - Absolute bioavailability
Absolute bioavailability (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Absolute control (no bacteria/no broth)
Control (only broth – no bacteria)
With L. acidophilus
With L. rhamnosus
With E. coli
Gastric
Intestinal



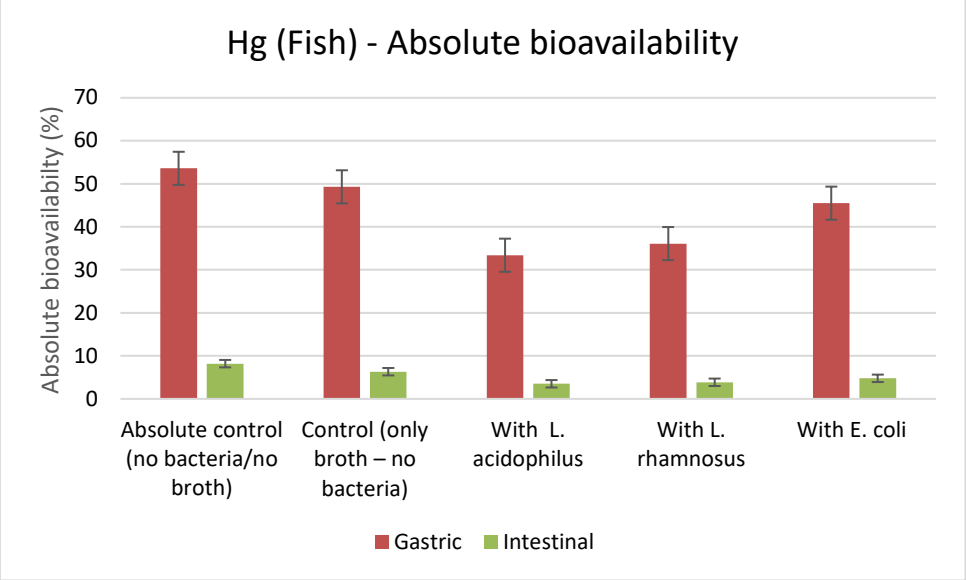


Figure 5.1 Effect of gut bacteria on bioaccessibility of metal(loid)s

Although a vast number of studies have examined the effect of gut microbes on nutrient absorption (Krajmalnik-Brown et al., 2012), there have been only limited studies on the effect of gut microbes on metal(loid) bioaccessibility. Probiotics have been shown to reduce the absorption of metal(loid) contaminants into human blood circulation (Monachese et al., 2012; Zhai et al., 2015, 2016). The solubility and intestinal absorption of metal(loid)s including Fe, Cd, and Pb have been shown to be decreased by dietary constituents such as oxalates,

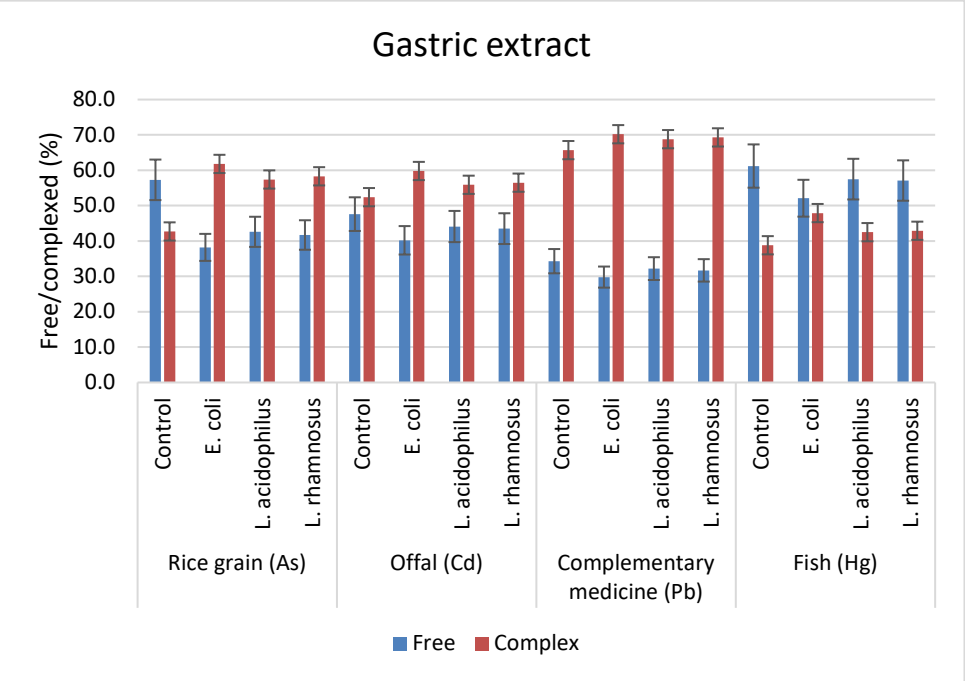
phosphates, and phytates (Diamond et al., 1998; Monachese et al., 2012). Gastrointestinal absorption of metal(loid)s is a complex process involving dissolution, absorption, and interactions with other dietary components (Diamond et al., 1998; Moser and McLachlan, 2002; Kilpin and Dyson, 2013). The microbial-induced decrease in bioaccessibility of metal(loid)s may lead to a decrease in its bioavailability. For example, Breton et al. (2013b) using germ-free mice confirmed the essential role of intestinal microbes to limit the absorption of Pb and Cd. The study found that in mice with intact microbiomes, exposure to Pb and Cd resulted in 5-10 times lower concentrations in tissue such as kidney and liver than mice without gut microbiota, indicating that the intestinal microbes decrease the bioavailability of heavy metal(loid)s. One of the implications of this observation is that lacking intestinal microbiota creates a more susceptible environment for the accumulation of heavy metal(loid)s into the blood and hence organs.

5.6.2 Distribution of metal(loid)s in the gastric and intestinal extracts

The distribution of solution metal(loid)s as free ions and complexes as impacted by gut microbes is presented in Table 5.5. The data indicate that the percentage of free metal(loid) ions is decreased with the addition of bacterial growing medium, and the effect was more pronounced in the presence than absence of gut microbes. Based on the bioaccessibility tests, the metal(loid)s distribution as free and complexed ions in gastric and intestinal phases varied between the control and bacterial species (Table 5.5; Figure 5.2). Among all the bacterial species, *E. coli* adsorbed more metal(loid) ions and hence the complexed ions were higher than the other two bacterial species. In terms of metal(loid)s, Pb was found to be high in the complexed form. The reduction in free metal(loid) concentration in solution was higher when metal(loid)s were added as a salt solution than those derived from the oral ingestion sources. Although the change in the distribution of free metal(loid) ions in solution caused by the presence of bacteria may not impact the bioaccessibility of metal(loid)s, it is likely to reduce the bioavailability of metal(loid)s because cellular uptake of most metal(loid)s occurs as free metal(loid) ions (Zhao et al., 2005; Dean et al., 2012; Bird, 2015). The effect of the distribution of metal(loid)s on their bioavailability as measured by intestinal permeability will be discussed in Chapter 7.

Table 5.5 Percentage of total solution concentration of metal(loid)s as free metal(loid) ionic species in bioaccessibility tests

Metal(loid) source	Gut bacteria	Gastric phase (%)		Intestinal phase (%)	
		Free ion	Complexed	Free ion	Complexed
Rice grain (As)	Control	57.3	42.7	18.9	81.1
	E. coli	38.2	61.8	14.2	85.8
	L. acidophilus	42.6	57.4	16.2	83.8
	L. rhamnosus	41.7	58.3	16.1	83.9
Offal (Cd)	Control	47.6	52.4	6.7	93.3
	E. coli	40.2	59.8	5.7	94.3
	L. acidophilus	44.1	55.9	6.2	93.8
	L. rhamnosus	43.5	56.5	5.9	94.1
Complementary medicine (Pb)	Control	34.3	65.7	8.1	91.9
	E. coli	29.8	70.2	6.3	93.7
	L. acidophilus	32.2	68.8	7.4	92.6
	L. rhamnosus	31.7	69.3	7.2	92.8
Fish (Hg)	Control	61.2	38.8	11.5	88.5
	E. coli	52.1	47.9	8.6	91.4
	L. acidophilus	57.5	42.5	9.3	89.7
	L. rhamnosus	57.1	42.9	9.3	89.7



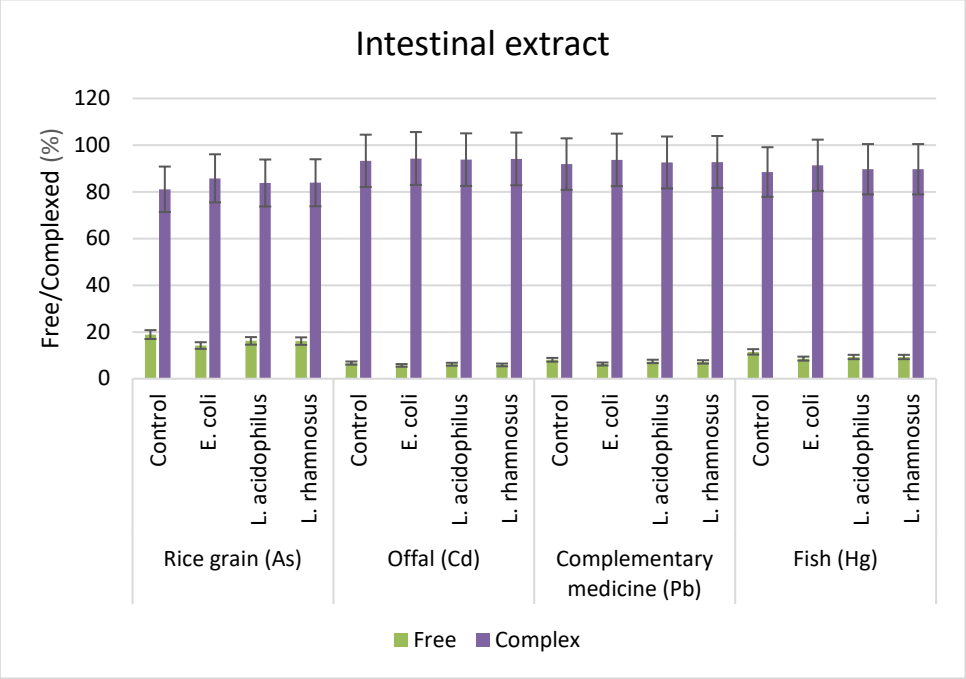


Figure 5.2 Effect of gut bacteria on the distribution of metal(loid)s (free ionic and complexed forms) in gastric and intestinal solutions

5.6.3 Metal(loid) adsorption

Metal(loid) adsorption followed Langmuir adsorption isotherm (Eq. 5.2) (Table 5.6). The maximum adsorption as indicated by X_m (Eq. 5.2) varied between both metal(loid) and bacterial species. However, the binding constant varied only amongst the metal(loid)s, and there was no effect of gut bacteria on this parameter. The maximum adsorption of metal(loid)s by bacteria followed: Pb > Cd > Hg > As (Figure 5.3). In general, *E. coli* adsorbed much more metal(loid)s than did *Lactobacillus* species. The difference in adsorption among the bacterial species increased with increasing pH, especially for Pb, Cd and Hg (Figure 5.3).

The X_m value for Pb, Cd and Hg increased with increasing pH, while it decreased for As (Figure 5.3). Various reasons have been attributed for the effect of pH on adsorption of metal(loid)s by microorganisms. Firstly, an increase in pH results in an increase in net surface negative charge in variable-charge surfaces, thereby increasing adsorption of cationic species such as Pb^{2+}, Cd^{2+} and Hg^{2+}, and decreasing the adsorption of anionic species such as As(V) (Dickson and Koohmaraie, 1989; Naidu et al., 1994; Mahimairaja et al., 2005; El Badawy et al., 2010; Krulwich et al., 2011). pH is the most important factor which controls surface properties of variable charge components including bacteria (Djeribi et al., 2013). In bacteria, the net

negative charge increases with pH due to the dissociation of protons (H^+) from weakly acidic functional groups (Kinnari et al., 2009). Secondly, an increase in pH results in hydroxyl species of cations such as Pb, Cd and Hg, which are preferentially adsorbed. For example, Perera et al. (2001) noticed that Pb forms four hydroxy species and these mononuclear complexes are likely to be important under typical environmental and biological conditions. Thirdly, precipitation of cationic species such as Pb and Cd as their respective hydroxide [$Pb(OH)_2$ or $Cd(OH)_2$] causes greater retention of these cationic species at high pH (Lee and Saunders, 2003).

Table 5.6 Parameters of Langmuir adsorption isotherms for gut bacteria

Metal(loid) source	**Parameters***	***Escherichia coli***	***Lactobacillus rhamnosus***	***Lactobacillus acidophilus***
As	X_m (mg/g)	54.3	23.4	35.2
	b (L/mg)	0.0012	0.0007	0.0009
	r^2	0.9651	0.9221	0.9516
Cd	X_m (mg/g)	540	265	230
	b (L/mg)	0.0056	0.0057	0.0060
	r^2	0.9651	0.9221	0.9516
Pb	X_m (mg/g)	600	270	220
	b (L/mg)	0.0144	0.0151	0.0142
	r^2	0.9425	0.9584	0.9171
Hg	X_m (mg/g)	510	249	213
	b (L/mg)	0.0078	0.0079	0.0077
	r^2	0.9653	0.9233	0.9483

**Metal(loid) adsorption followed Langmuir adsorption isotherm (Eq. 5.2).*

$$X = 1/[(1/X_m) + (1/C*X_m b)] \quad (5.2)$$

where, 'X' is uptake of metal(loid) (mg/g); 'C' is equilibrium concentration of metal(loid) in solution (mg/L); 'X_m' is the maximum uptake (mg/g); and 'b' is binding constant (L/mg).

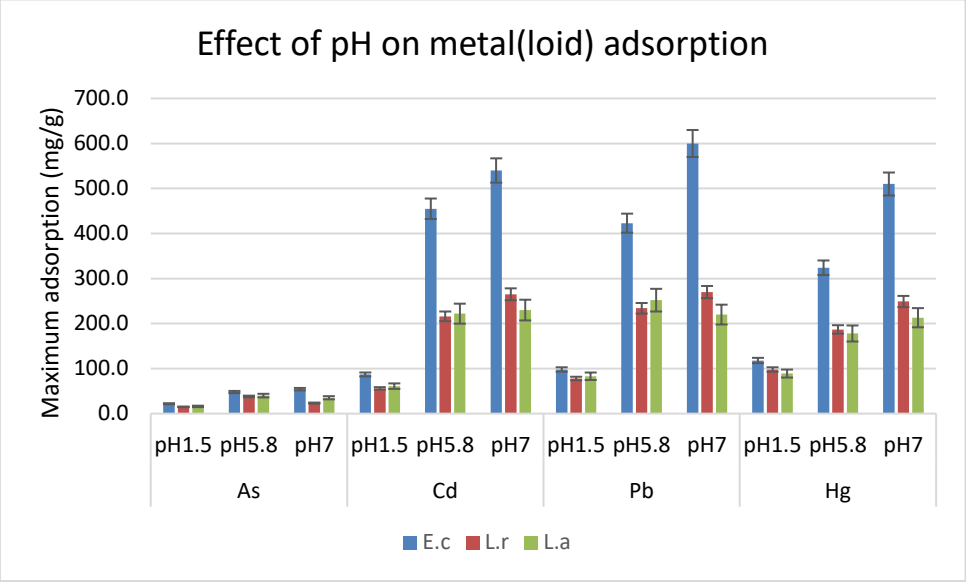


Figure 5.3 Effect of pH on the adsorption of metal(loid)s by gut bacteria

Whereas in the case of As(V), an increase in pH results in the reduction of As(V) to As(III) species which is less preferentially adsorbed. It has often been observed that the bioavailability of As(V) increases with increasing pH which is attributed to an increase in the conversion of As(V) to As(III) species which are not strongly adsorbed (Smith et al., 1998; Smith et al., 1999; Naidu et al., 2009; Bolan et al., 2015).

Halttunen et al. (2007b) examined the surface charge of a number native and chemically modified *lactobacillus* sp. The bacterial species were chemically modified to neutralize negative charge through methylation and amination of carboxyl groups and esterification of phosphoryl groups. The surface charge data as measured by microelectrophorosis indicated that the net surface surcharge of native bacteria was negative above pH 3.0, and the net negative charge increased with increasing pH. The net surface charge of chemically modified bacteria was positive even at higher pH values (pH < 5.0). Halttunen et al. (2003, 2007a,c) observed that the adsorption of Cd and Pb increased with increasing pH, which they attributed to an increase in net negative charge. They observed that As(V) adsorption was higher for chemically modified than native bacteria indicating the involvement of amino groups in As(V) adsorption. Schar-Zammaretti and Ubbink (2003) indicated that *L. acidophilus* and *L. crispatus* produced S-layer proteins, thereby retaining As(V). Singh and Sharma (2010) showed that *L. acidophilus* was effective in adsorbing As(V), thereby reducing As(V) concentration in aqueous medium. Similarly, Bhakta et al. (2012) noticed six strains of probiotic *Lactobacillus* sp (As99-1, As100-

2, As101-3, As102-4, As105-7, and As112-9) isolated from environmental samples were effective in the removal of As, Cd, and Pb from water.

5.6.4 Bacterial cell surface analysis by FTIR and TEM

The three bacterial species were analysed by FTIR to gain greater understanding of surface constituents of these bacteria (Figure 5.4; Table 5.7). Results indicated that the major functional groups of the bacterial surfaces tested are carboxyl, amide, phosphate, hydroxyl, and carbohydrate related moieties (Table 5.7). FTIR data also indicate that the majority of functional group chemistry of all three bacterial species are almost identical. Jiang et al. (2004) noticed that both Gram-negative (*Escherichia coli*) and Gram-positive (*Lactobacillus* sp.) bacterial surfaces have identical functional groups and variations in binding were related to the pH dynamics.

Table 5.7 Major functional groups in the bacteria tested and the changes in absorbance resulting from Pb adsorption (FTIR data)

Functional groups and major vibrational modes	**Absorption bands (cm^{-1})**					
	Without Pb adsorption			**With Pb adsorption**		
	E.C	**L.R**	**L.A**	**E.C**	**L.R**	**L.A**
Hydroxyl groups and water; OH stretching	3785	3782	3783	3801	3793	3794
Carboxylic acid group; Stretching of C=O	1723	1725	1726	1707	1713	1713
Amide I; Stretching of C=O	1641	1646	1651	1634	1639	1638
Amide II; N-H bending and C-N stretching	1543	1541	1546	1529	1534	1534
Carboxylate group; CH_2 scissoring	1451	1450	1451	1451	1450	1451
Asp and Glu side chain carboxylate group; Symmetric stretching of COO-	1397	1396	1397	1391	1407	1407
Esters; Vibrations of -COOH, and C-O-C	1146	1133	1135	1108	1123	1121
Phosphate group; Stretching of P=O bond in phosphate	1217	1211	1213	1204	1205	1204
Phosphate, polysaccharides & alcohols; Asymmetric & symmetric stretching of PO_2^- and $P(OH)_2$	1098	1090	1085	1102	1107	1096

*EC-*Escherichia coli; LR-Lactobacillus rhamnosus; LA-Lactobacillus acidophilus*

Since highest adsorption (X_m) values were observed for Pb adsorption by all gut bacteria species (Table 5.6; Figure 5.3), the FTIR spectra of the bacterial biomass with and without Pb loading were analysed to examine the probable functional groups contributing to Pb sorption (Figure 5.4). Although the maximum adsorption values for Pb varied between the bacterial species tested in this study, there was less difference in binding strength for adsorption between the bacterial species. Both the Pb adsorption behaviour and the FTIR functional group distribution findings are found to concur with the similar adsorption characteristics of different metal(loid)s onto Gram-positive and Gram-negative bacteria and their consortium (Yee and Fein, 2001, 2003; Borrok et al., 2004; Borrok et al., 2005).

The FTIR spectra of the bacterial biomass without Pb loading displayed diverse absorption peaks, indicating the complex nature of the bacterial biomass. The spectra of bacterial biomass with and without Pb loadings were compared and shifts in specific peaks were observed separately for *E. coli* and *Lactobacillus* sp. (Table 5.7). The hydroxyl (–OH) spectra representing the bonded and non-bonded hydroxyl groups and water ranged from 3782 to 3786 for the bacterial species grown in their respective media. In the presence of Pb, the shifts in the bands were found to be up to 3801 and 3794 for *E. coli* and *Lactobacillus* sp., respectively. This can be attributed to the association of the hydroxyl group with the Pb ions in the form of complexation reaction (Ray et al. 2005; Allievi et al. 2011). While the bands in methylene (CH_2) stretching carboxylate (COOH) groups remained stable for all the bacterial species used, shift in the absorption peaks was observed for aspartate (Asp) and glutamate (Glu) side chain COOH group (Table 5.7). Gerbino et al. (2011) also observed similar absorption peaks and they termed it to be metal(loid)/protein interaction with contribution from amine and amide (NH) groups in the peptide backbone. Several researchers showed the importance of amide groups I and II in Pb adsorption and complexation (Ha et al., 2010; Seki and Suzuki, 1999). In this study, amide I bands shifted from 1651 to 1638 cm^{-1} for *Lactobacillus* sp. and from 1641 to 1634 cm^{-1} for *E. coli,* in the presence of Pb. In the case of amide II containing both amine (N-H) bending and aromatic amine (C-N) stretching, the shift was less pronounced in *Lactobacillus* sp. and there was a significant shift in the peak for *E. coli* grown in the presence of Pb. The shift from 889.44 to 879.51 cm^{-1} corresponds to the carbonyl (O–C–O) in-plane bending (i.e., scissoring) vibration of polysaccharide (Merroun et al., 2003).

The changes in the FTIR spectra may be related to the interaction of Pb species with various surface functional groups of bacteria that include hydroxyl, amide, and amino groups. It is important to note these functional groups contribute to complexation and adsorption

reactions with Pb ions. Using electron microscopy and Fourier transform infrared spectroscopy (FTIR), Gerbino et al. (2011) observed metal(loid) precipitations in the S-layer and alteration in the secondary structure of the S-layer that for two strains of *L. kefir* CIDCA 8348 and JCM 5818.

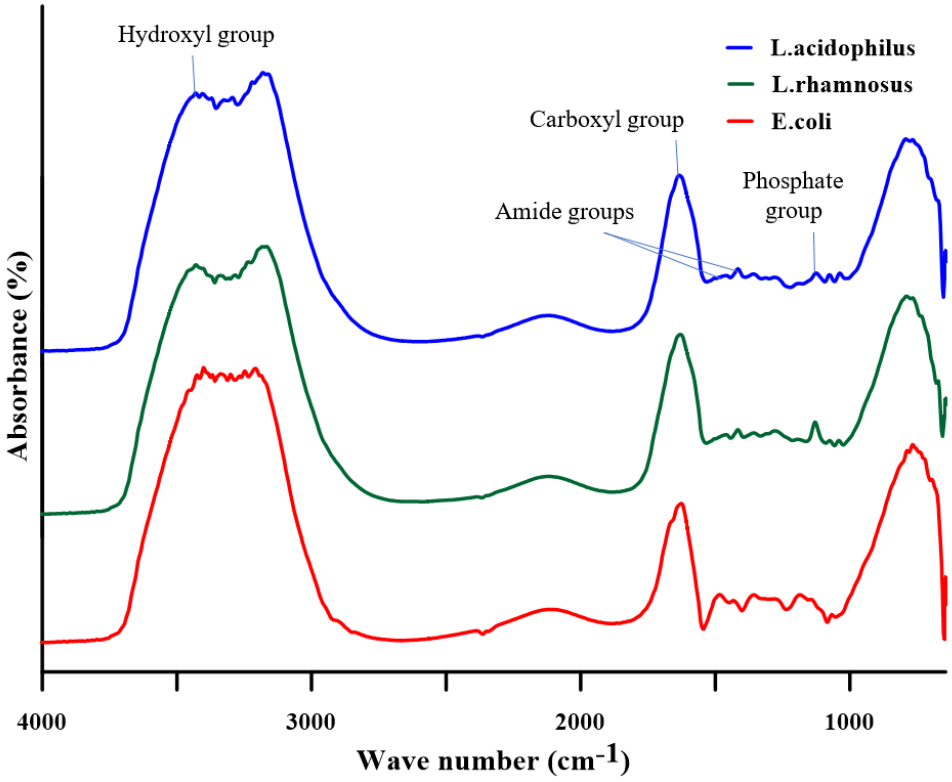


Figure 5.4 FTIR spectra of bacterial species in response Pb binding

Figure 5.5 shows scanning transmission election microscopy (STEM) micrographs of gut bacteria in the presence of Pb adsorption. Both STEM and EDX data provide evidence for Pb adsorption by gut bacteria. Dunham-Cheatham et al. (2011) used STEM to examine the distribution of Pb in Gram-positive *Bacillus subtilis* and Gram-negative *Shewanella oneidensis* under high and low saturation states of Pb adsorption. Although Pb precipitation occurred in bulk solution, there was no evidence for precipitation of Pb on the cell wall of these bacteria. Similarly, Muñoz et al. (2015) examined the distribution of the Pb within the cell was using HR-TEM-EDX technique. The data indicated Pb was largely fixed at the cell surface with some Pb accumulation within the cytoplasm, indicating bioaccumulation mechanism.

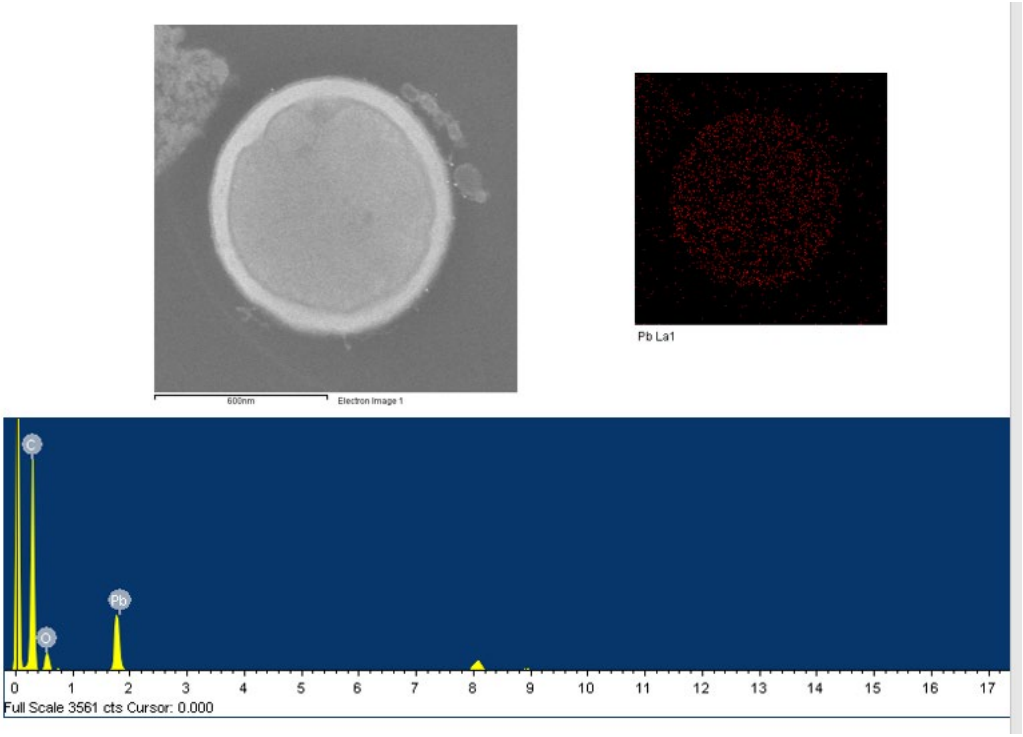


Figure 5.5 Transmission and scanning electron microscopy (TEM) observations of bacterial species in response to Pb binding

5.6.5 Mechanisms for microbial modulation of metal(loid) bioaccessibility

The reduction in the bioaccessibility of metal(loid)s in various sources by gut microbes could be attributed to the immobilization through adsorption, complexation, and precipitation reactions (Unz and Shuttleworth, 1996; Halttunen et al., 2007a,b,c; Monachese et al., 2012; Jarosławiecka and Piotrowska-Seget, 2014; Zoghi et al., 2014) (Figure 5.6). The microbial cell wall is a natural barrier for metal(loid)s, since the functional groups of several macromolecules are involved in the immobilization of metal(loid)s. In Gram-negative bacteria, lipopolysaccharide, a major component of the outer membrane, is effective in the immobilization of metal(loid) ions. In Gram-positive bacteria, peptidoglycan along with teichoic and teichuronic acids are involved in metal(loid) binding (Beveridge and Graham, 1991). For example, Cabuk et al. (2006) demonstrated that hydroxyl and carboxyl groups, along with nitrogen-based bio-ligands such as amide and sulfonamide, are responsible in the complexing Pb^{2+} by *Bacillus* sp.

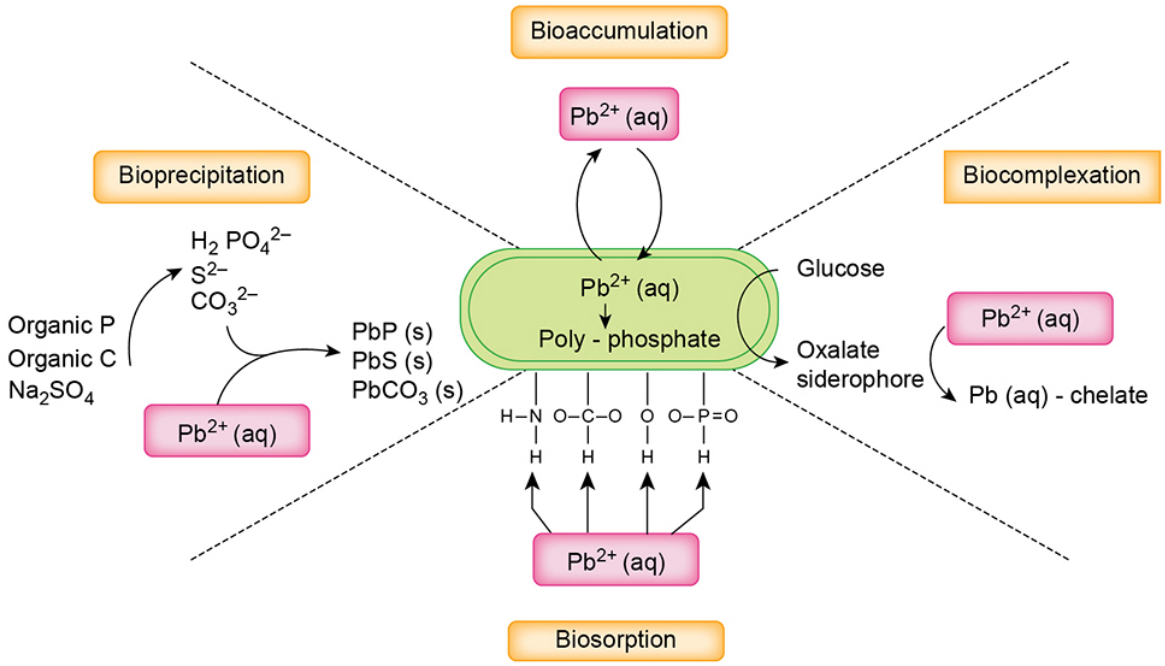


Figure 5.6 Schematic diagram illustrating the microbial metal(loid) immobilization processes

Most microorganisms excrete extracellular polymeric substances (ESPs) that bind toxic metal(loid) cations, thereby protecting metal(loid)-sensitive biochemical components (Figure 5.6) (Gupta and Diwan, 2017). The components of ESPs including proteins, polysaccharides, and nucleic acids, involve in the chelation of metal(loid)s (Guibaud et al., 2003; Pal and Paul, 2008). Binding of Pb^{2+}, Cd^{2+} and Hg^{2+} and other metal(loid)s by ESPs has been observed for bacteria (e.g., *Paenibacillus jamilae*; proteobacteria (*Halomonas* sp. and cyanobacteria) (Bae et al., 2003; Binet et al., 2003; Paperi et al., 2006; Perez et al., 2008; Chakravarty and Banerjee, 2012; Wei et al., 2014, 2016; Khan et al., 2016; Cruz et al., 2017; Kalita and Joshi, 2017; Wong et al., 2017). For example, The ESP produced by *Paenibacillus jamilae* (Perez et al., 2008) contains a high level of uronic acids, which plays an important role in metal(loid) binding. After crossing the cellular membrane, metal(loid) ions such as Pb^{2+} are bound to intracellular elements such as polyphosphate bodies (Pereira et al., 2011). Accumulation of Pb^{2+}, Cd^{2+} and Hg^{2+} and other metal(loid)s in the vacuolar structures of many bacterial and fungal species has also been observed (Li et al., 1997; Sauge-Merle et al., 2003; Prévéral et al., 2006; Pereira et al., 2011; Jaishankar et al., 2014). Metallothionein-like proteins bind metal(loid)s including Pb^{2+}, Cd^{2+} and Hg^{2+} in biota including microorganisms (Wong et al., 2017). Extracellular enzymes including superoxide dismutase excreted by microorganisms can also be involved in binding

metal(loid)s including Pb^{2+}, Cd^{2+} and Hg^{2+} (Wang et al., 1997; So et al., 2001; Rigden et al., 2003; Gonick, 2011; Chandrangsu et al., 2017).

Additionally, metal(loid) cations such as Pb^{2+}, Cd^{2+} and Hg^{2+} forms strong soluble and insoluble complexes with organic compounds including tryptone, cysteine, neopeptone, casamino acid, and succinic acid (Tan et al., 1994; Nigam et al., 2000; Mayer and Godwin, 2006; Gadd, 2010; Hajdu and Slaveykova, 2012; Ndu et al., 2012). Microorganisms release a number of organic compounds including short chain fatty acids and carboxylic acids (e.g., lactic acid) that are involved in nutrient absorption and energy regulation (Krajmalnik-Brown et al., 2012). These organic compounds form complexes with metal(loid)s, resulting in the removal of these metal(loid)s from solution. For example, Teemu et al. (2008) demonstrated that lactic acid producing bacteria, *Lactobacillus fermentum* ME3 and *Bifidobacterium longum* were found to be effective in the removal of Cd and Pb from solution, which they attributed to complexation of metal(loid) by lactic acid and also the direct adsorption of metal(loid)s by the bacteria. Similarly, Jadán-Piedra et al. (2017) explored the use of lactic acid producing bacteria to reduce the amount of Hg solubilized during gastrointestinal extraction and available for absorption. Ten strains of lactic acid producing bacteria were tested and noticed that all of the strains assayed reduced the soluble fraction from standards of Hg species (Hg(II), CH_3Hg and food samples) in gastrointestinal extraction (72-98%).

Metal(loid) cations such as Pb^{2+} also reacts with inorganic anions such as chlorides, carbonates, phosphates, and hydroxyl ions to form precipitates (Kumpiene et al., 2008; Gao et al., 2017). The precipitation of Pb^{2+} is employed by microorganisms to lower the free Pb^{2+} ion concentration by sequestering it in the form of both extracellular and intracellular phosphate salts (Figure 5.6). The composition of these precipitates varies amongst microbial strains. For example, *Citrobacter freundii* and *Enterobacter cloacae* precipitate Pb^{2+} as an extracellular phosphate ($PbHPO_4$). Lead precipitation in these bacterial strains results from the acid phosphatase-induced hydrolysis of organic phosphorus and the subsequent precipitation of Pb^{2+} on the cell surface (Park et al., 2011; Liang et al., 2016). Three stages of intracellular precipitation process of metal(loid)s including Pb^{2+} have been identified (Smeaton et al., 2009): (1) binding of metal(loid) ions to the cell surface; (2) metabolic intracellular uptake of metal(loid) ions, and (3) complexation and precipitation of metal(loid) as insoluble compounds. For example, *Staphylococcus aureus* precipitates metal(loid) inside the cell mainly as phosphate compounds. The marine bacterium, *Vibrio harveyi* has been shown to precipitate Pb^{2+} inside the cell in the form of $Pb_9(PO_4)_6$. This process is regulated partially by quorum sensing which controls the supply of inorganic phosphates (Mire et al., 2004). Precipitation of Pb^{2+} as

$Pb_9(PO_4)_6$ was demonstrated also in *Providencia alcalifaciens* 2EA due to the release of inorganic phosphates by phosphatase enzyme (Naik et al., 2012). The phosphate solubilizing *Enterobacter cloacae* (Park et al., 2011) and *Burkholderia cepacia* (Templeton et al., 2003) immobilize Pb^{2+} in the form of pyromorphite $Pb_5(PO_4)_3Cl$. Similarly, Dunham-Cheatham et al. (2011) observed that both Gram-positive *Bacillus subtilis* and Gram-negative *Shewanella oneidensis* MR-1 formed lead phosphate precipitation on cell surface through nucleation process. The high binding affinity of cell walls for aqueous metal(loid) cations creates nucleation sites for mineral precipitation reactions in saturated systems. These nucleation sites are likely to promote heterogeneous nucleation of metal(loid) phosphates on or in the cell wall through surface complexation reactions.

Macaskie and Dean (1982) noticed that cells of a strain of a *Citrobacter sp*., accumulated Cd extensively when suspended in a buffer that contained Cd^{2+} and glycerol 2-phosphate (Macaskie and Dean, 1982 and 1984). Using X-ray microanalysis and magic angle spinning NMR analysis, the accumulated compound was identified as cell-bound cadmium phosphate, $CdHPO_4$. Its accumulation is consistent with the activity of a phosphatase, induced during pre-growth, that continues to function in the resuspended cells to liberate HPO_4^{2-} from glycerol 2-phosphate. This anion then combines with Cd^{2+} to form insoluble cell-bound $CdHPO_4$. Bauda and Block (1990) showed that whole cells of *Pseudomonas fluorescens* bind less metal(loid) than other bacterial forms, suggesting the outer membrane acts as a barrier toward metal(loid)s.

Metal(loid)s such as Pb and Hg have been shown to be precipitated as respective sulphide. Sulfate-reducing bacteria have been shown to use anglesite ($PbSO_4$) as the electron acceptor, thereby reducing to poorly soluble galena (PbS) (Karnachuk et al., 2002)). Aiking et al. (1985) observed that *Klebsiella aerogenes* NCTC 418 was most sensitive to Cd under conditions of phosphate limitation and most sensitive to Hg under conditions of sulfate limitation indicating that these inorganic detoxification mechanisms generally depended on 'facilitated precipitation'. This suggests that heavy metal(loid)s are accumulated and precipitated near the cell perimeter due to the relatively high local concentrations of sulfide and phosphate. Xu et al. (2015) indicated that efficient stabilization of Hg can be achieved through co-precipitation of Hg as a sulphidic phosphate compound. Microbes have been employed to synthesize nanocrystals of Cd and Pb compounds (Dameron et al., 1989; Shenton et al., 1997; Ahmad et al., 2002; Kowshik et al., 2002; Marusak et al., 2018). For example, Sweeny et al. (2004) have noticed that *E. coli*, when incubated with cadmium chloride and sodium sulfide, have the capacity to synthesize intracellular cadmium sulfide (CdS) nanocrystals.

5.7 Conclusions

Bioaccessibility of contaminants, including heavy metal(loid)s, underpins their bioavailability and toxicity to biota including microorganisms and human being. Gut microbes play an important role in the absorption of nutrients and heavy metal(loid)s in human intestine through their effect on the bioaccessibility. This study demonstrated that gut microbes decreased bioaccessibility of metal(loid)s, which is likely to impact their bioavailability, intestinal absorption, and toxicity. The effect of gut microbes on bioaccessibility may be attributed to bioimmobilization of metal(loid)s through adsorption, precipitation, and complexation reactions. It is, therefore, imperative to undertake bioaccessibility and bioavailability measurements in the presence of gut microbes, especially for orally-ingested contaminants which will be discussed in Chapter 7. It is important to point out that the human gut hosts a large number of microbial species including bacteria, fungi, and archaea. Some of these microbial species are known to produce ESPs which are involved in the chelation of heavy metal(loid)s. The effect of chelates on bioaccessibility and bioavailability of heavy metal(loid)s will be covered in Chapters 6 and 7. In this study, the effect of only selected bacterial species on bioaccessibility, and bioavailability was examined. Future studies should focus on the effect of composite gut microbial culture on bioaccessibility and subsequent bioavailability of toxic metal(loid)s.

Chapter 6

BIOACCESSIBILITY OF HEAVY METAL(LOID)S AS IMPACTED BY GUT MICROBES IN THE PRESENCE OF CHELATING AGENTS

6.1 Introduction

The data in Chapter 3 indicate that heavy metal(loid)s are toxic to gut microbe species, and the extent of toxicity depends on both the nature of metal(loid)s and gut bacteria. In chapters 4 and 5, it has been demonstrated that gastro-intestinal bioaccessibility of heavy metal(loid)s varies amongst metal(loid) sources, and gut bacteria modulate the bioaccessibility of metal(loid)s. The modulation of bioaccessibility of metal(loid)s by gut bacteria has been attributed to the immobilization through adsorption, complexation, and precipitation reactions (Unz and Shuttleworth, 1996; Halttunen et al., 2007a,b,c; Monachese et al., 2012; Jarosławiecka and Piotrowska-Seget, 2014; Zoghi et al., 2014) (Figure 5.6). For example, most microorganisms excrete extracellular polymeric substances (ESPs) including proteins, polysaccharides, and nucleic acids, which are involved in the complexation and chelation of metal(loid)s (Guibaud et al., 2003; Pal and Paul, 2008).

Synthetic chelating compounds are used as therapeutic agents to detoxify metal(loid)s in humans (Goyer et al., 1995; Porru and Alessio, 1996; Blanuˇsa et al., 2005; Risher and Amler, 2005; Lynes et al., 2007; Flora and Pachauri, 2010; Sears, 2013; Aaseth et al., 2015). The chelating agents that are presently suitable for use in human beings can be classified in the following structurally related categories: polyaminocarboxylic acids, chelating agents with vicinal -SH groups, b-mercapto-alpha-amino acids, hydroxamic acids, orthohydroxy carboxylic acids or orthodiphenols, and miscellaneous agents (Haendler and Geyer, 1938; Schubert, 1981; Williams and Halstead, 1982; Flora and Pachauri, 2010).

All chelating agents, whether synthetic or natural, form chemical bonds with metal(loid) ions, rendering them much less chemically reactive. The resulting metal(loid)-chelate complex is water-soluble, which allows it to enter the bloodstream, and these complexes are readily excreted in the urine or faeces (Aposhian et al., 1992, 1996; Porru and Alessio, 1996; Flora

and Pachauri, 2010; Sears, 2013). Detoxification of heavy metal(loid)s depends on specific proteins and enzymes that bind the metal(loid)s and transport them out of cells into the bloodstream (Shen et al., 2013; Sharma et al., 2014). The chelating agents impact the metal(loid)-induced toxicity to gut microbes and also the bioaccessibility of metal(loid)s (Lowry, 2005; Robertson et al., 2012; Gerhardsson and Aaseth, 2016).

The most common synthetic chelating agents used to manage acute metal(loid) poisoning are ethylene diamine tetra acetic acid (EDTA), 2,3-dimercaptopropane-1-sulfonate (DMPS) and 2,3-dimercaptosuccinic acid (DMSA) (Goyer et al., 1995; Porru and Alessio, 1996; Shannon et al., 2001; Flora et al., 2004; Blanu˘sa et al., 2005; Risher and Amler, 2005; Lynes et al., 2007; Bradberry and Vale, 2009; Flora and Pachauri, 2010; Sears, 2013; Aaseth et al., 2015; Gerhardsson and Aaseth, 2016) (Table 6.1; Ferrero, 2016). EDTA is primarily used for the chelation of Pb, Hg and Cd metal(loid)s (Chisolm, 1992; Chappel, 1997; Roussel et al., 2009; Crinnion, 2011; Ferrero,, 2016). It is administered both intravenously and orally, and mostly be able to chelate extracellular metal(loid)s. The benefits of oral chelation may result from the synergistic effect of combining EDTA with numerous natural chelating agents, such as activated clays, bioflavonoids, chlorella, cilantro, L-cysteine, L-glutathione, etc. (Sears, 2013). Numerous animal and human studies have shown that DMSA administration increases urinary Hg excretion and reduces blood and tissue Hg concentration (Chisolm, 1992; Crinnion, 2011). The hydrophilic nature of DMSA causes considerable absorption in GI tract, and hence oral administration creates its distinct advantage over other chelating agents (Sears, 2013; Gerhardsson and Aaseth, 2016). DMSA is most commonly used for treating Pb, As and Hg toxicity (Aposhian, 1983; Asledu et al., 1995; Hoet et al., 2006; Adams et al., 2009). DMPS is available in intravenous and oral forms and is primarily used for the treatment of As and Hg poisoning (Aposhian et al., 1996; B¨ose-O'Reilly et al., 2003; George et al., 2004; Bradberry et al., 2009; Rafati-Rahimzadeh et al., 2014).

Table 6.1 Characteristics of common chelating agents used in clinical practice (Ferrero, 2016).

Chelate	**Route of administration**	**Adult dose**	**Route of metal complex excretion**	**Side effects**
BAL	IM	5 mg/kg/day	Urine, bile, faeces, lungs	Nausea, vomiting, hypertension, tachycardia, headache
Deferiprone	Oral	50–100 mg/kg/day	Urine	CNS toxicity, lenticular opacities, arthropathy
Deferoxamine	IV	50 mg/kg/day	Urine	

Chelate	Route of administration	Adult dose	Route of metal complex excretion	Side effects
	Subcutaneously	20 mg/kg/day		Nausea, weight loss, ocular toxicity
Deferasirox	Oral	35 mg/kg/day	Faeces, urine	Abdominal pain, nausea, vomiting, elevation of liver enzymes
DMPS	Oral	300 mg three times a day	Urine	Rash, nausea, leukopenia
	IV	until 1500 mg/day		
	IM	20 mg/kg/day		
DMSA	Oral	30 mg/kg/day	Urine, bile, faeces, lungs	Gastrointestinal disorders (GI), skin rashes, flu-like symptoms
	IV	10 mg/kg three times a day		
EDTA	IV	2 gr/week	Urine	-
D-Penicillamine	Oral	5–20 mg/kg/day	Urine	Degenerative dermopathy, thromboleukocytopenia, GI

BAL: 2,3-dimercatopropanol; DMPS: 2,3-dimercapto-1-propane sulphonic acid; DMSA: meso-2,3-dimercaptosuccinic acid; EDTA: $CaNa_2$ ethylenediaminetetraacetic acid. IM: Intra-muscular; IV: Intra-venous

In Chapter 5, it has been demonstrated that gut bacteria reduce the gastro-intestinal bioaccessibility of heavy metal(loid)s which is attributed mainly to the microbial adsorption of metal(loid)s. In this chapter (Chapter 6), the interactive effect of chelating agents and gut bacteria on the bioaccessibility of heavy metal(loid)s will be examined. Chelating agents impact the bioaccessibility of metal(loid)s, which is attributed mainly to increased solubilisation of metal(loid)s through chelation. The chelating agents themselves can be toxic to gut microbes while modulating the metal(loid)-induced toxicity to gut microbes (Oviedo and Rodríguez, 2003; Wenger et al., 2005; Smith, 2013). For example, Santos et al. (2012) and Smith et al (2017) noticed that EDTA, DMPS and DMSA were found to be toxic at high doses to bacteria, and EDTA was found to be more toxic than the other two chelating agents. The difference in toxicity amongst chelating agents is attributed to difference in the rate of absorption by the bacteria (Oviedo and Rodríguez, 2003).

It is important to examine effect of chelating agents on the bioaccessibility and bioavailability of metal(loid)s in relation to their beneficial value in the treatment of metal(loid) toxicity. However, most of the studies on the effect of chelation on bioaccessibility and bioavailability of metal(loid)s are related to metal(loid) toxicity and phytoavailability in metal(loid) contaminated soils (Barona and Romero, 1996; Cooper et al., 1999; Hong et al., 1999; Evangelou et al., 2007; Jiang et al., 2011). These studies have demonstrated that the

solubility and phytoavailability of metal(loid)s varied between the metal(loid) sources and the nature of chelating agents (Tandy et al., 2004; Mohamed et al., 2013; Lee and Sung, 2014). Although a vast number of studies have examined the effects of chelating agents on the solubility and phytoavailability of heavy metal(loid)s in soil (Evangelou et al., 2007), their effects on the bioaccessibility of orally ingested heavy metal(loid) sources haven't been examined in detail, which is the main focus of the research reported in this chapter. Similarly, most studies focussed on the mobilization of heavy metal(loid)s by chelating agents through chemical complexation (Flora, 2009; Sears, 2013). Chelating agents also impact the metal(loid)-induced toxicity to gut microbes and also the bioaccessibility of metal(loid)s (Lowry, 2005; Robertson et al., 2012; Gerhardsson and Aaseth, 2016). Chelating agents also influence gut microbes, thereby impacting the mobilization and bioavailability of metal(loid)s. The interactive effect of chelating agents and gut microbes on the bioaccessibility of heavy metal(loid)s has not been examined in detail which will also be covered in this chapter. The effect of gut microbes on the bioavailability of these metal(loid) sources as impacted by chelating agents will be covered in Chapter 7.

6.2 Objectives

The overall objective of this work was to examine the interactive effect of gut bacteria and chelating agents on the bioaccessibility of orally ingested As, Cd, Hg and Pb. The specific objectives in this chapter were to:

(i) Examine the effect of chelating agents on the growth of gut bacteria.
(ii) Monitor the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb in various sources as impacted by gut bacteria in the presence of chelating agents.
(iii) Monitor the distribution of various species of As, Cd, Hg and Pb in gastric and intestinal extractions as impacted by gut bacteria in the presence of chelating agents.

6.3 Hypothesis

(i) Chelating agents are toxic to gut microbes and the effect varied between the chelating agents and gut microbes.
(ii) Chelating agents influences bioaccessibility of metal(loid)s through their effects on the solubilisation of metal(loid) sources and subsequent interactions with metal(loid)s, and the effect depends on the nature of chelating agents and metal(loid)s sources.

6.4 Experiments

The major experiments and analyses conducted to test the hypothesis, and the treatments used in this chapter are listed in Table 6.2.

Table 6.2 The major experiments and treatments used in Chapter 6

No.	Title	Treatments
1	Effect of chelates on the growth of gut bacteria	3 gut bacteria species (*Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) – Table 5.2), 3 chelating agents (EDTA, DMPS, DMSA - Table 6.3); Bacterial growth.
2	Bioaccessibility of metal(loid)s	In the absence of gut bacteria: 4 metal sources (Table 4.3); 3 chelating agents (Table 6.3); Gastric and intestinal bioaccessibility tests. In the presence of gut bacteria: 4 metal sources (Table 4.3), 2 gut bacteria species (*Escherichia coli* (MG1655) and *Lactobacillus acidophilus* (IFO 13951) - Table 5.2); 2 chelating agents (EDTA, DMPS - Table 6.3), Gastric and intestinal bioaccessibility tests.
3	Speciation of metal(loid)s	In the absence of gut bacteria: 4 metal sources (Table 4.3); 3 chelating agents (Table 6.3); Gastric and intestinal extracts. In the presence of gut bacteria: 4 metal sources (Table 4.3), 2 gut bacteria species (*Escherichia coli* (MG1655) and *Lactobacillus acidophilus* (IFO 13951) - Table 5.2); 2 chelating agents (EDTA, DMPS - Table 6.3), Gastric and intestinal extracts.

6.5 Materials and Methods

6.5.1 Metal(loid) sources, chelating agents and gut bacteria

The metal(loid) sources included in this study are the common orally ingested sources, and are based on their heavy metal(loid) content derived from literature data (Table 4.2). The metal(loid) sources include offal pet food (cadmium), fish meal (mercury), complementary medicine (lead) and rice grain (arsenic) (Table 4.3), which are described in Section 4.5.1 (Chapter 4). Arsenic oxide, Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3).

The chelating compounds included in this study are based on their potential applications in the treatment of heavy metal(loid) toxicity derived from literature data (Table 6.1). The most common synthetic chelating agents used to manage acute metal(loid) poisoning in human are ethylene diamine tetra acetic acid (EDTA), 2,3-dimercaptopropane-1-sulfonate (DMPS) and 2,3-dimercaptosuccinic acid (DMSA) (Table 6.1). These three chelating agents are used to

examine their effect on the bioaccessibility of As, Cd, Pb and Hg in the respective heavy metal(loid) sources. The properties of these three chelating agents and the dosage used in clinical application are reported in Table 6.3.

The three gut bacteria, *Escherichia coli* (MG1655), *Lactobacillus rhamnosus* (BUCSAV 227) and *Lactobacillus acidophilus* (IFO 13951) (Table 5.2 in Chapter 5) were used in this chapter to examine their growth response to the three chelating agents. Since there was no significant difference in gut bacteria induced bioaccessibility between *lactobacillus* species (Chapter 5), only *E. coli and L. acidophilus* were used in this chapter to examine their effect on the bioaccessibility of metal(loid)s in the presence of chelating agents. Subcultures of these bacteria were inoculated from their respective mother cultures purchased from American Type Culture Collection (ATCC, Melbourne; https://www.atcc.org/).

The effect of all three chelating agents on the bioaccessibility of As, Cd, Pb and Hg in orally ingested sources was examined in the absence of gut bacteria. Since there was no significant difference between DMSA and DMPS on the growth of gut bacteria, only DMPS and EDTA were used to examine their effect on the bioaccessibility of metal(loid)s in the presence of gut bacteria.

Table 6.3 Chelating agents used in this study

Chelating agent	Properties	Metal(loid)s complexed	Treatment delivery	Chelation dose
EDTA	$C_{10}H_{16}N_2O_8$ 292.24 g·mol^{-1} 0.860 g cm^{-3} (at 20 °C) aminopolycarboxylic acid; colorless and water-soluble solid	Cd, Pb, Hg, As, Cu, Zn	Mainly Intravenous; sometimes oral	1.2–2 g/d
DMPS	$C_3H_8O_3S_3$ M.wt; 188.289g/mol Water soluble	Hg, As, Cd, Pb	Mainly oral; sometimes intravenous	10–30 mg/kg/d
DMSA	$C_4H_6O_4S_2$ 182.22 g/mol Melting point 125 °C	Hg, As, Cd, Pb, Sb	Mainly oral; sometimes intravenous	300–400 mg/d Common side effects include vomiting, diarrhea, rash, and low blood neutrophil levels

EDTA = Ethylene diamine tetra acetic acid; DMPS = 2,3-dimercaptopropane-1-sulfonate; DMSA = 2,3-dimercaptosuccinic acid

6.5.2 Effect of chelating agents on gut bacterial growth

The growth of the three bacterial species was studied in the presence of various concentrations (0, 0.1, 1.0, 5.0 and 10 mmol/L) of the three chelate solutions (EDTA, DMPS and DMSA). The bacterial species were inoculated in the growing media containing the chelating agents and monitored over a period of 24 hours in a 96-well round bottom microplate (Costar 3799, Corning Incorporated, USA) under sterile anaerobic conditions at 37°C. The bacterial growth was monitored by measuring optical density (@600 nm) over time in a microplate reader (BMG LABTECH FLUOstar OPTIMA Fluorescence Microplate Reader, Germany) (Koch, 1970; Stevenson et al., 2016).

6.5.3 *In vitro* gastrointestinal bioaccessibility tests

Bioaccessible metal(loid) contents in various metal(loid) sources were measured following the *in vitro* gastrointestinal (IVG) method by Rodriguez et al. (1999) which involves a two-step sequential extraction: a gastric followed by an intestinal extraction. Arsenic oxide, Lead acetate, Cadmium acetate and Mercuric chloride were used as the standard reference compounds for Pb, Cd, As and Hg, respectively (Table 4.3). The *in vitro* gastrointestinal bioaccessibility of these reference compounds were used to estimate the relative bioaccessibility of the metal(loid) sources.

The gastrointestinal extraction was carried out as described in Section 3.5.3 (Chapter 3). The extraction was carried out with the addition of 1 mL of 1mM of EDTA or DMPS or DMSA chelating solution. Preliminary tests have indicated that there was no effect of this concentration on the growth of gut bacteria. The extraction was also carried out with the addition of 1 mL of broth with and without (control) bacterium (Chapter 5). A negative control without the addition of broth was also used to determine the effect of broth on bioaccessibility and speciation of metal(loid). The gastric and intestinal phase samples were filtered through 0.2 μm syringe filters for metal(loid) analysis by ICP-MS.

6.5.4 Distribution of free and complexed metal(loid)s

The distribution of free and complexed metal(loid)s in the gastric and intestinal extracts was measured using cation/anion-exchange resin cartridge (Empore, iminodiacetate functionalized poly(styrene divinylbenzene) - 234877 Aldrich) (Pu and Fukushi, 2013). The method for the measurement of distribution of free and complexed metal(loid)s in the gastric and intestinal extracts is described in Section 3.5.6 (Chapter 3).

6.5.5 Data analysis

Both absolute (AB) and relative (RB) metal(loid) bioaccessibility values for gastric and intestinal phases were calculated using Eqs. 4.1 – 4.4 described in Section 4.5.6 (Chapter 4).

All experimental analyses were carried out using three replications. Statistical comparisons were made using analysis of variance (ANOVA) in Predictive Analytics SoftWare (PASW) statistics (version 18.0.0; SPSS, Inc., 2009, Chicago, IL) in order to examine the significant differences in various treatments. Duncan's multiple range test was also employed to compare the means of various treatments; variability in the data was presented as the standard deviation and a $p < 0.05$ was considered statistically significant.

6.6 Results and discussion

6.6.1 Effect of chelating agents on gut bacterial growth

The bacterial growth at various concentrations of chelates is presented in Table 6.2 and Figure 6.1. There was a significant decrease in bacterial growth above 1.0mmol/L chelate concentration, and the decrease in bacterial growth varied amongst the bacterial species and chelating agents. EDTA caused a greater decrease in bacterial growth than did DMPS and DMSA. There was no significant difference in bacterial growth between DMPS and DMSA. The inhibitory effect of bacterial growth by chelating agent was more pronounced in *E. coli* than the *lactobacillus* species, and was more pronounced at higher concentrations of 5.0 mM and 10.0 mM than in lower concentrations of the chelating agents.

Table 6.4 Effect of chelating agents on the growth of gut bacteria

	E. coli			***L. acidophilus***			***L. rhamnosus***		
Concentration	**EDTA**	**DMPS**	**DMSA**	**EDTA**	**DMPS**	**DMSA**	**EDTA**	**DMPS**	**DMSA**
0 mM*	100	100	100	100	100	100	100	100	100
0.1 mM	99.5	99.3	98.7	99.5	99.6	100.1	998	98.7	99.2
1.0 mM	98.7	98.2	97.4	98.1	97.8	98.2	97.7	98.3	97.4
5.0 mM	73.4	83.2	85.5	81.7	88.8	87.3	80.4	88.3	88.6
10.0 mM	62.6	73.4	75.4	69.2	78.4	77.5	67.7	78.2	78.5

**Growth at the control (0 mM Chelate concentration) as measured by OD values for E. coli, L. acidophilus and L. rhamnosus are kept as 100% growth to compare the relative growth at various concentrations of chelating agents*

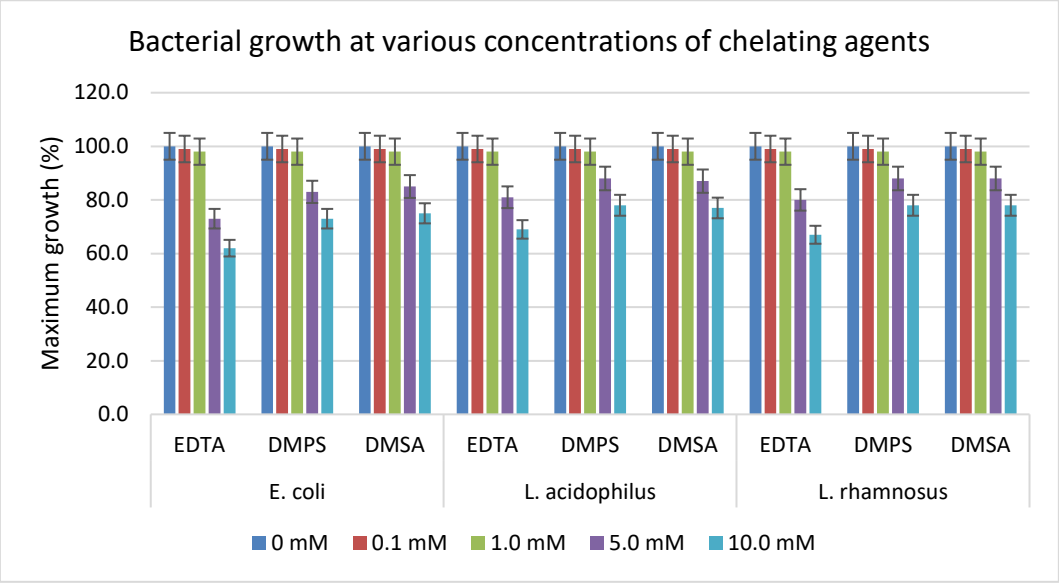


Figure 6.1 Effect of chelating agent on the growth of gut bacteria

Chelating agents have been shown to inhibit microbial growth (i.e., antimicrobial), which is attributed to their ability to bind free metal(loid) ions essential for bacterial metabolism and membrane stabilization (Root et al., 1988; Agh-Atabay et al., 2003; Thompson et al., 2012; Liu et al., 2017). For example, EDTA and DMSA have been shown to exhibit antimicrobial properties against both Gram-positive and Gram-negative organisms (Lambert et al., 2004; Rai et al., 2006; Yoshizumi et al., 2013; Finnegan and Percival, 2015). The release of lipopolysaccharide (LPS) and the destabilization of the LPS layer of the microbial cell membrane have been suggested as possible mechanisms involved in the antimicrobial effect of EDTA. For example, it has been proposed that EDTA solutions can be used to treat dairy cows' teat dips as a disinfection strategy in attempts to prevent the spread of mastitis pathogens such *Streptococcus agalactiae* and *Streptococcus uberis* (Reidmiller et al., 2006).

Santos et al. (2012) and Smith et al (2017) examined the effect of various chelating agents on bacteria and noticed that the toxicity of chelating agents as measured by LD_{50} value varied amongst both the bacterial species and the chelating agents. In their study, EDTA, DMPS and DMSA were found to be toxic at high doses to bacteria, and EDTA was found to be more toxic than the other two chelating agents. The difference in toxicity amongst chelating agents is attributed to difference in the rate of absorption by the bacteria and the ability of the different chelates in complexing the essential elements such as Fe, thereby decreasing their

bioavailability to bacteria (Oviedo and Rodríguez, 2003; Flora and Pachauri, 2010). Chelating agents cause the disruption of the outer membrane with the consequent loss of lipopolysaccharide, which in turn, make cells susceptible to lysis due to the action of many substances such as detergents, proteases, lipases and lysozymes.

A number of elements including iron (Fe) and cobalt (Co) are essential cofactors of biochemical pathways in both prokaryotic and eukaryotic species. Thus, limiting the bioavailability of these nutrients is likely to inhibit microbial growth (Ohland and Jobin, 2015). Most microorganisms have developed efficient methods of absorbing Fe from the environment and many microorganisms secrete siderophores in order to scavenge Fe (Hider and Kong, 2010). Such methods of uptake can be circumvented by the introduction of high-affinity iron-selective chelating agents. However, the affinity of these agents for Fe must be high, enabling them to compete efficiently with siderophores.

Numerous studies have assessed the potential viability of Fe chelators as therapeutic agents against various microbes (Collins et al., 2002; Fernandes et al., 2010; Qiu et al., 2011). For example, Thompson et al. (2012) examined the activities of Fe chelators (deferoxamine, deferiprone, Apo6619, and VK28) against type strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*. Deferiprone, Apo6619, and VK28 each inhibited growth of these bacteria in tissue culture medium, while deferoxamine had no effect.

Since the chelate-induced bacterial growth inhibition occurred only above 1.0mmol/L concentration, the effect of cheating agents on bioaccessibility of metal(loid)s in the presence of bacterial species was examined at chelate concentration of 1.0mmol/L. There was no difference in the inhibition of gut bacteria between DMPS and DMSA, and hence only EDTA and DMPS were used in the bioaccessibility assay in this Chapter.

6.6.2 Effect of chelating agents on the bioaccessibility of metal(loid)s

There was no significant effect of the addition of chelating agents on the solubility of reference heavy metal(loid) samples in the gastric phase. However, the addition of chelating agents slightly increased solubility of reference heavy metal(loid) samples in the intestinal phase (Table 6.5). The addition of chelating agents increased the solubility of heavy metal(loid)s in the respective sources both in the gastric and intestinal phases, and the effect varied between the chelating agents and also amongst the heavy metal(loid)s sources (Table 6.5). The metal(loid) extracted by the gastric solution was greater than the metal(loid) extracted by the intestinal solution for the reference compounds and also for the metal(loid) sources both with

and without the addition of chelating agents (Table 6.5). As discussed in Chapter 4, the reduction of measured metal(loid) bioaccessibility between gastric and intestinal phases can be related to the decreased solubility of metal(loid)s in the higher pH of the intestinal solution compared with the gastric phase (pH 5.8 vs. 1.8). It has been observed that a major proportion of solubilized metal(loid) in the gastric phase is readsorbed onto media components or precipitated at the higher intestinal phase pH, thereby reducing the bioaccessibility in the intestinal phase (Jayawardene et al., 2010; Juhasz et al., 2016; Boros et al., 2017). However, the addition of chelating agents increased the solubility of heavy metal(loid)s in the intestinal phase indicating that chelating agents remobilized the readsorbed heavy metal(loid)s in the intestinal phase.

Table 6.5 Effect of chelating agents on absolute and relative bioaccessibility of metal(loid)s in standard metal(loid) and test samples..

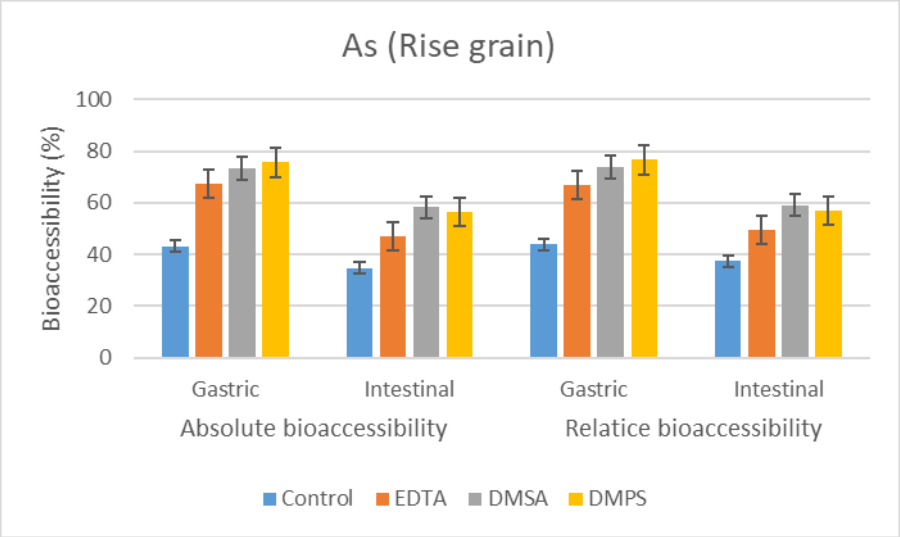
Metal(loid) sources	Chelating agent	Absolute bioaccessibility*		Relative bioaccessibility*	
		Gastric	Intestinal	Gastric	Intestinal
Standard samples					
Arsenic oxide	Control	98.4	91.4	NA	NA
	EDTA	101.3	95.6		
	DMSA	99.5	98.5		
	DMPS	98.6	99.2		
Cadmium acetate	Control	98.3	75.6	NA	NA
	EDTA	100.2	99.4		
	DMSA	98.2	98.2		
	DMPS	99.4	100.2		
Mercuric chloride	Control	98.7	69.3	NA	NA
	EDTA	101.1	78.9		
	DMSA	99.1	95.6		
	DMPS	99.6	97.8		
Lead acetate	Control	100.2	65.4	NA	NA
	EDTA	100.8	98.7		
	DMSA	99.5	98.2		
	DMPS	98.4	97.8		
Test samples					
Rice grain (As)	Control	43.2	34.6	43.87	37.36
	EDTA	67.5	47.2	66.63	49.37
	DMSA	73.4	58.2	73.76	59.08
	DMPS	75.6	56.5	76.67	56.95

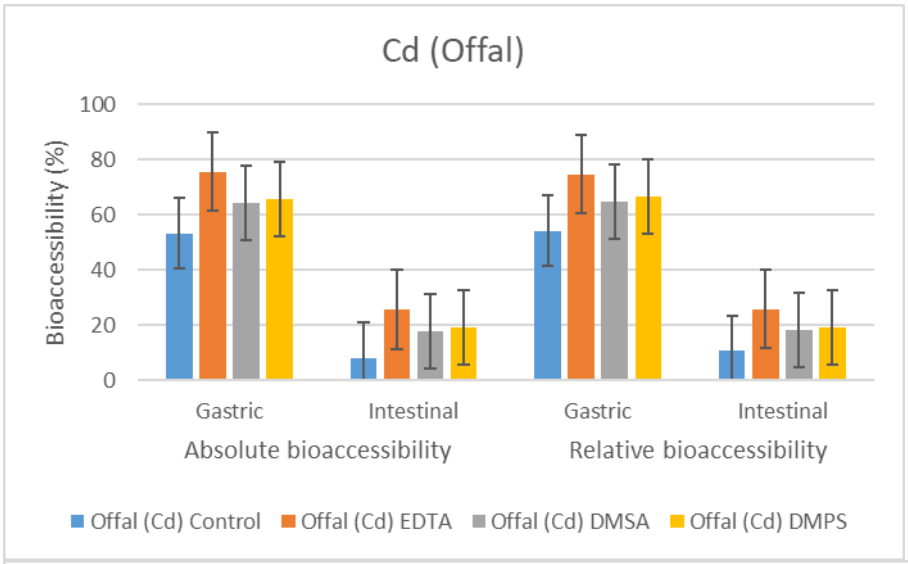
Offal (Cd)	Control	53.2	8.14	54.08	10.66
	EDTA	75.6	25.6	74.62	25.75
	DMSA	64.5	17.8	64.81	18.12
	DMPS	65.8	19.2	66.67	19.16
Fish (Hg)	Control	72.3	14.6	73.46	20.28
	EDTA	77.5	23.4	76.50	29.65
	DMSA	84.7	37.8	85.12	39.53
	DMPS	81.3	39.2	82.45	40.08
Complementary medicine (Pb)	Control	61.7	6.13	61.00	9.23
	EDTA	81.2	28.3	80.55	28.67
	DMSA	67.6	16.7	61.93	17.00
	DMPS	71.4	17.2	72.41	17.58

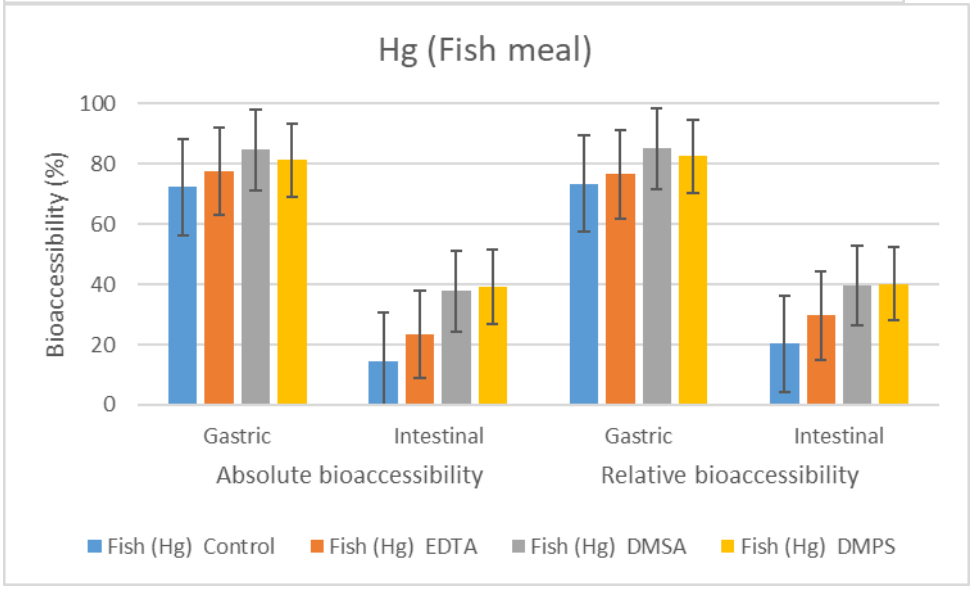
**Absolute bioaccessibility (%) = (in vitro metal(loid)/ total metal(loid)) × 100*
where, in vitro metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by gastric phase or intestinal phase solutions, total metal(loid) = total metal(loid) content in the source material or reference metal sample added to the in vitro assay.

Relative metal(loid) bioaccessibility (%) = ($AB_{metal(loid)\ source}$/$AB_{meta(loid)l\ ref}$) x 100
where, $AB_{metal(loid)\ source}$ = absolute bioaccessibility for metal(loid) source and $AB_{metal(loid)\ ref}$ = absolute bioaccessibility for reference metal(loid) compound.

In the present study, the addition of chelating agents has resulted in a significant increase in both gastric and intestinal bioaccessibility, and the effect of chelating agents on the intestinal and gastric bioaccessibility varied between the chelating agents and metal(loid) species. Generally, DMPS caused a greater increase in the solubility and bioavailability of heavy metal(loid)s than did DMSA and EDTA. The increase in solubility of heavy metal(loid) sources varied between the chelating agents. It followed Cd > Pb > Hg > As for DMPS, Pb > Cd > Hg > As for DMSA, and As> Cd > Pb > Hg for EDTA (Table 6.5; Figure 6.2).




Cd (Offal)
Bioaccessibility (%)
100
80
60
40
20
0
Gastric
Intestinal
Gastric
Intestinal
Absolute bioaccessibility
Relative bioaccessibility
Offal (Cd) Control
Offal (Cd) EDTA
Offal (Cd) DMSA
Offal (Cd) DMPS


Hg (Fish meal)
Bioaccessibility (%)
100
80
60
40
20
0
Gastric
Intestinal
Gastric
Intestinal
Absolute bioaccessibility
Relative bioaccessibility
Fish (Hg) Control
Fish (Hg) EDTA
Fish (Hg) DMSA
Fish (Hg) DMPS

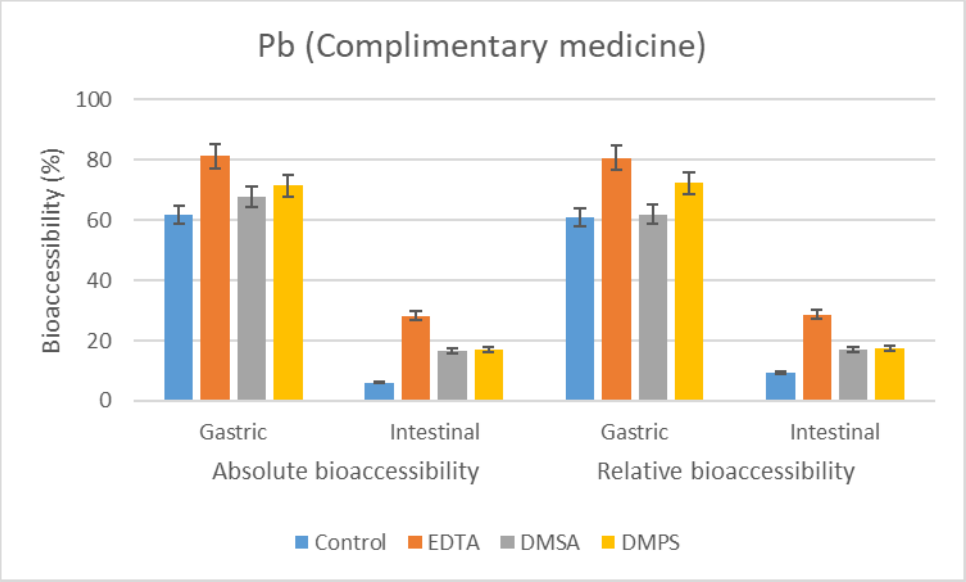


Figure 6.2 Effect of chelating agents on the gastric and intestinal bioaccessibility of As, Cd, Hg and Pb from respective sources

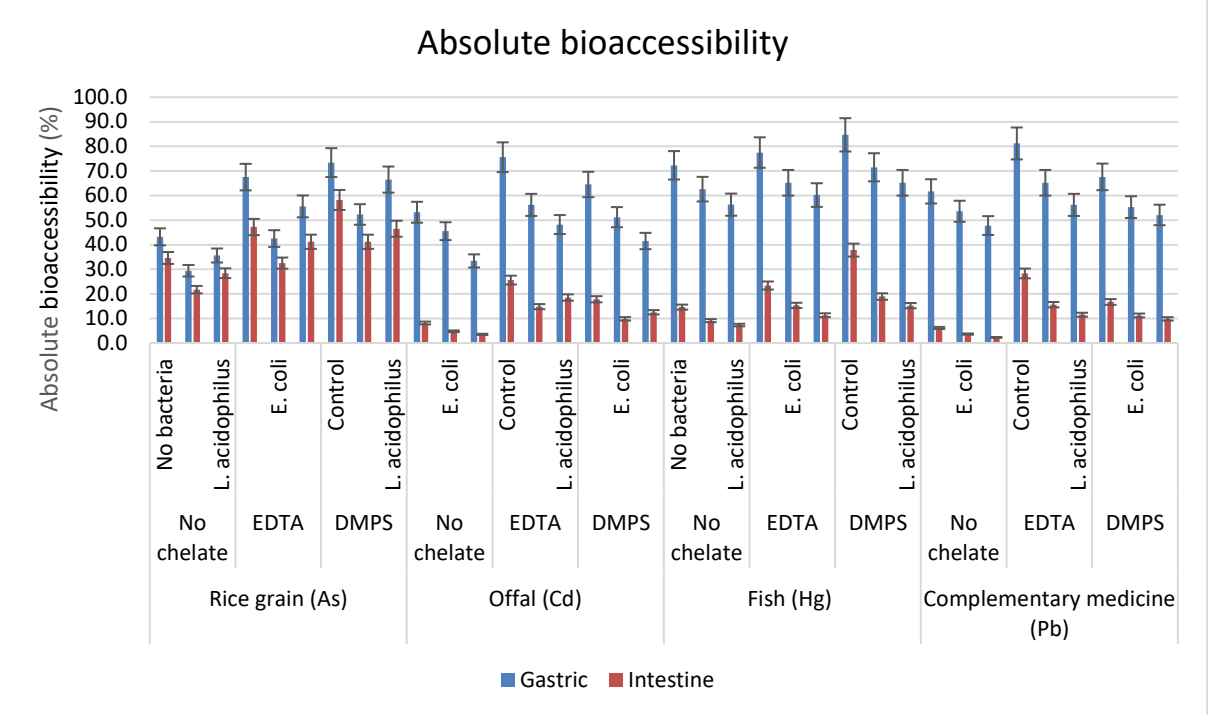
In the gastric phase, the relative bioaccessibility was comparable to absolute bioaccessibility both in the presence and absence of chelating agents (Table 6.5). However, in the intestinal phase, the relative bioaccessibility was slightly higher than the absolute bioaccessibility (Table 6.5), which is attributed to the decrease in the solubility of the metal(loid) salts at the higher pH (pH 5.8) in the intestinal phase. For example, Juhasz et al. (2016) observed that the relative Pb bioaccessibility values for soils in the intestinal phase (pH 6.5) estimated from the solubility of Pb acetate as a reference compound were approximately 10-fold greater than the corresponding absolute bioaccessibility values.

Although a vast number of studies have examined the effect of chelating agents on the mobilization of nutrient elements such as Fe and Zn (Ashmead, 2001; Krajmalnik-Brown et al., 2012; Dayyani et al., 2013), there have been only limited studies on the effect of chelating agents on metal(loid) bioaccessibility (Hogstr and Haux, 1991; Hughes and Poole, 1991). For example, a number of organisms including gut microbes produce metallothionein compounds which serve as a chelating agent (Vignesh and Deepe, 2017). Proteins with chemical structures similar to that of chelating agents have been isolated and characterized from a wide range of organisms (Demain and Vaishnav, 2009). The chelate-induced increase in bioaccessibility of metal(loid)s may lead to an increase in their bioavailability. For example, a number of studies showed that the administration of chelating agents produced marked effects

on the excretion of endogenous metal(loid)s (Cantilena Jr. and Klaassen, 1982; Tandon et al., 1994; Flora and Pachauri, 2010; Sears, 2013), confirming that these chelating agents increased the bioavailability and subsequent excretion of these metal(loid)s.

6.6.3 Interactive effect of chelating agents and gut bacteria on the bioaccessibility of metal(loid)s

The gastric and intestinal bioaccessibility data for both reference heavy metal(loid) samples and the orally ingested sources as impacted by chelating agents and gut bacteria are presented in Table 6.6 and Figure 6.3. In the absence of gut bacteria, there was no significant effect of the addition of chelating agents on the solubility of reference heavy metal(loid) samples. However, chelating agents increased the solubility of heavy metal(loid)s in the orally ingested sources, and the effect varied between the chelating agents and also amongst the heavy metal(loid) sources (Table 6.6). However, in the absence of chelating agents, there was a significant decrease in both gastric and intestinal bioaccessibility with the addition of bacteria, and the effect was more pronounced for the gastric bioaccessibility. There was a greater decrease in both gastric and intestinal phases in the presence of *L. acidophilus* than *E. coli.* The bacterial-induced reduction in the bioaccessibility followed: Pb > Cd > Hg > As (Figure 6.3).



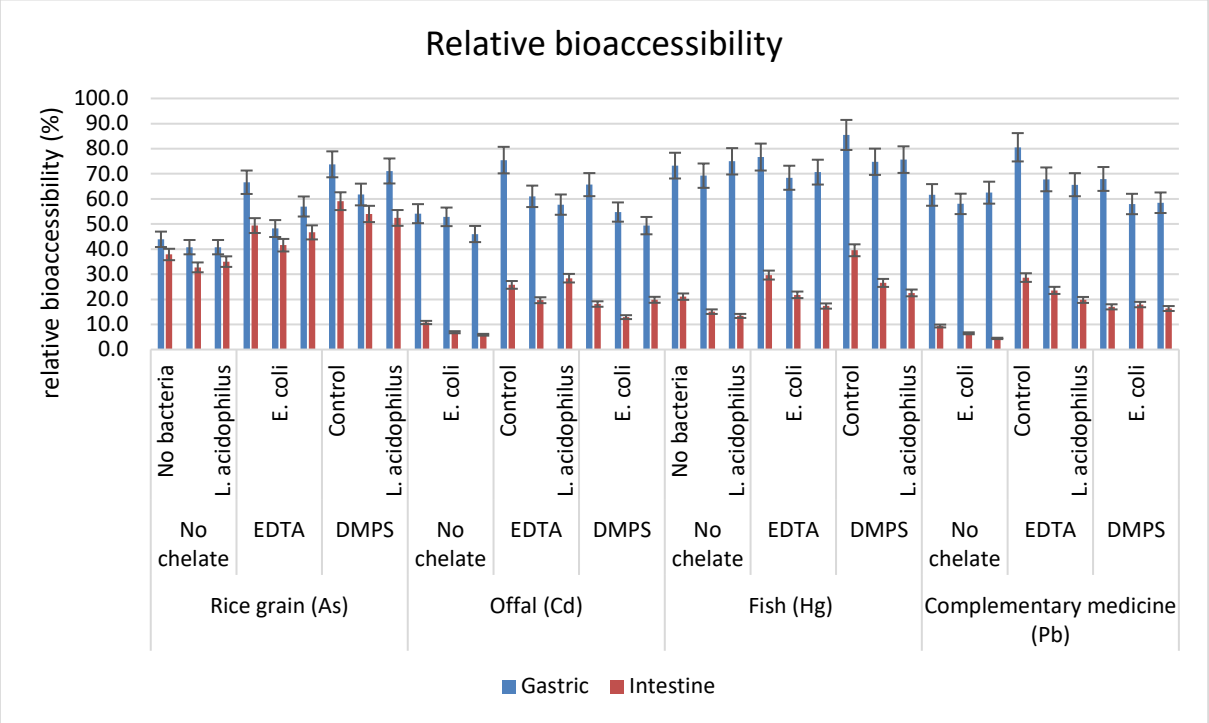


Figure 6.3 The interactive effect of gut bacteria and chelating agents on the gastric and intestinal bioaccessibility of heavy metal(loid)s

The interactive effects of chelating agents and gut bacteria on the gastric and intestinal bioaccessibility are presented in Figure 6.3. While the chelating agents increased the bioaccessibility, gut bacteria decreased the bioaccessibility of heavy metal(loid)s. The net interactive effect of their addition together was an increase in the bioaccessibility over the control treatment in the absence of either chelating agents or gut bacteria. The interactive effect of chelating agents and gut bacteria in increasing the bioaccessibility was more pronounced in the case of *L. acidophilus* than *E. coli* and also more pronounced in the presence of DMPS than EDTA. This indicates the metal(loid) complexes formed in the presence of chelating agents are not readily adsorbed by bacteria, which may contribute to the net increase in the bioaccessibility of metal(loid)s in the presence of both chelating agents and gut bacteria.

Table 6.6 Interactive effect of chelating agents and gut bacteria on absolute and relative bioaccessibility of metal(loid)s in standard metal(loid) and test samples

Metal(loid) sources	**Chelate**	**Gut bacteria**	**Absolute bioaccessibility***		**Relative bioaccessibility***	
			Gastric	**Intestinal**	**Gastric**	**Intestinal**
Standard reference samples						
Arsenic oxide	Control#	Control	98.4	91.4	NA	NA
		E. coli	72.1	66.3	NA	NA
		L. acidophilus	87.3	81.1	NA	NA
	EDTA	Control	101.3	95.6	NA	NA
		E. coli	88.2	78.2	NA	NA
		L. acidophilus	97.6	88.3	NA	NA
	DMPS	Control	99.5	98.5	NA	NA
		E. coli	84.7	76.3	NA	NA
		L. acidophilus	93.5	88.7	NA	NA
Cadmium acetate	Control	Control	98.3	75.6	NA	NA
		E. coli	86.1	69.3	NA	NA
		L. acidophilus	72.6	59.6	NA	NA
	EDTA	Control	100.2	99.4	NA	NA
		E. coli	92.1	75.3	NA	NA
		L. acidophilus	83.5	65.1	NA	NA
	DMPS	Control	98.2	98.2	NA	NA
		E. coli	93.5	76.2	NA	NA
		L. acidophilus	84.1	63.1	NA	NA
Mercuric chloride	Control	Control	98.7	69.3	NA	NA
		E. coli	90.4	60.3	NA	NA
		L. acidophilus	75.1	54.5	NA	NA
	EDTA	Control	101.1	78.9	NA	NA
		E. coli	95.3	70.2	NA	NA
		L. acidophilus	85.2	65.2	NA	NA
	DMPS	Control	99.1	95.6	NA	NA
		E. coli	95.6	71.2	NA	NA
		L. acidophilus	86.2	67.4	NA	NA

Lead acetate	Control	Control	100.2	65.4	NA	NA
		E. coli	92.4	56.1	NA	NA
		L. acidophilus	76.5	51.2	NA	NA
	EDTA	Control	100.8	98.7	NA	NA
		E. coli	96.2	66.2	NA	NA
		L. acidophilus	85.6	58.2	NA	NA
	DMPS	Control	99.5	98.2	NA	NA
		E. coli	95.4	62.5	NA	NA
		L. acidophilus	89.1	60.1	NA	NA
Test samples						
Rice grain (As)	Control	Control	43.2	34.6	43.90	37.86
		E. coli	29.4	21.7	40.78	32.73
		L. acidophilus	35.6	28.4	40.78	35.02
	EDTA	Control	67.5	47.2	66.63	49.37
		E. coli	42.5	32.5	48.19	41.56
		L. acidophilus	55.6	41.2	56.97	46.66
	DMPS	Control	73.4	58.2	73.77	59.09
		E. coli	52.3	41.2	61.75	54.00
		L. acidophilus	66.5	46.5	71.12	52.42
Offal (Cd)	Control	Control	53.2	8.14	54.12	10.77
		E. coli	45.5	4.78	52.85	6.90
		L. acidophilus	33.4	3.51	46.01	5.89
	EDTA	Control	75.6	25.6	75.45	25.75
		E. coli	56.2	14.8	61.02	19.65
		L. acidophilus	48.2	18.5	57.72	28.42
	DMPS	Control	64.5	17.8	65.68	18.13
		E. coli	51.2	9.84	54.76	12.91
		L. acidophilus	41.5	12.5	49.35	19.81
Fish (Hg)	Control	Control	72.3	14.6	73.25	21.07
		E. coli	62.6	9.1	69.25	15.09
		L. acidophilus	56.3	7.3	74.97	13.39
	EDTA	Control	77.5	23.4	76.66	29.66
		E. coli	65.2	15.3	68.42	21.79
		L. acidophilus	60.2	11.3	70.66	17.33
	DMPS	Control	84.7	37.8	85.47	39.54
		E. coli	71.5	18.9	74.79	26.54
		L. acidophilus	65.2	15.2	75.64	22.55
Complementary medicine (Pb)	Control	Control	61.7	6.13	61.58	9.37
		E. coli	53.6	3.63	58.01	6.47
		L. acidophilus	47.8	2.27	62.48	4.43
	EDTA	Control	81.2	28.3	80.56	28.67
		E. coli	65.2	15.6	67.78	23.56
		L. acidophilus	56.2	11.5	65.65	19.76
	DMPS	Control	67.6	16.7	67.94	17.01
		E. coli	55.3	11.2	57.97	17.92
		L. acidophilus	52.1	9.82	58.47	16.34

**Absolute bioaccessibility (%) = (in vitro metal(loid)/ total metal(loid)) × 100*
where, in vitro metal(loid) = Metal(loid) extracted from standard reference compounds or metal(loid) sources by gastric phase or intestinal phase solutions, total metal(loid) = total metal(loid) content in the source material or reference metal sample added to the in vitro assay.

Relative metal(loid) bioaccessibility (%) = ($AB_{metal(loid)\ source}$ /$AB_{meta(loid)l\ ref}$) x 100
where, $AB_{metal(loid)\ source}$ = absolute bioaccessibility for metal(loid) source and $AB_{metal(loid)\ ref}$ = absolute bioaccessibility for reference metal(loid) compound.
#The data for control (in the absence of gut bacteria and chelating agents) were used in Chapter 5.

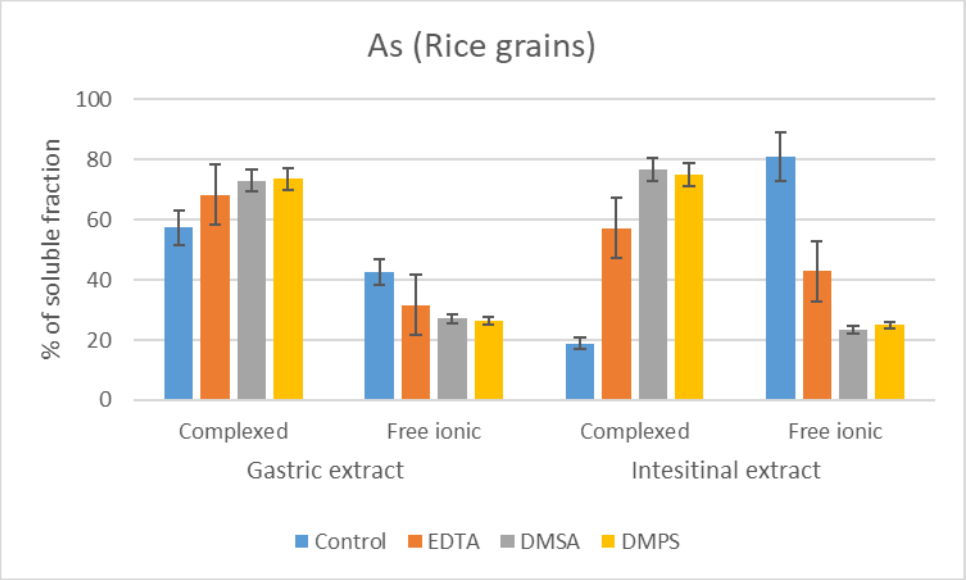
6.6.4 Effect of chelating agents on the distribution of metal(loid)s in the gastric and intestinal extracts

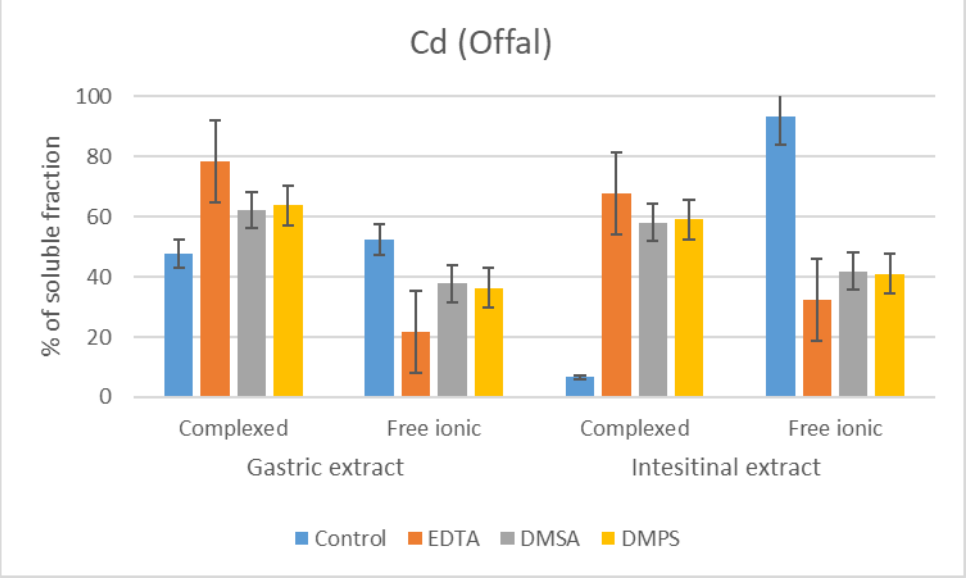
The distribution of solution metal(loid)s as free ions and complexes in the gastric and intestinal extracts as impacted by gut bacteria is presented in Table 6.7. Based on the bioaccessibility tests, the distribution of metal(loid)s as free and complexed ions in gastric and intestinal phases varied between the control and chelating agents (Table 6.7; Figure 6.4). Addition of chelating agents increased the percentage of complexed metal(loid)s both in gastric and intestinal extracts, and the effect was more pronounced in intestinal than gastric extracts. There was some difference in the extent of complexing of metal(loid)s amongst the chelating agents. Generally, DMPS caused a greater increase in the percentage of complexed heavy metal(loid)s than did DMSA and EDTA. It followed Cd > Pb > Hg > As for DMPS, Pb > Cd > Hg > As for DMSA, and As> Cd > Pb > Hg for EDTA (Figure 6.4).

Table 6.7 Percentage of total solution concentration of metal(loid)s as free metal(loid) ionic species in bioaccessibility tests

Metal(loid) sources	Chelating agent	Gastric		Intestinal	
		Complexed	Free ionic	Complexed	Free ionic
Standard samples					
Arsenic oxide	Control	73.4	26.6	45.6	54.4
	EDTA	88.9	11.1	58.9	41.1
	DMSA	92.1	7.9	69.2	30.8
	DMPS	93.2	6.8	71.2	28.8
Cadmium acetate	Control	67.3	32.7	23.6	76.4
	EDTA	93.9	6.1	64.5	35.5
	DMSA	82.1	17.9	54.2	45.8
	DMPS	83.2	16.8	53.4	46.6
Mercuric chloride	Control	72.1	27.9	27.6	72.4
	EDTA	82.9	7.1	57.4	42.6
	DMSA	93.2	6.8	67.1	32.9
	DMPS	94.1	5.9	66.2	33.8
Lead acetate	Control	43.7	56.3	19.8	80.2
	EDTA	93.5	6.5	68.2	31.8
	DMSA	89.4	10.6	56.7	43.3
	DMPS	90.2	9.8	55.4	44.6
Test samples					
Rice grain (As)	Control	57.3	42.7	18.91	81.09
	EDTA	68.3	31.7	57.2	42.8
	DMSA	72.9	27.1	76.5	23.5
	DMPS	73.5	26.5	75.1	24.9
Offal (Cd)	Control	47.6	52.4	6.72	93.28
	EDTA	78.2	21.8	67.8	32.2

	DMSA	62.3	37.7	58.1	41.9
	DMPS	63.7	36.3	59.0	41.0
Fish (Hg)	Control	61.2	38.8	11.5	88.5
	EDTA	78.2	21.8	45.6	54.4
	DMSA	85.1	14.9	65.7	34.3
	DMPS	86.3	13.7	64.8	35.2
Complementary medicine (Pb)	Control	34.3	65.7	8.19	91.81
	EDTA	68.9	31.1	58.2	41.8
	DMSA	56.2	43.8	49.8	50.2
	DMPS	57.1	42.9	49.1	50.9





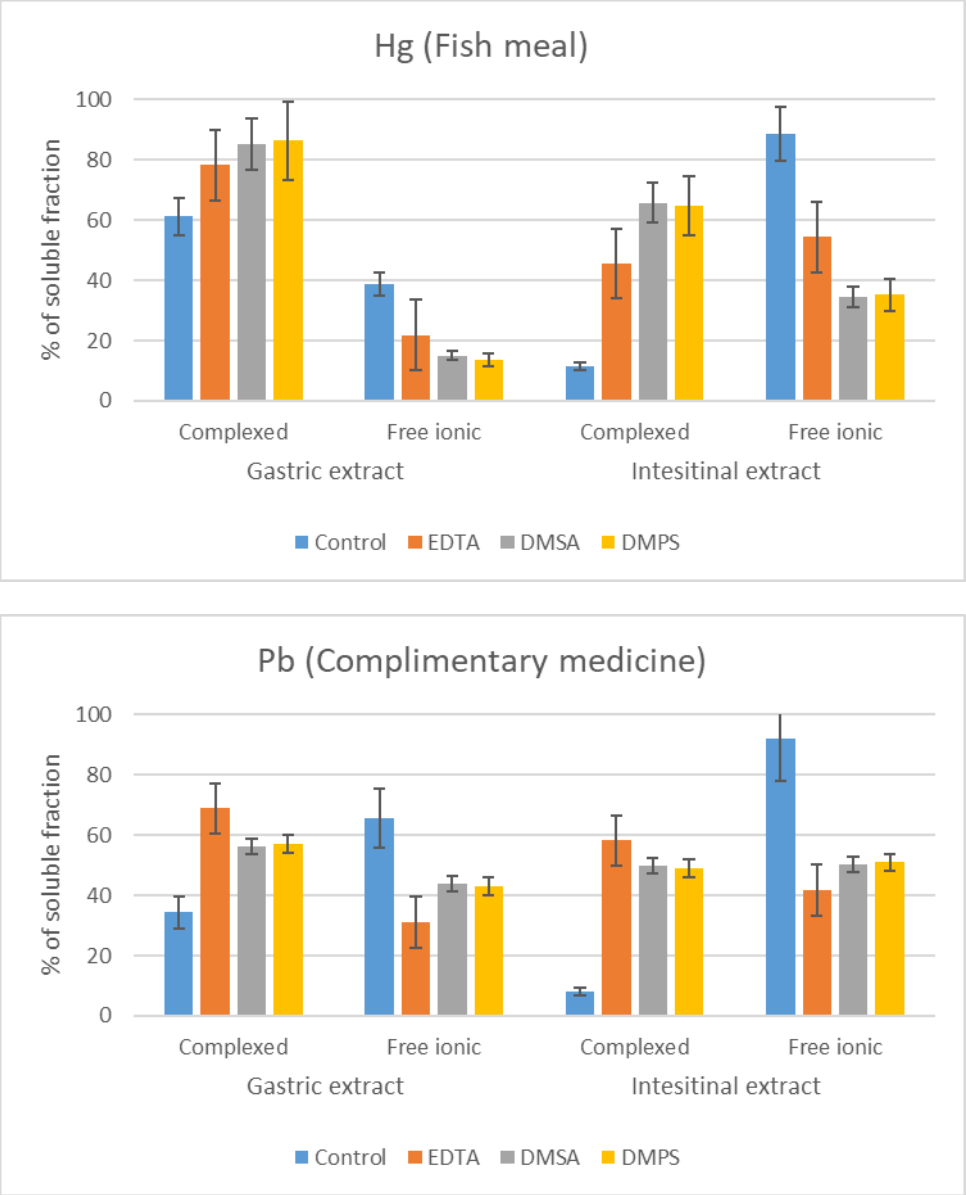


Figure 6.4 Effect of chelating agents on relative distribution of free ionic and complexed heavy metal(loid)s in the gastric and intestinal extracts

Chelating agents have been well known for complexing metal(loid)s thereby increasing their solubility and bioavailability. The increase in complexed metal(loid) concentration in solution due to the addition of chelating agent was higher when metal(loid)s were added as a salt solution than those derived from the oral ingestion sources. Although the change in the redistribution of complexed metal(loid) ions in solution caused by the presence of chelating agents may not impact the bioaccessibility of metal(loid)s, it is likely to impact the bioavailability of metal(loid)s because cellular uptake of most metal(loid)s occurs as free metal(loid) ions

(Zhao et al., 2005; Dean et al., 2012; Bird, 2015). However, there have been debate on the intestinal permeability of chelated metal(loid)s and their subsequent circulation in the blood (Aronson and Rogerson, 1972; Yeung et al., 2005; Flora and Pachauri, 2010). The effect of the chelate-induced distribution of metal(loid)s on their bioavailability as measured by intestinal permeability will be discussed in Chapter 7.

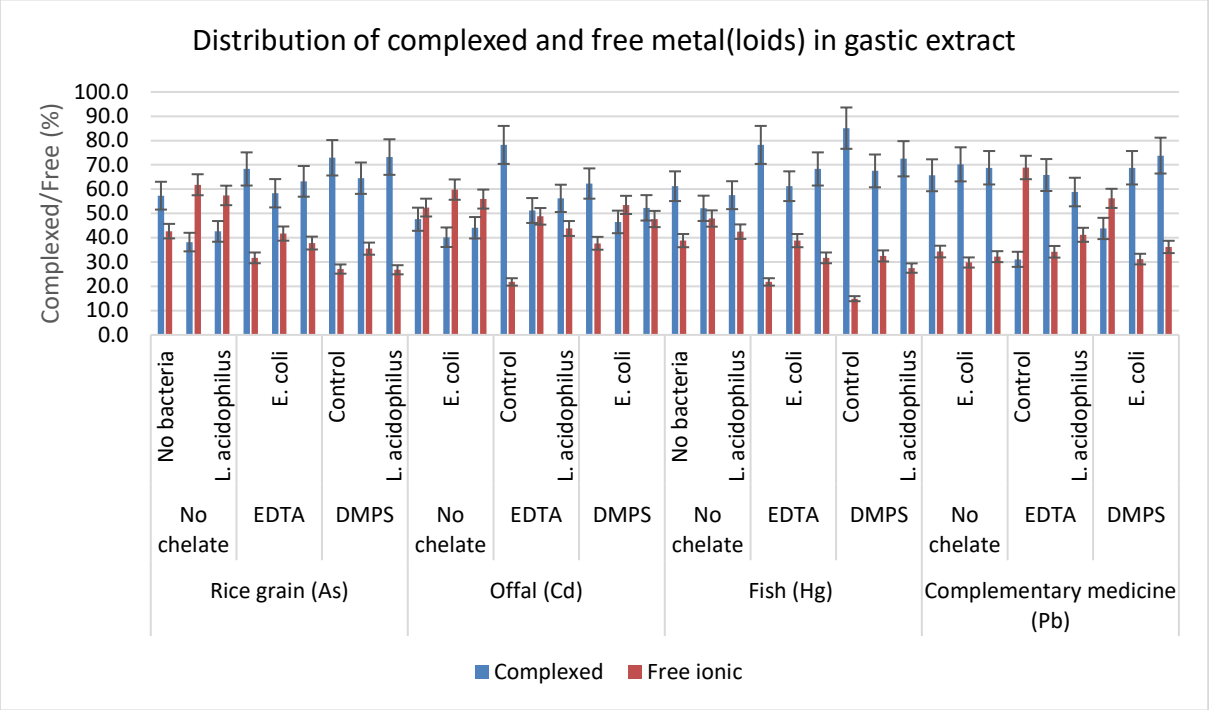
6.6.5 Interactive effect of chelating agents and gut bacteria on the distribution of metal(loid)s in the gastric and intestinal extracts

The distribution of solution metal(loid)s as free ions and complexes in the gastric and intestinal extracts as impacted by chelating agents and gut bacteria is presented in Table 6.8. As shown in Chapters 5, the distribution of metal(loid)s as free and complexed ions in gastric and intestinal phases varied between the gut bacteria (Chapter 5) and also between the chelating agents (Chapter 6) (Table 6.8; Figure 6.5). Addition of chelating agents increased the percentage of complexed metal(loid)s both in gastric and intestinal extracts, and the effect was more pronounced in intestinal than gastric extracts. In the absence of chelating agents, both gut bacteria increased the percentage of complexed metal(loid)s in the gastric and intestinal extracts. The concentration of complexed metal(loid)s was higher in the presence of *E. coli* than *L. acidophilus.* The interactive effects of chelating agents and gut bacteria on the distribution of solution metal(loid)s as free ions and complexes in the gastric and intestinal extracts are presented in Figure 6.5. The interactive effect of chelating agents and gut bacteria in increasing the concentration of complexed metal(loid)s was more pronounced in the case of *L. acidophilus* than *E. coli* and also more pronounced in the presence of DMPS than EDTA. This indicates both chelating agents and gut bacteria facilitated the complexation of metal(loid)s in gastric and intestinal extracts.

Table 6.8 Percentage of total solution concentration of metal(loid)s as complexed and free metal(loid) ionic species in gastric and intestinal extracts

Metal(loid) sources	Chelate	Gut bacteria	Gastric		Intestinal	
			Complexed	Free ionic	Complexed	Free ionic
Test samples						
Rice grain (As)	Control#	Control	57.3	42.7	18.9	81.1
		E. coli	38.2	61.8	14.2	85.8
		L. acidophilus	42.6	57.4	16.2	83.8
	EDTA	Control	68.3	31.7	57.2	42.8
		E. coli	58.3	41.7	23.8	77.2
		L. acidophilus	63.2	37.8	29.3	70.7
	DMPS	Control	72.9	27.1	76.5	23.5
		E. coli	64.5	35.5	36.2	63.8
		L. acidophilus	73.2	26.8	42.1	57.9
Offal (Cd)	Control	Control	47.6	52.4	6.72	93.2
		E. coli	40.2	59.8	5.7	94.3
		L. acidophilus	44.1	55.9	6.2	93.8
	EDTA	Control	78.2	21.8	67.8	32.2
		E. coli	51.2	48.8	15.2	84.8
		L. acidophilus	56.2	43.8	19.2	80.8
	DMPS	Control	62.3	37.7	58.1	41.9
		E. coli	46.5	53.5	11.2	88.8
		L. acidophilus	52.3	47.7	16.5	83.5
Fish (Hg)	Control	Control	61.2	38.8	11.5	88.5
		E. coli	52.1	47.9	8.6	91.4
		L. acidophilus	57.5	42.5	9.3	89.7
	EDTA	Control	78.2	21.8	45.6	54.4
		E. coli	61.2	38.8	11.6	88.4
		L. acidophilus	68.3	31.7	15.8	84.2
	DMPS	Control	85.1	14.9	65.7	34.3
		E. coli	67.5	32.5	19.8	80.2
		L. acidophilus	72.5	27.5	22.4	77.6
Complementary medicine (Pb)	Control	Control	34.3	65.7	8.1	91.8
		E. coli	29.8	70.2	6.3	93.7
		L. acidophilus	32.2	68.8	7.4	92.6
	EDTA	Control	68.9	31.1	58.2	41.8
		E. coli	34.2	65.8	17.2	82.8
		L. acidophilus	41.2	58.8	25.6	74.4
	DMPS	Control	56.2	43.8	49.8	50.2
		E. coli	31.2	68.8	11.5	88.5
		L. acidophilus	36.2	73.8	15.6	84.4

#The data for control (in the absence of gut bacteria and chelating agents) were used in Chapter 5.



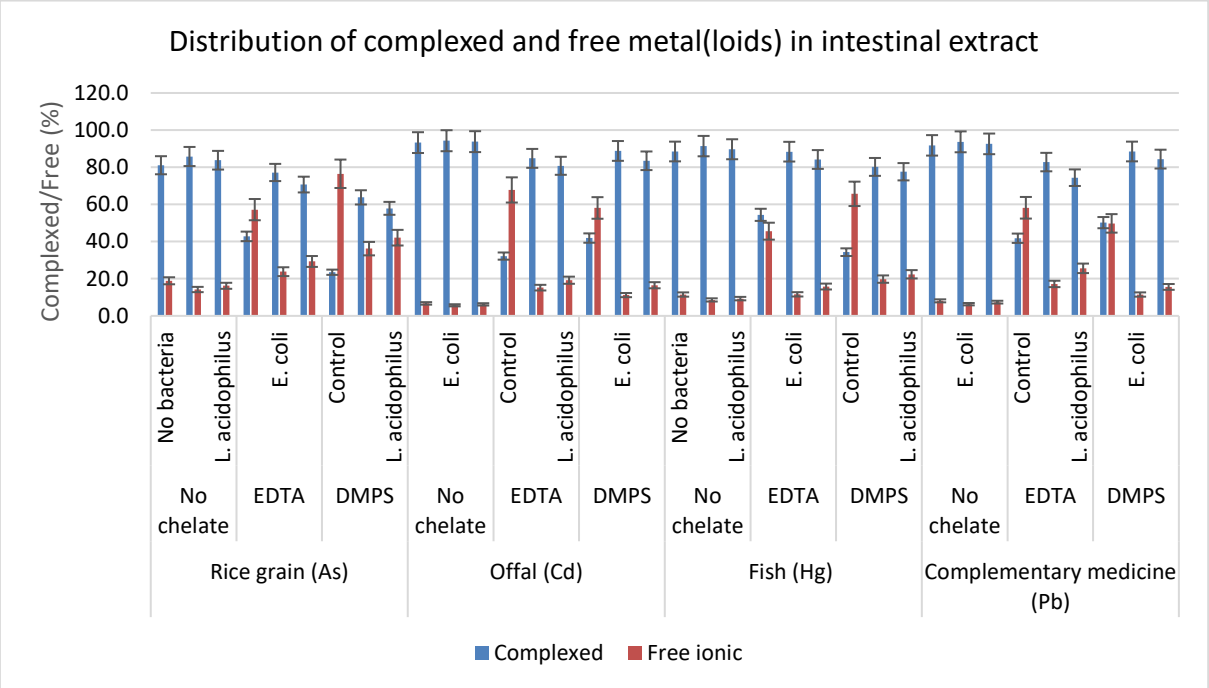
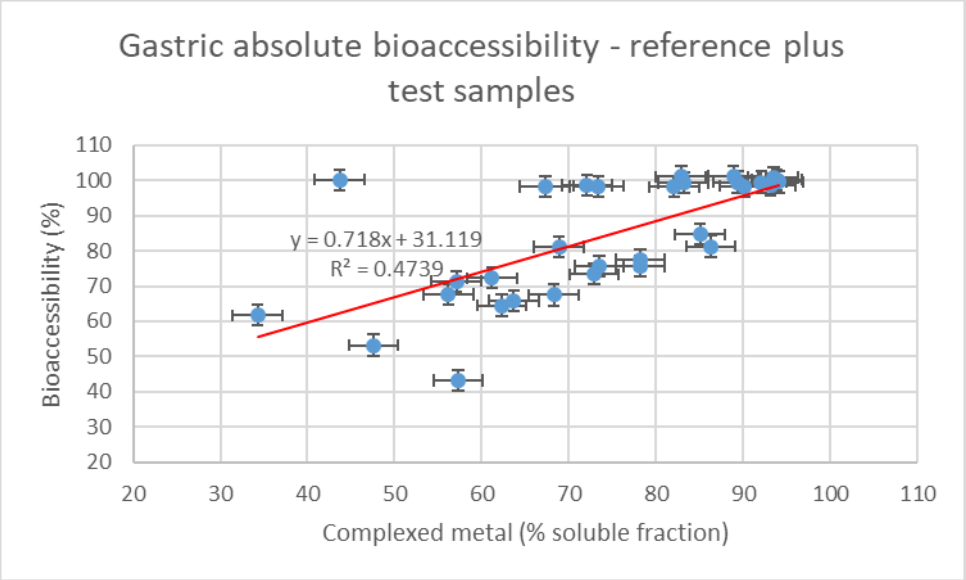


Figure 6.5 The interactive effect of gut bacteria and chelating agents on the distribution heavy metal(loid)s in the gastric and intestinal extracts

6.6.6 Mechanisms for chelate-induced modulation of metal(loid) bioaccessibility

The effect of chelating agents on the increase in the bioaccessibility of metal(loid)s in various sources could be attributed to the mobilization through solubilisation and complexation (Flora and Pachauri, 2010; Sears, 2013). There was a significant relationship between percentage complexed metal(loid)s and absolute gastric bioaccessibility (Figure 6.6). This indicates that the increase in the bioaccessibility caused by chelating agents is attributed to the complexation/chelation of metal(loid)s by the chelating agents and the subsequent increase in the solubilisation of these metal(loid)s from the respective metal(loid) sources.



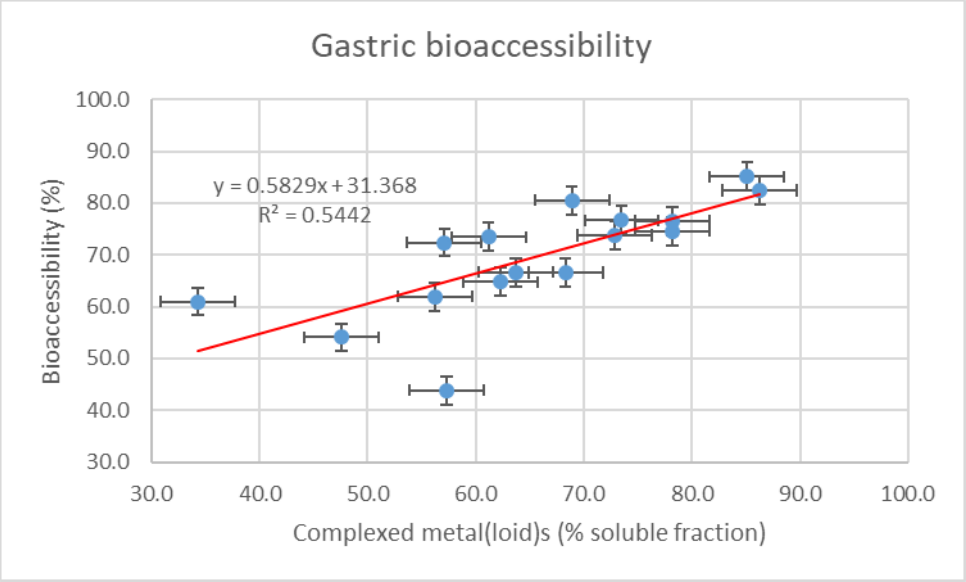


Figure 6.6 Relationships between percentage complexed metal(loid)s in the gastric extract and the absolute gastric bioaccessibililty as impacted by chelating agents in the absence of gut bacteria.

The complexing capacity of chelating agents depends on the nature of chelating agents, nature of metal(loid) ions and the environmental factors including pH and ionic strength of the substrate medium. For example, Ekholm et al. (2000) examined the effect of six chelating agents common in food (citric, lactic, malic, and ascorbic acids, glucose and xylitol) on the solubility of mineral elements from oat bran and flake samples after enzymatic digestion of starch and proteins. Adding citric, malic, or lactic acids increased the solubility of the mineral elements studied, and citric acid which is a tricarboxylic acid was found to be the most efficient chelating agent in solubilizing the mineral elements. Thus, the intestinal availability of mineral elements may be affected by dietary hydroxy acids such as citric and malic acids in high dietary fibre diets.

As discussed in Chapter 5, most microorganisms excrete extracellular polymeric substances (ESPs) that bind toxic metal(loid) cations, thereby protecting metal(loid)-sensitive biochemical components (Gupta and Diwan, 2017). The components of ESPs including proteins, polysaccharides, and nucleic acids, involve in the chelation of metal(loid)s (Pal and Paul, 2008; Guibaud et al., 2003). Additionally, metal(loid) cations such as Pb^{2+}, Cd^{2+} and Hg^{2+} forms strong soluble complexes with both natural and synthetic chelating compounds (Sears, 2013).

In biological systems, important factors that determine the functional attributes of metal(loid) chelates include: (i) intrinsic stability of the chelated metal(loid) complex; (ii)

chelating agent:metal(loid) ratios; (iii) stability chelated metal(loid) complex as a function of pH; (iv) competition from other metal(loid)s, and endogenous and exogenous chelating ligands; (v) charge and lipophilicity of chelating agents and chelated metal(loid) complex; and (vi) rates of hydrolysis and chelated metal(loid) complex formation (Sears, 2013).

Chelating compounds carry functional groups which are capable of combining with a metal(loid) by donating a pair of electrons followed by the formation of a ring with the metal(loid) ion as the closing member (Schubert, 1981; Goyer et al., 1995; Sears, 2013). Since most chelating agents contain protons (H^+), these compete with the metal(loid) ion for the chelating agent:

$$M + H_nL = ML + nH \qquad (6.1)$$

where, L represents the ligand and M the metal(loid) ion.

The fraction of the metal(loid) present as a chelated complex (ML), derives from mass action:

$$ML/M = K_fL \qquad (6.2)$$

where, K_f represents the formation constant or stability constant of the metal(loid) in chelate form (ML).

The equation shows that the fraction of metal(loid) (M) in chelate form {(ML)/[(M) + (ML)]}, is enhanced either by increasing the concentration of the chelating agent ligand (L), or by using chelating ligands producing chelated metal(loid)s with higher stability constant (K_f). It may not always be possible to increase the chelating agent ligand concentrations because some of the chelating agents used for treating metal(loid) poisoning, become toxic at high concentrations. However, in the choice of chelating agents for biological use, the stability constant (K_f) values offer greater flexibility for the release and excretion of bound metal(loid)s.

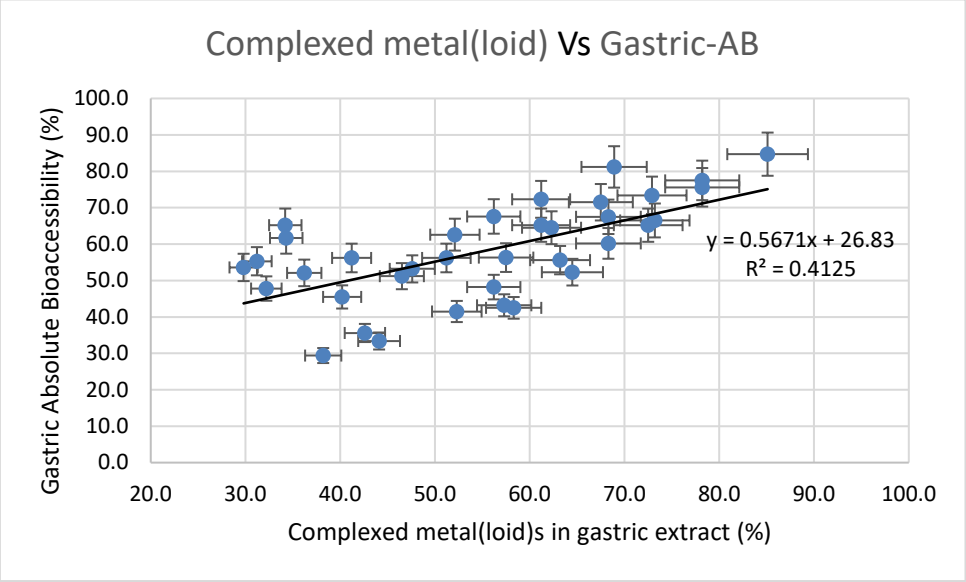
While the net ionic charge of the chelating agent defines its absorption, distribution and ability to reach the metal(loid) ion for binding, the net ionic charge of the chelated metal(loid) complex controls the elimination of the metal(loid) species from the specific site and excretion from the body (Sears, 2013). Thus, it is important that therapeutic chelating agents satisfy criteria that allow them to (Flora and Pachauri, 2010; Sears, 2013): (i) transport across physiological barriers into compartments where a toxic metal(loid) ion is concentrated, (ii) form a stable complex with the metal(loid) after removing it from the biological system, and (iii) form a chelation complex whose properties render it non-toxic and facilitate its excretion from the site of deposition and eventually from the body. Chelating agents are also used to increase the absorption of essential elements such as Fe and Zn in humans (Liu and Hider, 2002; Hider and Zhou, 2005; Hatcher et al., 2009; Udechukwu et al., 2016). The effect of chelating agents

generally depends on the stability of the chelating compounds in the intestine and their solubility in water or lipids.

6.6.7 Mechanisms for the interactive effect of chelate and gut bacteria on the modulation of metal(loid) bioaccessibility

The addition of gut bacteria in the absence of chelating agents decreased the bioaccessibility of heavy metal(loid)s which has been attributed mainly to the adsorption and precipitation reactions (Chapter 5). Whereas the addition of chelating agents in the absence of gut bacteria increased the bioaccessibility of heavy metal(loid)s which is attributed to the solubilisation and complexation reactions (Chapter 6). The addition of gut bacteria and chelating agents together resulted in a net increase in the bioaccessibility which indicates that the chelated metal(loid) species are not readily adsorbed by gut bacteria (Chapter 6). There was a significant relationship between percentage complexed metal(loid)s and absolute gastric bioaccessibility (Figure 6.7). In the presence of both gut bacteria and chelating agents, there was a net increase in the bioaccessibility of metal(loid)s. This indicates that the increase in the bioaccessibility caused by combined effects of chelating agents and gut microbes is primarily attributed to the complexation/chelation of metal(loid)s by the chelating agents and the subsequent increase in the solubilisation of these metal(loid)s from the respective metal(loid) sources.

Chelating agents have been well known for complexing metal(loid)s thereby impacting their solubility and bioavailability. Compared to free meta(loid)s ions, chelated metal(loid) species are less readily adsorbed by microbes, soil and sediments (Nowack et al., 1996; Macrellis et al., 2001; Martinez et al., 2001; Wood et al., 2016). Chelating agents are often used to extract metal(loid)s adsorbed to these environmental samples (Peters, 1999; Jiang et al., 2011; Palma et al., 2011; Sanderson et al., 2017; Beiyuan et al., 2018). For example, EDTA is often used to desorb metal(loid)s adsorbed onto soil samples, thereby facilitating the mobilization of these metal(loid)s and subsequent removal using soil washing techniques (Hong et al., 1995; Tandy et al., 2004; Jiang et al., 2011; Karthika et al., 2016). Similarly, it has been observed that chelate complexed heavy metal(loid)s are less toxic to microbes than free metal(loid)s ions which is attributed to the decrease in the uptake of former metal(loid) species by the microbes (Sterritt and Lester, 1980; Mazidji et al., 1992; Gadd, 2010).



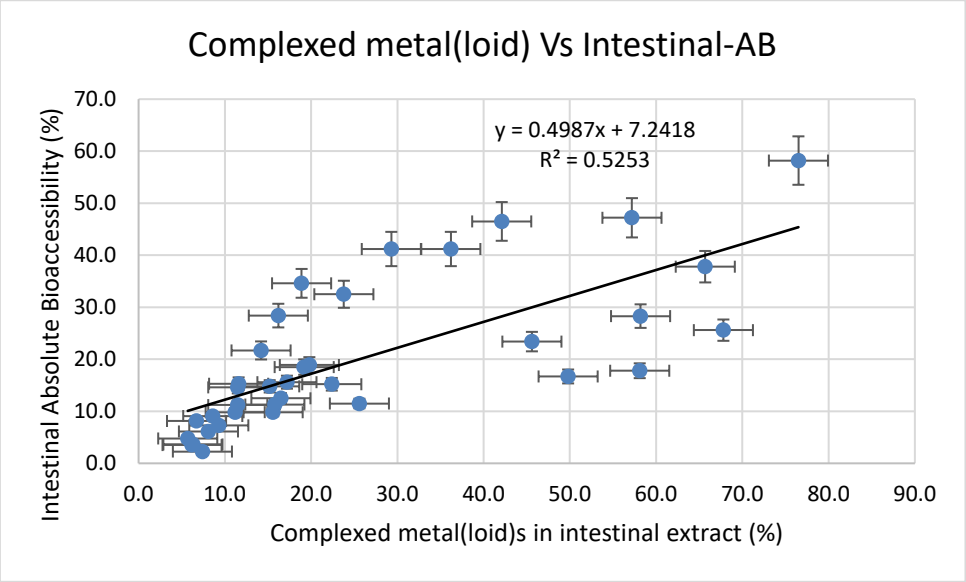


Figure 6.7 Relationship between percentage complexed metal(loids) and gastric and intestinal bioaccessibility as impacted by the interactive effect of gut bacteria and chelating agents

In chapter 5, it has been shown that the presence of gut bacteria in the gastric and intestinal extracts decreases the bioaccessibility of metal(loid)s which is attributed to the adsorption and precipitation of metal(loid)s by gut bacteria. However, there was an increase in the bioaccessibility of metal(loid)s in the presence of both gut bacteria and chelating agents. This has been attributed to the complexation of metal(loid)s by the chelating agents, and the

subsequent ‘unavailability’ of these chelated metal(loid) complexes to adsorption by gut bacteria. Chelating compounds are used to reduce the metal toxicity of microorganisms (Jin et al., 2017). For example, Malakul et al. (1998) quantified the inhibitory effect of Cd on the growth of *Pseudomonas putida* as impacted by modified-clay complex or a commercial chelating resin (Chelex). They have demonstrated that the toxicity of Cd to *P. putida* can be greatly reduced by the addition of the modified-clay complex or the chelating resin (Chelex). The reduction in Cd toxicity to *P. putida* can be quantitatively related to the Cd adsorption and complexation characteristics of modified-clay complex and chelating resin. The addition of these materials decreased the bioaccessibility of Cd, thereby decreasing the adsorption/absorption by *P. Putida.*

Although the change in the redistribution of complexed metal(loid) ions in solution caused by the presence of chelating agents and gut microbes may not impact the bioaccessibility of metal(loid)s, it is likely to impact the bioavailability of metal(loid)s because cellular uptake of most metal(loid)s occurs as free metal(loid) ions (Zhao et al., 2005; Dean et al., 2012; Bird, 2015). However, there have been debate on the intestinal permeability of chelated metal(loid)s and their subsequent circulation in the blood (Aronson and Rogerson, 1972; Yeung et al., 2005; Flora and Pachauri, 2010), which will be covered in Chapter 7.

6.7 Conclusions

The effect of three chelating agents on the solubility and bioaccessibility of four heavy metal(loid) sources in the absence and presence of gut bacteria was examined in this study. The results indicated that all the three chelating agents increased both gastric and intestinal bioaccessibility of As, Cd, Hg and Pb. The increase in bioaccessibility of metal(loid)s varied amongst the heavy metal(loid)s and also the chelating agents. The effect of chelating agents generally depends on the stability of the chelated metal(loid) complex in the intestine and their solubility in water or lipids. Chelating agents bind to heavy metal(loid)s in the body and they all have a very rapid mobilising activity (Sears, 2013). Metal(loid)s enter the bloodstream rapidly, which can overwhelm the organs of excretion such as the liver and kidneys. Consequently, instead of being excreted, they may be redistributed and reabsorbed by vital organs, potentially damaging these organs (Gerhardsson and Aaseth, 2016). Heavy metal(loid) chelating agents also bind to and remove essential elements such as Zn, Cu, Mn, Fe, Se and Mg which lead to mineral deficiency (Shah, 1981; Schwalfenberg and Genuis, 2015). The chelating agents also

impact the gut bacteria, thereby affecting the intestinal permeability of heavy metal(loid)s, which will be covered in Chapter 7.

The results also indicated that while the addition of chelating agents alone in the absence of gut bacteria increased both gastric and intestinal bioaccessibility of As, Cd, Hg and Pb, the addition of gut bacteria in the absence of chelating agents decreased the bioaccessibility of these metal(loid)s. The effect of chelating agents in increasing the bioaccessibility is attributed to the solubilisation and complexation reactions, and the gut bacteria-induced decrease in bioaccessibility is attributed to the adsorption and precipitation reactions (Chapter 5). When the chelating agents and gut bacteria were added together there was a net increase in the bioaccessibility of these metal(loid)s indicating that chelated metal(loid) species are not readily adsorbed by gut bacteria. There was a significant relationship between percentage complexed metal(loid)s and absolute gastric bioaccessibility which indicates that the increase in the bioaccessibility caused by combined effects of chelating agents and gut bacteria is primarily attributed to the complexation/chelation of metal(loid)s by the chelating agents and the subsequent increase in the solubilisation of these metal(loid)s.

Chapter 7

BIOAVAILABILITY OF HEAVY METAL(LOID)S AS MEASURED BY INTESTINAL PERMEABILITY

7.1 Introduction

Bioavailability is the fraction of a compound that reaches the systemic circulation after intestinal absorption, and it can be evaluated by means of *in vivo* or *in vitro* assays (Naidu et al., 2008; Kulkarni and Hu, 2011). Several *in vitro* cell-line-based (e.g., Caco-2, Human colorectal adenocarcinoma Tumour cell line with epithelial morphology (HT-29), and Madin-Darby canine kidney (MDCK)) or tissue-based (e.g., Everted intestinal ring) systems, and artificial membrane (e.g., Parallel artificial membrane permeability assay (PAMPA)) techniques are available to assess the potential intestinal permeability of nutrients, drug compounds and metal(loid)s (Sambuy et al., 2005: Polli, 2008; de Angelis and Turco, 2011; Lee et al., 2018). Intestinal epithelial cells are regarded as the first barrier for nutrients and contaminants entering the circulation system via the oral route. Caco-2 cells have been widely used to examine absorption mechanisms and to estimate permeability of drugs, nutrients, and minerals (Sambuy et al., 2005; Sarmento et al., 2012; Larregieu and Benet, 2014). The *in vitro* approach to the study of bioavailability can be determined by monitoring bioaccessibility, which is the maximum concentration soluble in simulated gastrointestinal media that is available for subsequent process of absorption into the intestinal mucosa (Naidu et al., 2008). Bioaccessibility of metal(loid)s is covered in Chapters 4 – 6 in this thesis. This approach of bioavailability can be improved with the Caco-2 cell model which mimics the process of intestinal cell retention and transport (van Breemen and Li, 2005; de Angelis and Turco, 2011) (Figure 7.1).

The Caco-2 intestinal cell line is established from human colon adenocarcinoma cells. When cultured, the cells spontaneously differentiate into monolayers of polarised enterocytes (Sambuy et al., 2005; Larregieu and Benet, 2014). The differentiated cell monolayer is polarised, with microvilli on the apical border, intercellular tight junctions (TJ), secretion of enzymes inherent to the brush border membrane, and the expression of transporters

characteristic of the small intestine in the apical and basolateral membranes (Dulanta et al., 2011; Ivanov, 2012; Lee, 2015). While this cell line is extensively used in drug and nutrient absorption research (Delie and Rubas, 1997; Artursson et al., 2001), more recently, it is also being used to assess the intestinal permeability and absorption of environmental contaminants (Oomen et al., 2003; Lefebvre et al., 2015; Zhai et al., 2016). The flux of drug compounds that is transported across a Caco-2 monolayer has been demonstrated to show good correlation with human *in vivo* absorption (Artursson et al., 2001; Sambuy et al., 2005; Larregieu and Benet, 2014).

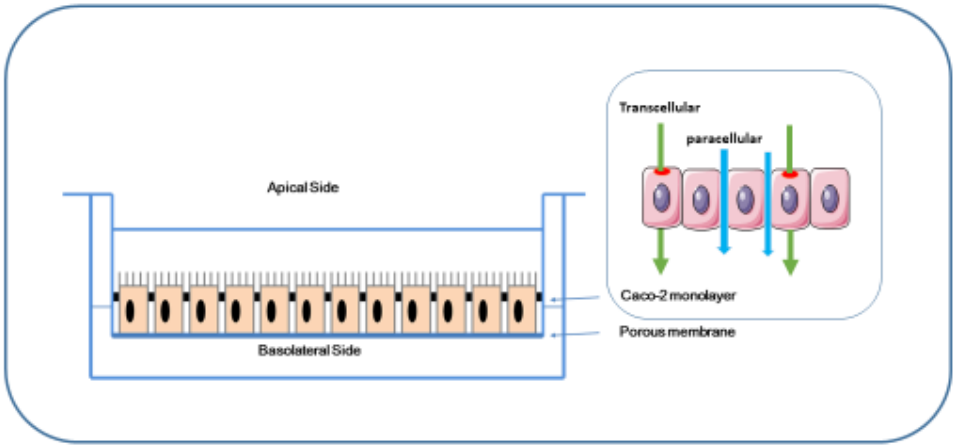


Figure 7.1 Schematic diagram of Caco 2 technique for monitoring bioavailability of heavy metal(loid)s as measured by intestinal permeability

The ability of a metal(loid) ion to pass through the gastrointestinal barrier is a key property to consider when examining the bioavailability and toxicity of heavy metal(loid)s (Foulkes, 1984; Citi et al., 1988; Foulkes, 2016). The mechanisms of metal(loid) permeation through biological barriers include passive diffusion (or paracellular) and active (or transcellular) transport pathways (Powell et al., 1994; Foulkes, 2016; Kiela and Ghishan, 2016). Passive diffusion of metal(loid)s is a physicochemical process that depends on properties such as lipophilicity, hydrogen bonding, stability constant (pKa) of the metal(loid) complex, molecular weight and test conditions, for example, the pH gradient and permeation time. In passive, paracellular absorption, the metal(loid) ions diffuse through tight junctions (TJ) into the basolateral spaces around enterocytes, and hence into blood (Schneeberger and Lynch, 1992; Tang and Goodenough, 2003). Active, transcellular absorption involves import of metal(loid)s

into the enterocyte, transport across the cell, and export into extracellular fluid and blood. Active transport involves active carrier mediated transportation and the use of energy to transport specific substrates across barriers, even against the concentration gradient (Kiela and Ghishan, 2016).

Intestinal absorption could be amplified after chronic metal(loid) exposures. For instance, cell death after chronic Cd exposure may cause leakage in the epithelial layer, resulting in larger amounts of Cd permeation (Zhai et al., 2016). Furthermore, Cd-induced disruption of TJs may lead to an intercellular leakage, allowing Cd to pass through the intestinal barrier (Zhai et al., 2016). Tight junctions are located in the apical part of the intestinal epithelial cells and are composed of a large group of proteins, including the scaffolding proteins zonula occludens-1 (ZO-1), and the transmembrane proteins, occludin and claudins, which are crucial in maintaining the barrier function (Schneeberger and Lynch, 1992; Arrieta et al., 2006; Karasov, 2017). When the expression of the TJ proteins is altered, the functionality of this physical barrier is compromised (Schneeberger and Lynch, 1992) and may lead to a leaky gut which is characterized by having an epithelium with increased permeability to compounds that diffuse from the lumen to the lamina propria (Arrieta et al., 2006; Michielan and D'Incà, 2015; Mu et al., 2017).

Results in Chapter 5 and 6 have demonstrated that both gut bacteria (Chapter 5) and chelating agents (Chapter 6) influence the bioaccessibility of heavy metal(loid)s. In this chapter, the effect of gut bacteria and chelating agents on the bioavailability of heavy metal(loid)s as measured by intestinal permeability will be reported. The process of intestinal absorption of heavy metal(loid)s may be affected by their binding with compounds like chelating agents that reduce their passage through the epithelium, and also with their binding or interaction with gut microorganisms (Shah, 1981; Sears, 2013). The gut microbes provide benefits to the host gut and prevent intestinal barrier dysfunction by: (i) modulating immune responses, (ii) alleviating oxidative stress (iii) reducing intestinal permeability by maintaining intestinal barrier integrity through expression and distribution of TJ proteins, and (iv) inhibiting abnormal necrosis of epithelial cells (Michielan and D'Incà, 2015; Mu et al., 2017; Citi, 2014). Chelating agents such as ethylenediaminetetraacetic acid (EDTA), 2,3-dimercapto-1-propanesulfonic acid (DMPS) and dimercaptosuccinic acid (DMSA) have been shown to increase metal(loid) bioaccessibility, thereby influencing the absorption and bioavailability of metal(loid)s in the intestine (Chapter 6). While Caco-2 cell technique involving intestinal epithelial cells has been widely used to study drug and nutrient absorption, it has been less

used to understand the intestinal permeability of metal(loid)s in the presence of gut microbes and chelating agents, which is the main focus of this chapter.

7.2 Objectives

The overall objective of this work reported in this chapter was to examine the bioavailability of orally ingested As, Cd, Hg and Pb as measured by intestinal permeability using a Caco-2 cell model. The specific objectives in this chapter were to:

(i) Evaluate the intestinal permeability of As, Cd, Hg and Pb in the presence of intestinal extract.

(ii) Examine the effect of gut microbes (*Escherichia coli, Lactobacillus acidophilus*) on the intestinal permeability of As, Cd, Hg and Pb in the presence of intestinal extract.

(iii) Investigate the impact of chelating agents (EDTA and DMPS) on the intestinal permeability of As, Cd, Hg and Pb in the presence of intestinal extract.

7.3 Hypothesis

(i) Bioavailability of heavy metal(loid)s as measured by intestinal permeability is impacted by metal(loid) binding with compounds or gut microbes that reduce their solubility (i.e., bioaccessibility) or their passage through the epithelium.

(ii) Gut bacteria modulate bioaccessibility of metal(loid)s as measured by intestinal permeability through their interactions with metal(loid)s via adsorption and speciation processes.

(iii) Chelating agents alter the bioaccessibility of metal(loid)s by forming complexes with metal(loid)s, thereby influencing the intestinal absorption of metal(loid)s.

7.4 Experiments

The major experiments and analyses conducted to test the hypotheses, and the treatments used in this chapter are listed in Table 7.1.

Table 7.1 The major experiments and treatments used in Chapter 7

No.	Title	Treatments
1	Intestinal permeability of metal(loid)s	4 metal sources (Arsenic oxide (As), Cadmium chloride (Cd), Lead acetate (Pb), Mercuric chloride (Hg)), with and without intestinal extract solution; Coca 2 cell permeability test
2	Effect of bacteria	2 gut bacteria (*Escherichia coli* and *Lactobacillus acidophilus*) with and without intestinal extract solution; Coca 2 cell permeability test
3	Effect of chelates	2 chelating agents (*Ethylene diamine tetraacetic acid (EDTA)* and 2,3-dimercapto-1-propanesulfonic acid (DMPS)) with and without intestinal extract solution; Coca 2 cell permeability test

7.5 Materials and Methods

7.5.1 Metal(loid) sources

The metal(loid) sources included in this study are arsenic oxide (As III), cadmium chloride (Cd), lead acetate (Pb), mercuric chloride (Hg) (Table 3.3, 4.3). These metal(loid) sources were selected because these are readily soluble and have often been used for *in vivo* metal(loid) bioaccessibility assessment (Bannon et al., 2009; Juhasz et al., 2016), and also in toxicity studies in the Integrated Risk Information System (IRIS, 2004). These metal(loid) sources were also used as reference samples for monitoring the bioaccessibility in Chapter 4-6. In this study, the orally ingested metal(loid)s sources tested for bioaccessibility (Chapters 4-6) were not used because of the interference from other components in these sources on both the Caco-2 cell growth and intestinal permeability.

7.5.2 Chelating agents and Gut microbes

Escherichia coli and *Lactobacillus acidophilus* were selected as gut microbes to study their effect on heavy metal(loid) bioavailability in the presence of intestinal extracts as measured by intestinal permeability test using Caco-2 cells. Ethylene diamine tetraacetic acid (EDTA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS) (both chelates at 1 mM concentration) were selected to study their influence on heavy metal(loid) bioavailability in the presence of intestinal extracts as measured by intestinal permeability test using Caco-2 cells (Table 7.1).

7.5.3 Cell culture

The Caco-2 cells were acquired from Hunter Medical Research Institute, University of Newcastle, Australia. The Caco-2 cells were routinely grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 containing glucose (4.5 g L^{-1}) and L-glutamine (0.6 g L^{-1}) and supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) nonessential amino acids, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid), 100 U mL^{-1} of penicillin, 0.1 mg mL^{-1} of streptomycin, 0.0025 mg mL^{-1} of fungizone, and 1 mM sodium pyruvate (Artursson et al., 2001; Phillips and Arena, 2003). The cell lines were incubated at 37°C, in a humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every 2−3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 mg L^{-1}) followed by reseeding at a density of 0.5 × 10^6 cells/cm^2 (Figure 7.2).

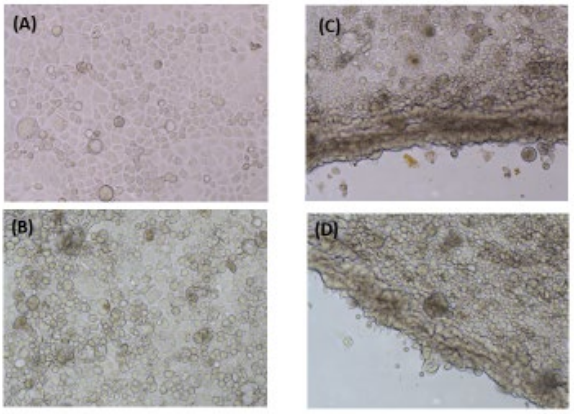


Figure 7.2 Coca-2 epithelial cell organisation. Cells were cultured on permeable membrane filter support for 20 days. Healthy cells (A) and their attachment to the membrane plate (B) in the presence of reference metal(loid) samples. Damaged cells (C) and their attachment to the membrane plate (D) in the presence of metal(loid) source samples used in bioaccessibility tests. Hence only reference metal(loid) samples were used for the bioavailability tests as measured by intestinal permeability using Caco-2 cell technique

7.5.4 Cell retention, transport and permeability test

The cell retention, transport, and permeability tests were performed in two chamber wells with polyester membranes (diameter 24 mm; pore size 0.4 μm; Transwell, Costar Corp., NY) (Phillips and Arena, 2003). In this system, the cells are kept on a porous support that separates the well into two compartments: apical and basal (or basolateral) chamber wells. The Caco-2 cells were seeded at 0.5×10^6 cells/cm^2. The media, Dulbecco's Modified Eagle's medium (DMEM) with 5% FBS (0.5 mL in apical and 1.5 mL in basolateral compartments), was changed every 2 days until cell differentiation was achieved, with mature Caco-2 cells obtained after 17–19 days post seeding. Metal(loid) permeability/transport test was performed 21 days post seeding of Caco-2 cells. The filter insert (i.e. apical chamber) was rinsed with DMEM (without Phenol Red) pH 7.2 supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and 15 mM L-glutamine, and allowed to equilibrate at 37°C for 15 min in the incubator. The test solutions contained 250 mg mL^{-1} Fluorescein isothiocyanate (FITC)-dextran (Mw 4400) (FD-4) as a paracellular marker. The test solutions also contained gut bacteria (*E. coli* and *L. acidophilus*) and chelating agents (1 mM EDTA and DMPS). For the uptake (retention and transport) assay with cells, the intestinal extract of metal(loid)s was heated for 4 min at 100 °C to inhibit sample proteases and then cooled by immersion in an ice bath.

The permeability tests were initiated by replacing the apical (0.5 mL) buffer with the test intestinal extract metal(loid) solutions. The test solutions were diluted with DMEM medium (1:3) before adding to the apical compartment. To diminish the unstirred water layer, transport experiments was carried out under agitation (70 Hz) in a plate shaker maintained at 37°C. A 500 μL sample was collected from the basolateral (1.5 mL) chamber at every 20min and replaced with fresh buffer. Sampling of basolateral solution was continued for 120min period. At the end of the assay, the cells were recovered using phosphate buffered solution, scraped, and lysed with 1% Triton X-100 (Merck, Germany). The metal(loid)s in the basolateral compartment and in the cells were quantified. The cell surfaces of the monolayers were washed three times with phosphate buffered saline (PBS), detached with a trypsin solution, and recovered with 0.5 mL of PBS (Calatayud et al., 2012b). The metal(loid) retention and transport percentages were calculated with respect to the initial quantity of metal(loid) added to the Caco-2 cell cultures. The respective samples were analysed for metal(loid) concentrations using ICP-MS as described in Chapter 3.

7.5.5 Distribution of metal(loid)s

The distribution of free and complexed metal(loid)s in the intestinal extract test solutions added to the apical compartment to measure intestinal permeability was measured using chelate/ion-exchange disk/cartridge (Empore, iminodiacetate functionalized poly(styrene divinylbenzene) - 234877 Aldrich) (Pu and Fukushi, 2013). The method for the measurement of distribution of free and complexed metal(loid)s in the intestinal extracts is described in Section 3.5.6 (Chapter 3).

7.5.6 Data analysis

The apparent or absolute permeability coefficient (P_{app} = cm/s) can be calculated from concentration-time profiles using the following equation (Youdim et al., 2003):

$$P_{app}\ (cm/s) = dC/dt * 1/A * V/C_o \qquad (7.1)$$

where, dC/dt (μg mL^{-1} s^{-1}) represents the flux across the monolayer (metal(loid) concentration (μg mL^{-1}) at various time (t in seconds) period); A (cm^2) the surface area of the monolayer; V (cm^3) the volume of the receiver chamber; and C_o (μg mL^{-1}) the initial metal(loid) concentration in the donor compartment.

The relative permeability values (P_{rel}) were estimated using Eq 7.2 to examine the effect of various treatments (gut microbes and chelating agents) on intestinal apparent permeability values (P_{app}).

$$P_{rel}\ (\%) = (P_{app}\ \text{treatment} / P_{app}\ \text{Control}) * 100 \qquad (7.2)$$

where P_{app} treatment is apparent permeability value for the test solution with a particular treatment (gut microbe or chelate addition) and P_{app} Control is apparent permeability value for the control treatment.

All experimental analyses were carried out using three replications. The permeability tests were conducted using Coca-2 cells grown for three passages. The passage number of a cell culture is a record of the number of times the culture has been subcultured, i.e. harvested and reseeded into multiple ‘daughter’ cell culture flasks (Phelan, 1998).

Statistical comparisons were made using analysis of variance (ANOVA) in Predictive Analytics SoftWare (PASW) statistics (version 18.0.0; SPSS, Inc., 2009, Chicago, IL) in order to examine the significant differences in various treatments. Duncan's multiple range test was also employed to compare the means of various treatments; variability in the data was presented as the standard deviation and a $p < 0.05$ was considered statistically significant.

7.6 Results and discussion

7.6.1 Transport of metal(loid)s and apparent permeability

The transport of metal(loid)s in the direction of apical to basolateral compartment of Caco-2 monolayer was assessed. Mass balance calculations were carried out to estimate the distribution of metal(loid)s in the basolateral well (permeable fraction), apical well, and Caco-2 cells (cell retention) (Table 7.2 and 8.3; Figure 7.3). The mass balance indicated that the total recovery of metal(loid) in the Coca-2 technique ranged from 89.7% to 105.3%, and there was a slight decrease in the recovery in the presence of gut bacteria. The total uptake values (cell retention plus basolateral transferred) of As, Cd, Hg and Pb in the Caco-2 cells were 81.9%, 32.9, 65.6% and 18.9%, respectively in the absence of intestinal extract and 67.3%, 17.3%, 61.2% and 3.45%, respectively in the presence of intestinal extract (Table 7.3) indicating that the intestinal extract decreased the uptake of metal(loid)s.

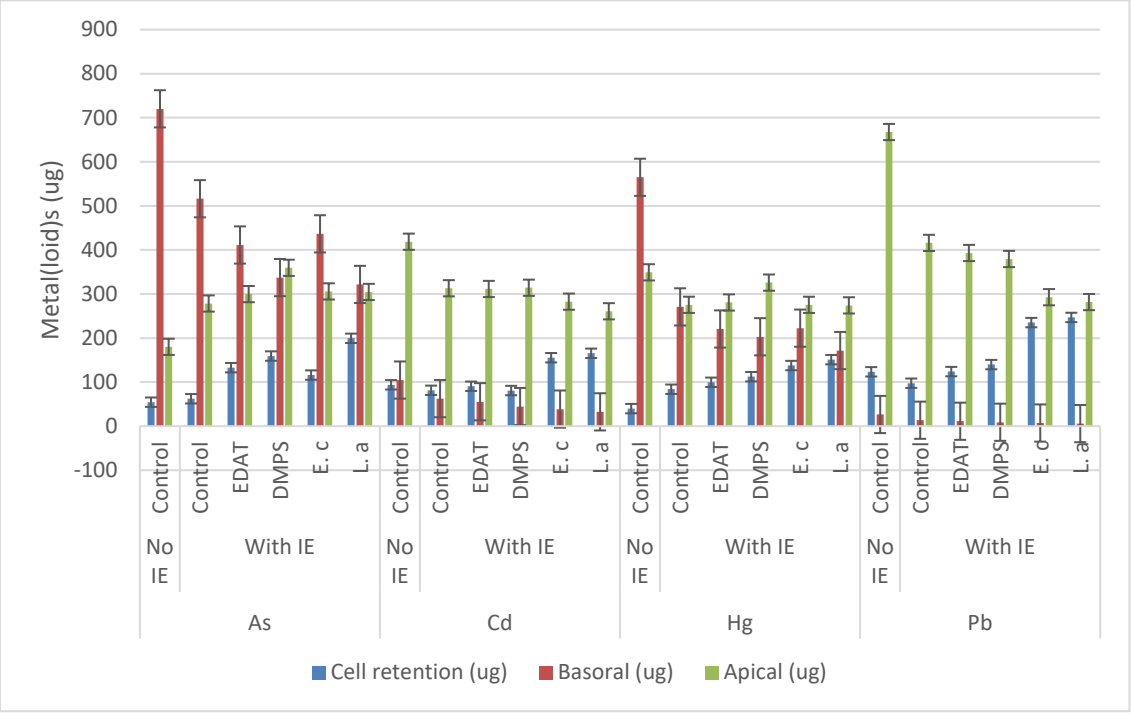


Figure 7.3 Distribution of metal(loid)s in the apical chamber, basolateral chamber and retention by cells during permeability test using caco-2 cell technique

Table 7.2 Mass balance of metal(loid)s during permeability test using caco-2 cell technique

Metal(loid) sources*	Chelating agents/Gut Bacteria	Metal(loid)				
		Total input (µg)**	Apical (µg)	Membrane retention (µg)	Basolateral (µg)	Total (% of input)
As – No IE	Control	945	180	54.2	720	101.1
As – with IE	Control	860	278	62.7	516	99.6
	EDTA	868	300	133	411	96.7
	DMPS	865	359	159	337	98.7
	E. coli	872	306	116	437	98.2
	L. acidophilus	856	305	199	322	95.4
Cd – No IE	Control	606	419	94.5	105	102.2
Cd – with IE	Control	460	313	81.2	62.6	99.2
	EDTA	472	311	91.4	55.2	96.2
	DMPS	465	314	81.8	45.5	93.4
	E. coli	481	283	155	39.6	98.7
	L. acidophilus	470	261	165	32.4	96.3
Hg – No IE	Control	923	349	40.6	565	103.5
Hg – with IE	Control	635	276	84.5	271	99.1
	EDTA	647	281	100	221	91.6
	DMPS	653	326	112	203	97.8
	E. coli	640	275	138	222	99.1
	L. acidophilus	648	274	151	172	89.7
Pb – No IE	Control	796	668	123	27.5	103.2
Pb – with IE	Control	530	416	98.2	14.5	99.3
	EDTA	553	393	124	11.3	94.2
	DMPS	546	379	140	9.35	95.6
	E. coli	538	293	235	7.56	99.0
	L. acidophilus	546	282	247	6.85	96.1

**No IE = No Intestinal Extract; with IE = with Intestinal Extract*
*** Based on the measured concentrations in the test metal(loid) samples in the presence and absence of intestinal extract*
Ethylene diamine tetraacetic acid (EDTA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS); Escherichia coli and Lactobacillus acidophilus

The time-course transport of metal(loid)s from the apical to basolateral compartment of Caco-2 monolayer is shown in Figure 7.4. The amount of metal(loid) transported from apical to basolateral compartment increased linearly with time for all the metal(loid)s. The apparent permeability (P_{app}) values of metal(loid)s were estimated using Eq 7.1 from the time-course of relationship metal(loid) transport shown in Figure 7.4. The relative permeability values (P_{rel}) were estimated using Eq 7.2 to examine the effect of various treatments (gut microbes and chelating agents) on intestinal apparent permeability values (P_{app}).

Table 7.3 Percentage distribution of metal(loid)s during permeability test using caco-2 cell technique

Metal(loid) sources*	Chelating agents/Gut microbes	Total input (µg)**	Percentage of total metal(loid)		
			Apical	Membrane retention	Basolateral
As – No IE	Control	945	19.05	5.74	76.19
As – with IE	Control	860	32.33	7.29	60.00
	EDTA	868	34.56	15.32	47.35
	DMPS	865	41.50	18.38	38.96
	E. coli	872	35.09	13.30	50.11
	L. acidophilus	856	35.63	23.25	37.62
Cd – No IE	Control	606	69.14	15.59	17.33
Cd – with IE	Control	460	68.04	17.65	13.61
	EDTA	472	65.89	19.36	11.69
	DMPS	465	67.53	17.59	9.78
	E. coli	481	58.84	32.22	8.23
	L. acidophilus	470	55.53	35.11	6.89
Hg – No IE	Control	923	37.81	4.40	61.21
Hg – with IE	Control	635	43.46	13.31	42.68
	EDTA	647	43.43	15.46	34.16
	DMPS	653	49.92	17.15	31.09
	E. coli	640	42.97	21.56	34.69
	L. acidophilus	648	42.28	23.30	26.54
Pb – No IE	Control	796	83.92	15.45	3.45
Pb – with IE	Control	530	78.49	18.53	2.74
	EDTA	553	71.07	22.42	2.04
	DMPS	546	69.41	25.64	1.71
	E. coli	538	54.46	43.68	1.41
	L. acidophilus	546	51.65	45.24	1.25

**No IE = No Intestinal Extract; with IE = with Intestinal Extract*

*** Based on the measured concentrations in the test metal(loid) samples in the presence and absence of intestinal extract*

Ethylene diamine tetraacetic acid (EDTA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS); Escherichia coli and Lactobacillus acidophilus

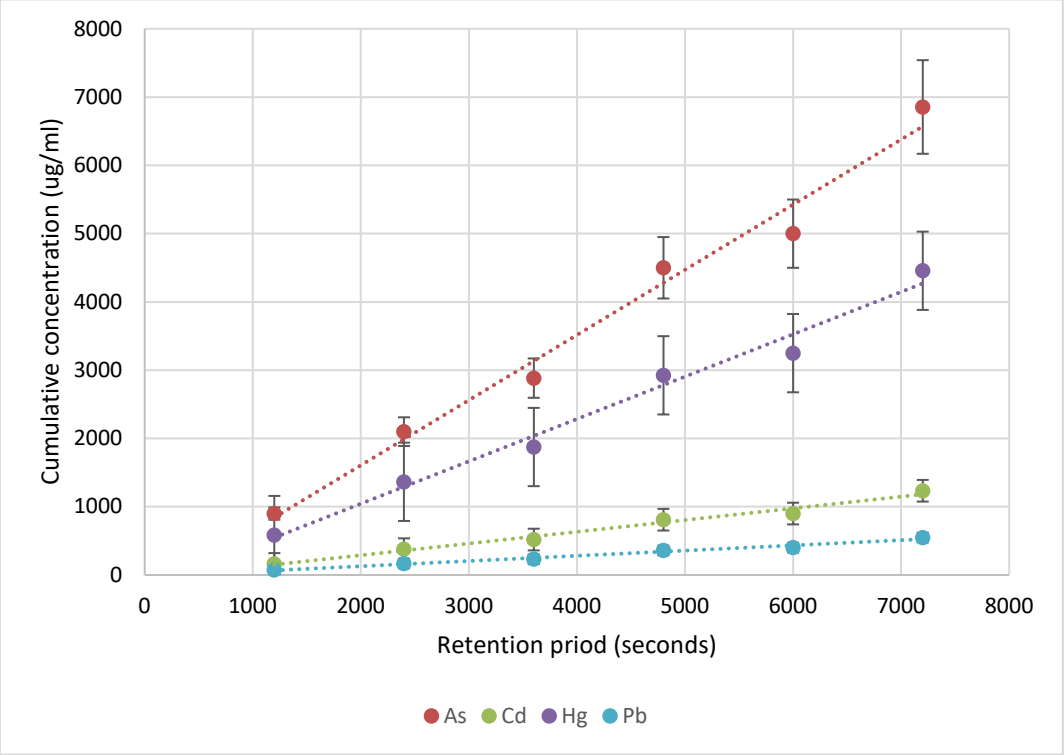


Figure 7.4 Time course of metal(loid) transport trough Caco-2 cells for estimation of Apparent permeability (App) of metal(loid)s

The apparent permeability (P_{app}) values of metal(loid)s as measured by Caco-2 cell model using Eq 7.1 are presented in Table 7.4 and Figure 7.5. The correlation between the absorbed fraction in humans (*in vivo*) and permeability across the Caco-2 monolayer (P_{app}) (*in vitro*) has been evaluated in many studies (Artursson and Karlsson, 1991; Yee, 1997; Artursson et al., 2001; Dahan et al., 2012; Bittermann and Goss, 2017). Yee (1997) suggests that a drug compound with P_{app} < 1 x 10^{-6} cm/s shows low absorption *in vivo* (0–20%), while a P_{app} of between 1–10 x 10^{-6} cm/s indicates moderate absorption (20–70%), and P_{app} > 10 x 10^{-6} cm/s suggests high absorption (70–100%).

Table 7.4 Absolute and relative Apparent Permeability of metal(loid)s as measured by Coca-2 cell technique and percentage of metal(loid)s complexed

Metal(loid) sources*	Chelating agents/Gut bacteria	Absolute App Permeability (x 10^6 cm/s)**	Relative App Permeability***	% Complexed metal(loid)s
As – No IE	Control	3.5600	100	10.2
As – with IE	Control	2.8907	81.2	40.4

	EDTA	2.5739	72.3	45.2
	DMPS	2.1542	60.5	48.1
	E. coli	2.6166	73.5	57.6
	L. acidophilus	2.2855	64.2	63.2
Cd – No IE	Control	0.8900	100	7.03
Cd – with IE	Control	0.7405	83.2	22.4
	EDTA	0.6728	75.6	35.3
	DMPS	0.5527	62.1	41.4
	E. coli	0.5367	60.3	65.4
	L. acidophilus	0.4931	55.4	68.7
Hg – No IE	Control	2.7500	100	8.01
Hg – with IE	Control	2.2055	80.2	22.2
	EDTA	1.9580	71.2	26.1
	DMPS	1.7078	62.1	24.3
	E. coli	1.9883	72.3	34.3
	L. acidophilus	1.7133	62.3	37.6
Pb – No IE	Control	0.1700	100	10.2
Pb – with IE	Control	0.1399	82.3	33.2
	EDTA	0.1229	72.3	36.4
	DMPS	0.1022	60.1	37.3
	E. coli	0.1059	62.3	63.3
	L. acidophilus	0.0904	53.2	65.6

NO IE = No Intestinal Extract; with IE = with Intestinal Extract; **Absolute Apparent permeability is calculated from Eq (8.1) (x 10^6 cm/s); * Absolute Apparent permeability is calculated from Eq (8.2); absolute permeability for metal(loid) alone (No IE) is taken as 100%*
Ethylene diamine tetraacetic acid (EDTA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS); Escherichia coli and Lactobacillus acidophilus

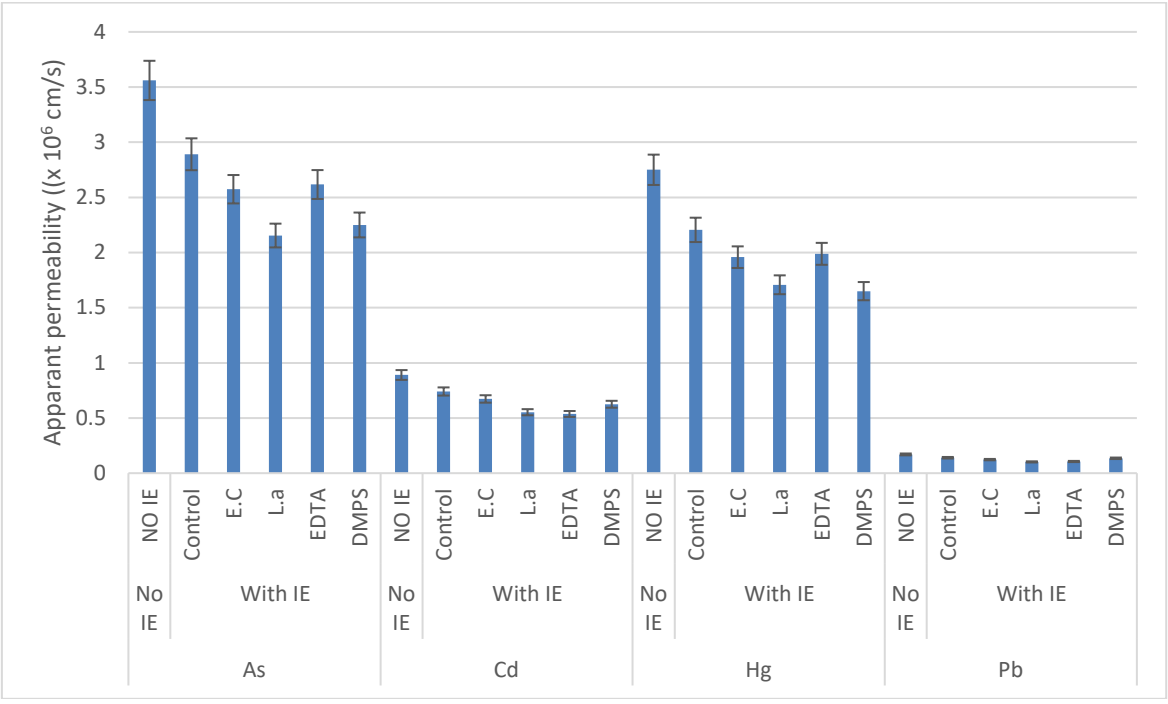


Figure 7.5 Effect of gut bacteria and chelating agents on the intestinal permeability of As, Cd, Hg and Pb

Addition of intestinal extract slightly decreased the transport of metal(loid)s from apical to basolateral compartment while increasing their cellular retention (Table 7.3; Figure 7.5). The apparent permeability coefficient (P_{app}) evaluates the velocity with which a solute crosses the cell monolayer. The P_{app} values for As, Cd, Hg and Pb were decreased by 7.5%, 6.3%, 7.9% and 8.2% in the presence of intestinal extract indicating less metal(loid) permeability. The P_{app} values varied between the metal(loid)s, and followed: As(III) > Hg(II)> Cd(II) > Pb(II).

Calatayud et al. (2011) found a linear increase of As transport in Caco-2 cells with increasing input concentration (1 µM - 67 µM), which suggests no saturable component in the transport within the concentration range tested in their study. The P_{app} values for As(III) and As(V) at 2 h for a concentration of 67 µM was 4.6 ± 0.3 × 10^{-6} cm/s and 1.00 ± 0.05 × 10^{-6} cm/s, respectively (Calatayud et al., 2011; Calatayud et al., 2012a). This indicates that As(III) species is more readily permeable through intestinal epithelial cells than As(V), which may contribute to higher toxicity of the former species to biota (Liu et al., 2016). However, Laparra et al. (2003, 2007) noticed a decrease in P_{app} value when the As(III) concentration in the donor compartment was increased suggesting the existence of a saturable intestinal transport system for As(III). The P_{app} value was 1.1 ± 0.8 × 10^{-6} cm/s after 2 h of incubation at a concentration of 67 µM which was lower when compared to Calatayud et al. (2011). Similarly, Liu et al. (2016) observed lower P_{app} values for As(V) (4.6 ± 0.2 × 10^{-7} cm/s) and As(III) (1.6 ± 0.1 × 10^{-6} cm/s) after 2 h of incubation at a concentration of 3 µM. Variations in apparent permeability coefficients amongst various studies were attributed to the differences in transport medium and cell conditions (e.g., culture conditions, passage).

The transport and absorption of Cd across Caco-2 monolayers in combination with the Ussing chamber technique was investigated by Schar et al. (2004). They have demonstrated that the exposure of Caco-2 cells to different Cd concentrations caused a reduction of Cd accumulation in cells from 38% (at 1 µM) to 13% (at 10 µM) indicating saturation of Cd binding sites at the outer apical or basolateral membrane. An earlier *in vivo* study by Foulkes (1991) showed a saturation of Cd-binding sites in the rat jejunum. The Cd transport across the Caco-2 monolayers in the present study was linear (Figure 7.4). This is in agreement with a study on Cd transport across Caco-2 cells by Blais et al. (1999). They found that Cd transport into the basolateral compartment was much slower and was undetectable during a lag time of about 60 min indicating a linear transport. This also suggests that Cd uses the cellular or carrier

pathway to move across the intestinal epithelium. In addition, after 24 h only a small part of the Cd accumulated in the Caco-2 cells (6% to 12%) and the remainder was found in the basolateral compartment.

In an *in vitro* digestion/Caco-2 cell model study, Chunhabundit et al. (2011) found that the cellular Cd uptake of inorganic Cd from $CdCl_2$ solution was significantly higher than that of the soluble Cd from food (pig kidney/kale) or $CdCl_2$ digests. Earlier studies reported that 25% of Cd was taken up (both retained in the cells and transferred through cells) by Caco-2 cells from $CdCl_2$ solution, while only 4–16% and 3.8–6.3% of Cd were taken up from leafy vegetables and infant food (Eklund et al., 2003; Chan et al., 2007; Fu and Cui, 2013). The lower Cd uptake from food suggests that the interaction or exchange between Cd and ligands in each food digest affect the intestinal Cd uptake.

In the present experiment, lowest apparent permeability values were obtained for Pb (Table 7.4; Figure 7.5). Using an *in vitro* digestion/Caco-2 cell model, Fu and Cui et al. (2013) studied the bioaccessibility and bioavailability of Pb in raw/cooked pakchoi (*Brassica rapa* L.) and Malabar spinach (*Basella rubra* L.). The Pb bioavailability was 9.4% in raw vegetables, and found to be significantly higher than the cooked vegetables (3.2%) after 4 hours of incubation. The Pb bioavailability in raw spinach was 4 times that of the cooked vegetable, and in the case of pakchoi, the difference was 2 times. Overall, the Pb bioavailability ranged from 2.0% to 13.0% for the leafy vegetables.

Differences exist in the bioavailability of metal(loid)s from assays chosen (*in vivo/in vitro*), food components, digested solution components, and the period of incubation for the chosen cell culture assay (e.g. Caco-2 cells). In rats, 1.4% and 0.9% of fish meal Pb were observed in kidney and liver, respectively (Yannai and Sachs, 1993). The absolute bioavailability values of ingested Pb acetate and mining waste Pb in rats were estimated to be 15% and 2.7%, respectively, based on blood Pb concentration measurements (Freeman et al., 1992). For Caco-2 cells, 30% of Pb was absorbed (Pb associated and transported by Caco-2 cells) from the digested soil solution (Oomen et al., 2003). After 24 hours, approximately 27% of Pb was retained in the cells and 3% were transported across the cell monolayer, without signs of approaching equilibrium, and a transcellular pathway was considered as the main mechanism of transport across the epithelial layer. Furthermore, since the free Pb^{2+} concentration in small intestinal fluid/chyme was negligible, results revealed the contribution of Pb phosphate and Pb bile complexes in chyme to the Pb flux towards the cells (Oomen et al., 2003).

Vázquez et al. (2015) evaluated the accumulation and transport of Hg(II) using Caco-2 cells as an intestinal epithelium model. The P_{app} values for Hg(II) after 120 min of exposure

increased with increasing concentration tested, though the increase was only significant for the 1 mg L^{-1} concentration (P_{app} 0.1 mg L^{-1} = 1 ± 0.13 × 10^{-6} cm/s; 0.5 mg L^{-1} = 1.4 ± 0.5 × 10^{-6} cm/s; 1 mg L^{-1} = 3.8 ± 0.32 × 10^{-6} cm/s). The metal(loid) showed moderate absorption, and its transport fundamentally took place via a carrier-mediated transcellular mechanism. A major observation was the cellular accumulation of up to 51% (21–51%) with respect to the Hg(II) initially added to the apical media, which was far greater than the transport to the basolateral side (9–20%). A similar observation of cell retention of Hg was found in the present study. Vázquez et al. (2015) noted that the *in vivo* studies using Hg(II) exhibits an absorption of <15%, which is lower than that deduced from the assays using Caco-2 cell line.

While Vázquez et al. (2014a,b) and Calatayud et al. (2012b) also observed increased cellular uptake of Hg added as a pure metal(loid) solution, the presence of luminal factors (e.g. bile salts, food components) reduces Hg transport across the intestinal epithelial cells as in the case of *in vivo* studies (Vázquez et al., 2013). Similarly, in a Caco-2 cell model, Calatayud et al. (2012b) found a higher cell retention (49–69%) and a much lower transport of bioaccessible fraction of swordfish Hg to the basal compartment (3–14%) after 2 and 4 hours. In a study by Vázquez et al. (2013), the components solubilised during gastrointestinal digestion of swordfish reduced the entry of CH_3Hg into Caco-2 monocultures and hence, resulted in reduced cellular accumulation. They demonstrated that in the case of inorganic $HgCl_2$ standard prepared in the gastrointestinal digestion blank, the presence of food matrix significantly increased the non-absorbed percentage (from 55% to ≥73%) and greatly reduced cell uptake (from 33% to 11%) during a period of 60 min.

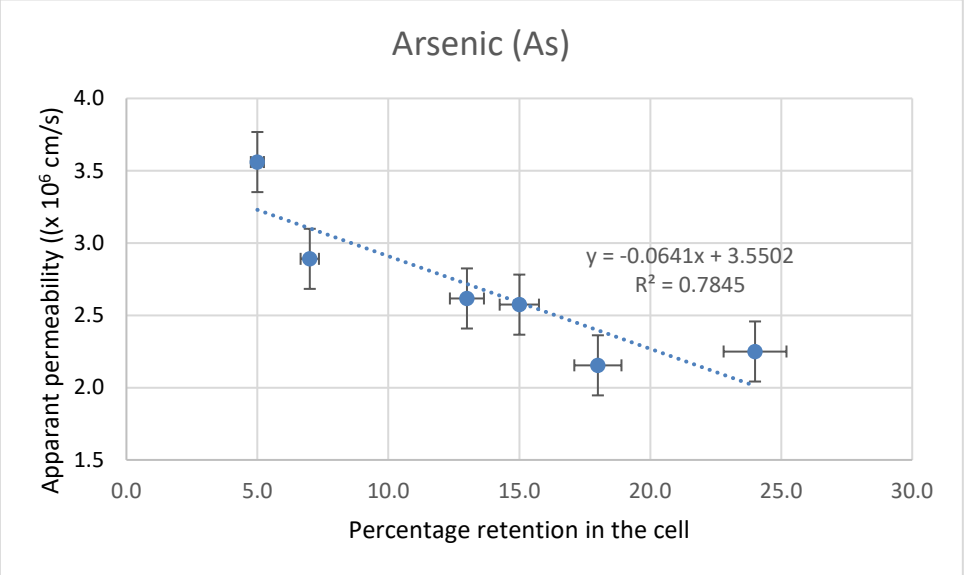
Overall, the results in the present study demonstrated lower metal(loid) transport in the presence of intestinal extracts which is related to some complexing components such as bile salts in the intestinal solution that affect metal(loid) absorption because of competition for transport or due to the formation of complexes with metal(loid), which has a lower transport rate (Jan et al., 2015). High retention of metal(loid)s in Caco-2 cells indicate that the intestinal epithelium acts as a barrier for metal(loid) absorption. The apparent permeability of metal(loid)s was in the order of: As(III) > Hg(II)> Cd(II) > Pb(II). While the anionic As transport can be passive and fast, the transport of remaining metal(loid)s which are cations, mostly occur by active transport, and hence can be slower than As.

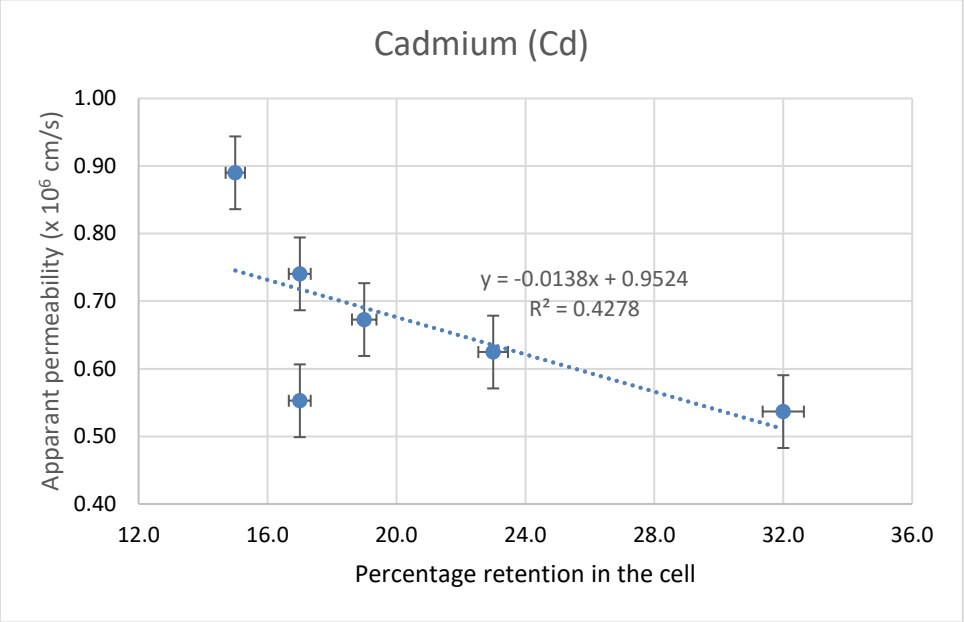
7.6.2 Effect of gut microbes on permeability of metal(loid)s

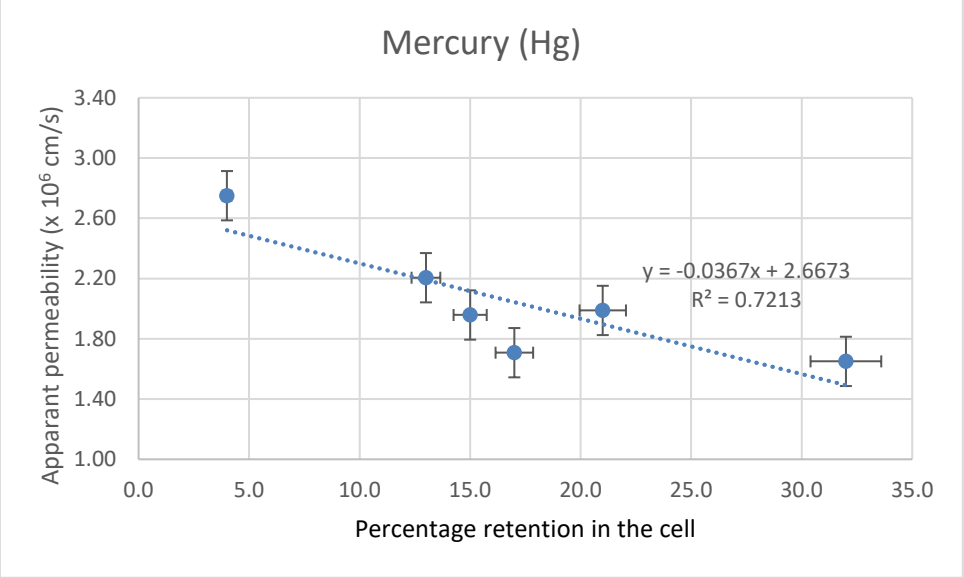
The ability of gut bacteria to adhere to mucus and/or intestinal epithelial cells is one of the major mechanisms protecting the host from contaminant invasion and adhesion (Thursby and

Juge, 2017). The effect is observed even if the bacterial adhesion is transient and does not lead to permanent intestinal colonisation (de Vos, 2015; Baumler and Sperandio, 2016; Sicard et al., 2017). One of the major objectives in this study was to determine the amount of metal(loid) transport across the Caco-2 cell monolayer in the presence of gut bacteria.

Treatment with gut microbes significantly reduced the permeability of metal(loid)s in Caco-2 cells as seen from the relative permeability (P_{rel}) values reported in Table 7.4. The apparent permeability (P_{app}) values calculated from Eq 7.1 were markedly reduced in the presence of gut microbes for all the metal(loid)s indicating low intestinal absorption (Table 7.4; Figure 7.5). The percentages of metal(loid) retained in the Caco-2 cell membrane and the metal(loid) complexed are presented in Table 7.4. There were significant relationships between the apparent permeability (P_{app}) values and the amount of metal(loid)s retained in the Caco-2 epithelial cells (Figure 7.6) and the amount of metal(loid) complexed (Figure 7.7). This indicates that metal(loids) retained by the epithelial cells may not be transported across the cells, and also only free metal(loid) species are transported across the cells (Mukhopadhyay et al., 2013; Jan et al., 2015).




Cadmium (Cd)
Apparant permeability (x 10⁶ cm/s)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
y = -0.0138x + 0.9524
R² = 0.4278
12.0
16.0
20.0
24.0
28.0
32.0
36.0
Percentage retention in the cell


Mercury (Hg)
Apparant permeability (x 10⁶ cm/s)
3.40
3.00
2.60
2.20
1.80
1.40
1.00
y = -0.0367x + 2.6673
R² = 0.7213
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
Percentage retention in the cell

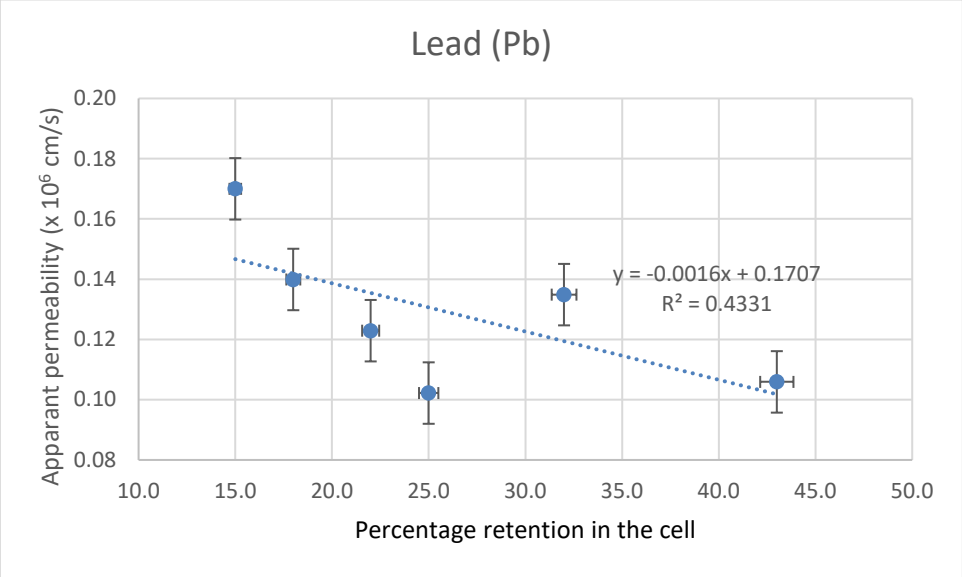


Figure 7.6 Relationships between percentage retention in the cell membrane and intestinal permeability of As, Cd, Hg and Pb

The effect of gut microbes on P_{app} varied both between the gut bacteria and also amongst the metal(loid)s. The adsorption of metal(loid)s by gut microbes was in the order of Pb > Cd > Hg > As (Chapter 5). In the presence of *L. acidophilus and E. coli*, the transport of metal(loid)s to the basolateral compartment decreased from 60.0% to 37.6% and 50.1% for As, from 13.6% to 6.89% and 8.23% for Cd, from 42.6% to 26.5% and 34.7% for Hg, and from 2.74% to 1.25% and 1.41% for Pb, respectively (Table 7.3). Correspondingly, the cellular retention of metal(loid)s was higher in the presence of gut microbes (Table 7.3). The results may be attributed to a direct protection of the intestinal barrier against the metal(loid)s or indirectly via intestinal metal(loid) sequestration by the gut microbes (Conlon and Bird, 2015; Thursby and Juge, 2017).

Using Caco-2 cells, Monachese (2012) compared the amount of Pb and Cd in the basolateral chamber in non-treated wells to *Lactobacilli* pre-treated wells and noticed a significant reduction (50% and 90% reduction in Pb and Cd, respectively) in measured metal(loid)s after a period of 5 hours. This observation greatly supports metal(loid) binding by *Lactobacilli* and reduced absorption by the Caco-2 cell line. Muhammad et al. (2018) recently demonstrated a notable Pb binding capacity and tolerance capability of *L. plantarum* KLDS 1.0344. Oral administration of both free and encapsulated KLDS 1.0344 significantly provided protection against induced chronic Pb toxicity by increasing faecal Pb levels and by decreasing blood Pb levels in mice.

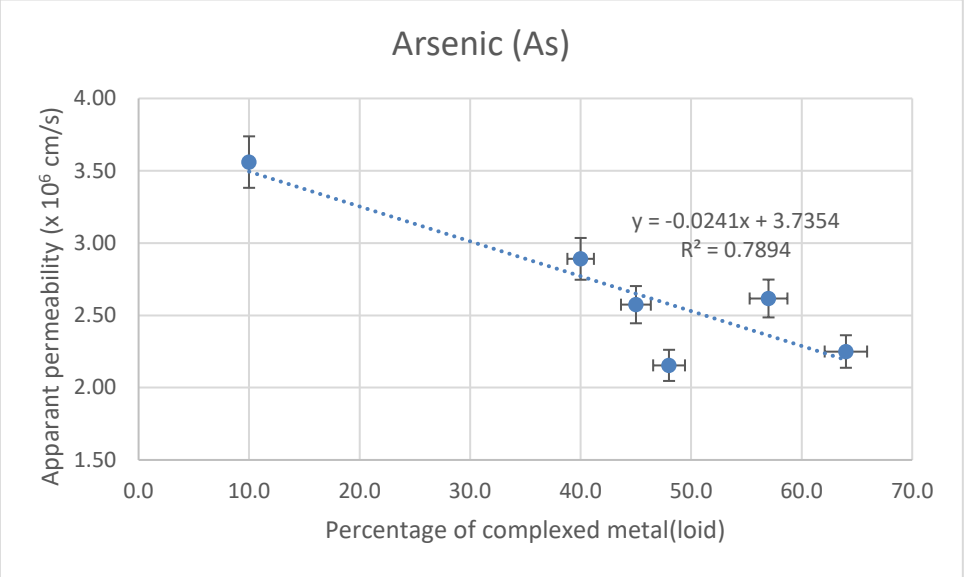
Caco-2 cell cultures have been widely used to investigate the adhesion of various gut microorganisms including *Lactobacillus* strains to epithelial cells (Lehto and Salminen, 1997; Gratz et al., 2004; Trinder et al., 2016). The gut microbes adhere to human intestinal cells via mechanisms which involve different combinations of carbohydrates and proteins on the bacterial cell surface (Greene and Klaenhammer, 1994; Sicard et al., 2017). The adhesion ability of gut microbes may differ in various cellular models used for examining the intestinal permeability of drugs, nutrients and metals. For example, Sarem et al. (1996) noticed varying degrees of *Lactobacillus* strain adhesion in two cellular models – human epithelial intestinal Caco-2 and Int-407 cell lines. Depending on the origin and the dose, the gut bacteria represent different adhesive properties (Boekhorst et al., 2006; Piątek et al., 2012). For instance, while one study reported *L. rhamnosus* as a strain with low ability to adhere to the epithelial cells, few other studies indicated the adhesive properties of *L. rhamnosus* in the range of 7.2–14.4% (Ahrne and Hagslatt, 2011) and at the level of 20% (Roos and Johnsson, 2002; Le et al., 2013; Slizova et al., 2015).

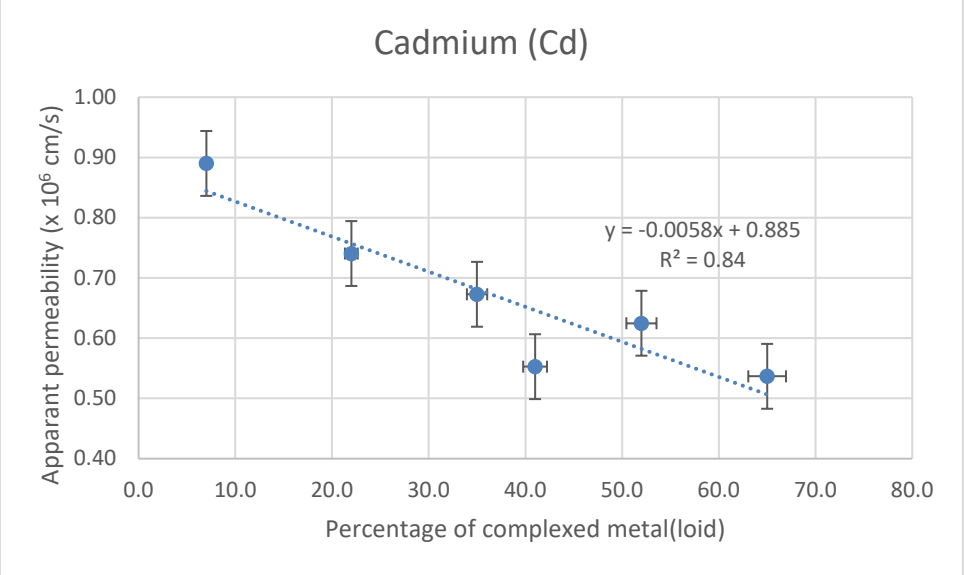
Exposure to contaminants including metal(loid)s is associated with an increase in gut permeability, leading to 'leaky gut syndrome' (Michielan and D'Incà, 2015; Mu et al., 2017). Exposure to Cd, for instance, causes significant damage to the gut barrier, including the toxicity of enterocytes, induction of inflammatory response, and disruption of tight junctions, as demonstrated by Zhai et al. (2016). However, gut bacteria can help in the modulation of contaminants-induced leaky gut syndrome through their effect on sequestering contaminants such as heavy metal(loid)s (Claus et al., 2016; Rosenfeld, 2017). For example, *L. plantarum* strains markedly decreased the permeability of Cd, thereby mitigating the Cd-induced leaky gut syndrome (Zhai et al., 2016). In their study, a clear protection against damage of HT-29 cells was observed when *L. plantarum* CCFM8610 gut bacteria was introduced simultaneously with Cd exposure (intervention assay) which they partly attributed to the intestinal Cd sequestration by gut bacteria thereby attenuating Cd exposure. Treatment with CCFM8610 significantly alleviated Cd-induced cytotoxicity and reversed the disruption of tight junctions in HT-29 cells. They further confirmed that the bacteria can inhibit Cd absorption by protecting the intestinal barrier in Cd-exposed mice. The presence of *Lactobacillus* sp. demonstrated significantly increased faecal Cd levels and decreased Cd accumulation in the tissues of Cd-exposed mice, and also a notable decrease in the intestinal permeability of Cd. This suggests that modulating the gut microbiota can serve as a potential strategy for regulating intestinal permeability and may help to alter the course of autoimmune diseases in susceptible individuals (Bischoff et al., 2014; Mu et al., 2017).

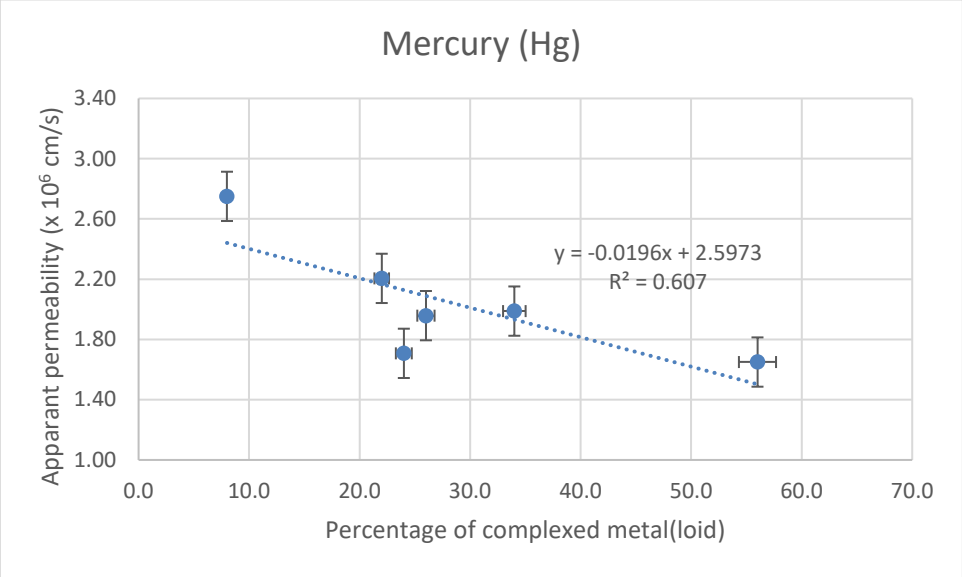
The metal(loid) adsorption capacities of three gut bacteria, *E. coli, L. rhamnosus and L. acidophilus* for As(III), As(V), Cd(II), Pb(II) and Hg(II) are reported in Chapter 5. The results have demonstrated that gut bacteria differed in their ability to adsorb metal(loid) which is attributed mainly to difference in the nature of functional groups between the bacteria. The reduction in the bioaccessibility of metal(loid)s in various sources by gut microbes as shown in Chapter 5 could be attributed to the immobilization through adsorption, complexation, and precipitation reactions (Jarosławiecka and Piotrowska-Seget, 2014). The microbial cell wall is a natural barrier for metal(loid)s, since the functional groups of several macromolecules are involved in the immobilization of metal(loid)s. In Gram-negative bacteria, lipopolysaccharide, a major component of the outer membrane, is effective in the immobilization of metal(loid) ions. As discussed in Chapter 5, in Gram-positive bacteria, peptidoglycan along with teichoic and teichuronic acids are involved in metal(loid) binding.

7.6.3 Effect of chelating agents on permeability of metal(loid)s

The results showed a significant reduction in the metal(loid) permeability in the presence of chelating agents (Table 7.4). The P_{app} values were lower in the presence of chelants indicating low intestinal absorption. However, the effect of chelants on the decrease in permeability of heavy metal(loid)s depended on the nature of metal(loid)s. While it was found that EDTA formed complexes with Cd and Pb more readily thereby decreasing the permeability of metal(loid)s, DMPS readily formed complexes with As and Hg. In the presence of EDTA and DMPS, the transport of metal(loid)s to the basolateral compartment decreased from 60.0% to 47.3% and 38.9% for As, from 13.6% to 11.7% and 9.8% for Cd, from 42.6% to 34.1% and 31.1% for Hg, and from 2.74% to 2.04% and 1.71% for Pb, respectively (Table 7.4). Correspondingly, the cellular retention of metal(loid)s were higher in the presence of chelants (Table 7.4).


Arsenic (As)
Apparant permeability (x 10⁶ cm/s)
4.00
3.50
3.00
2.50
2.00
1.50
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
Percentage of complexed metal(loid)
y = -0.0241x + 3.7354
R² = 0.7894


Cadmium (Cd)
Apparant permeability (x 10⁶ cm/s)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
Percentage of complexed metal(loid)
y = -0.0058x + 0.885
R² = 0.84



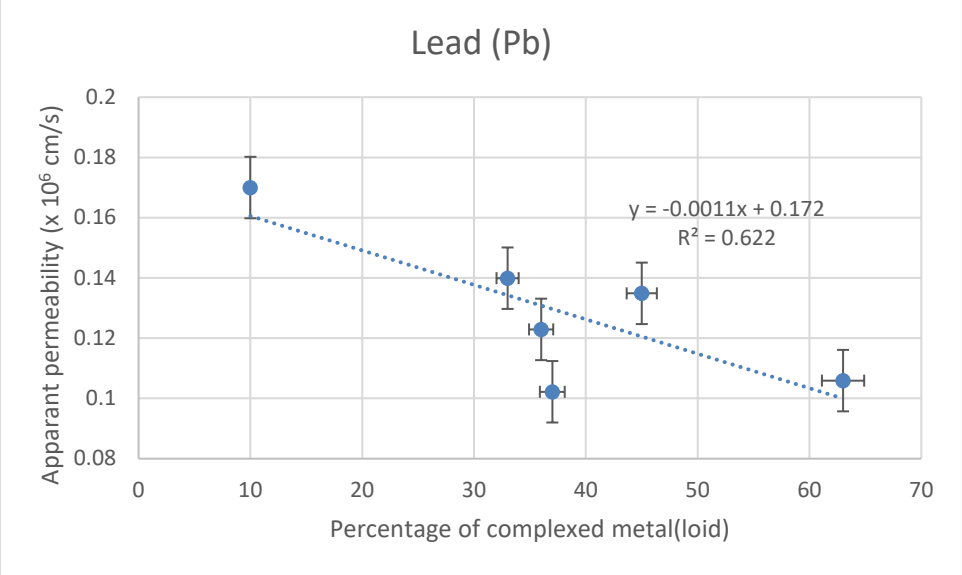


Figure 7.7 Relationships between percentage complexed metals and intestinal permeability of As, Cd, Hg and Pb.

The effect of chelate on the bioaccessibility of As, Cd, Pb and Hg is reported in Chapter 6. The results indicated that all the three chelating agents including EDTA, DMPS and DMPA increased both gastric and intestinal bioaccessibility of As, Cd, Hg and Pb. The increase in bioaccessibility varied amongst the heavy metal(loid)s and also the chelating agents. The increase in the bioaccessibility caused by chelating agents is attributed to the complexation/chelation of metal(loid)s by the chelating agents and the subsequent increase in the solubilisation of these metal(loid)s from the respective metal(loid) sources.

Dietary fibres, thiol-containing compounds such as cysteine, homocysteine, albumin and glutathione, and phytochemicals present in natural foods such as tea, can also act as chelants in lowering the intestinal absorption of contaminants including heavy metal(loid)s (Heaney, 2001; Flora et al., 2003; Gochfeld, 2003; Shim et al., 2009; Mori et al., 2012; Rubino, 2015). One of the mechanisms for reducing metal(loid) permeability is the formation of soluble metal(loid) complexes whose transport is less than that of the free forms of the metal(loid) species (Jadán-Piedra et al., 2018). The effect of dietary compounds on the transport of Hg present in the bioaccessible fraction (CH_3Hg) of swordfish was examined by Jadán-Piedra et al. (2016) using a Caco-2 model. The P_{app} values of Hg in the presence of cysteine and homocysteine were reduced by 38% and 35%, respectively. Similarly, Vázquez et al. (2014a,b) showed a decrease in the cellular accumulation of CH_3Hg by up to 55% in the presence of cysteine derivatives via Caco-2 cell model.

Clemente et al. (2017) examined the influence of dietary compounds including phytochemicals such as cysteine and glutathione, on the bioavailability of As(III) as measured by intestinal permeability using colon-derived human cells (NCM460 and HT-29MTX). Their findings demonstrated significant decreases in the quantity of As(III) transported across the epithelial monolayer in the presence of dietary compounds with a marked decrease in the presence of cysteine. The permeability of As(III) was reduced by 70%, 59%, 45% and 44% by cysteine, glutathione, epicatechin and homocysteine, respectively. The P_{app} values were decreased by 63% in the presence of cysteine. The binding of inorganic As to sulfhydryl groups is considered as one of the main mechanisms for As toxicity because its binding to a protein through cysteine residues alters the conformation and function of the protein (Shen et al., 2013). Also, the complexes formed between As(III) and free cysteine are insoluble in the pH range of 4−8 which partially explains the decrease in the transport of inorganic As across the intestinal cell monolayer in the presence of cysteine (Alonzo et al.,1983).

7.7 Conclusions

The ability of a metal(loid) to pass through the gastrointestinal barrier is an essential process when investigating the bioavailability and toxicity of heavy metal(loid)s. The process of absorption may be impacted by the metal(loid) binding with gut microbes or by competition with compounds that reduce its solubility or its passage through the epithelium. In this study, the intestinal permeability of metal(loid)s (As, Hg, Cd and Pb) as influenced by gut microbes

and chelating agents using an *in vitro* gastrointestinal/Caco-2 cell intestinal epithelium model was examined. The results showed that in the presence of gut microbes or chelating agents, there was a significant decrease in the permeability of metal(loid)s, with differences in metal(loid) retention and complexation amongst the chelants and the gut microbes. The decrease in metal(loid) permeability varied amongst the metal(loid)s. Chelating agents reduce intestinal absorption of metal(loid)s by forming complexes thereby making them less permeable. In the case of gut bacteria, the decrease in the intestinal permeability of metal(loid)s may be associated to a direct protection of the intestinal barrier against the metal(loid)s or indirect intestinal metal(loid) sequestration by the gut bacteria. Thus, both gut microbes and chelating agents can be used to decrease the intestinal permeability of heavy metal(loid)s, thereby mitigating their toxicity.

Chapter 8

SUMMARY AND CONCLUSIONS

8.1 Introduction

This chapter focusses on the summary of results obtained from all the research chapters covering heavy metal(loid) toxicity to gut microbes (Chapter 3), the bioaccessibility of heavy metal(loid)s as impacted by gut microbes and chelates (Chapter 4 - 6), and bioavailability of heavy metal(loid)s as measured by intestinal permeability tests (Chapter 7). It also includes the major conclusions derived from this study and also possible future research directions in this area.

The gut microbes not only can be affected by environmental contaminants but they themselves can alter the speciation and bioavailability of these contaminants. These interactions between gut microbes and environmental contaminants including heavy metal(loid)s can have both positive and negative consequences for the host. While essential metal(loid)s such as copper (Cu) and zinc (Zn) are involved in various metabolic functions, excess of these essential metal(loid)s and toxic heavy metal(loid)s such as arsenic (As) and lead (Pb) interfere with various functions of organ systems. Chelation therapy is an important clinical treatment for managing metal(loid) toxicity in human. Chelating agents can affect metal(loid) toxicity by mobilizing the toxic metal(loid) mainly into urine. Chelating agents forming stable complexes with toxic metal(loid)s may shield biological targets from the metal(loid) ion, thereby reducing the local toxicity. This thesis provides a greater understanding of the interactions of selected gut microbes (*Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Escherichia coli*) and toxic heavy metal(loid)s (As, Pb, Cd, Hg) in relation to metal(loid) toxicity to gut microbes, and bioaccessibility and bioavailability of these heavy metal(loid)s.

In this work, gastrointestinal bioaccessibility is defined as the amount of metal(loid)s that become solubilized in the gastric and intestinal system, and bioavailability as the amount of metal(loid)s that passes through the intestinal epithelial cells thereby reaching the blood circulation (Naidu et al., 2008). Bioaccessibility of heavy metal(loid)s was measured using *in-vitro* gastro-intestinal bioaccessibility test. Bioavailability of heavy metal(loid)s was monitored

by intestinal permeability test as measured by caco-2 cell technique. Bioaccessibility of contaminants, including heavy metal(loid)s, underpins their bioavailability and toxicity to biota including microorganisms and human being (Figure 2.3).

8.2 Research concept

The overall concept of the study is presented in Figure 8.1. The primary objective of the thesis is to examine the interactions between gut microbiome and heavy metal(loid)s in relation to metal(loid) toxicity to gut microbes, and bioaccessibility and bioavailability of these metal(loid)s. The specific objectives of the study include: (i) to demonstrate the effect of arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) on the growth of selected gut microbes; (ii) to examine the effect of selected gut microbes on the bioaccessibility of these heavy metal(loid)s as measured by gastrointestinal bioaccessibility test; (iii) to examine the effect of chelating therapeutic agents on bioaccessibility of heavy metal(loid)s; and (iv) to examine the effect of gut microbes and chelating therapeutic agents on the bioavailability of heavy metal(loid)s as measured by intestinal permeability test.

Firstly, the toxicity of four major heavy metal(loid)s that include As, Cd, Pb and Hg on three gut microbes that include *L. rhamnosus, L. acidophilus* and *E. coli* was examined (Chapter 3). In the subsequent experiments, the gastrointestinal bioaccessibility of these four metal(loid)s from orally ingested sources as impacted by gut microbes and chelating agents was examined (Chapters 4 - 6). In the final experiment, the bioavailability of these metal(loid)s as measured by intestinal permeability was monitored using Caco-2 permeability test (Chapter 7).

8.3 Heavy metal(loid) toxicity to gut microbes

The study demonstrates the relationship between concentration of selected heavy metal(loid)s and growth of gut microbes. The growth of all three gut bacteria (*L. rhamnosus, L. acidophilus* and *E. coli*) decreased with increasing metal(loid) (As, Cd, Pb and Hg) concentrations, indicating metal(loid) toxicity to gut bacteria. Heavy metal(loid) toxicity to gut microbes varied between both the metal(loid) species and gut microbes. While, the toxicity of all the cationic metal(loid)s (Cd, Pb and Hg) to gut bacteria decreased with pH, the anionic As species exhibited an opposite effect. The decrease in toxicity of former metal(loid)s with increasing pH is attributed to a decrease in their solubility and bioaccessibility. The increase in As toxicity

with increasing pH is attributed to the conversion of As(V) to As(III), which is more toxic to bacteria. The order of metal(loid) toxicity, as measured by the lethal dose concentration (LD_{50}), was Hg>Cd>Pb>As(III)>As(V) for *E. coli*; and Hg>Cd>As(III)>Pb>As(V) for the two *Lactobacillus sp*. Arsenite (AsIII) is more toxic than arsenate (AsV) to gut bacteria. There is clear evidence that bacteria-dependent metabolism of pollutants modulates the toxicity for the host. The toxicity of these metal(loid)s to the bacteria also depends on their bioavailability and bioaccessibility. The difference in toxicity between metal(loid) species may be attributed to the difference in the adsorption and permeability of metal(loid)s.

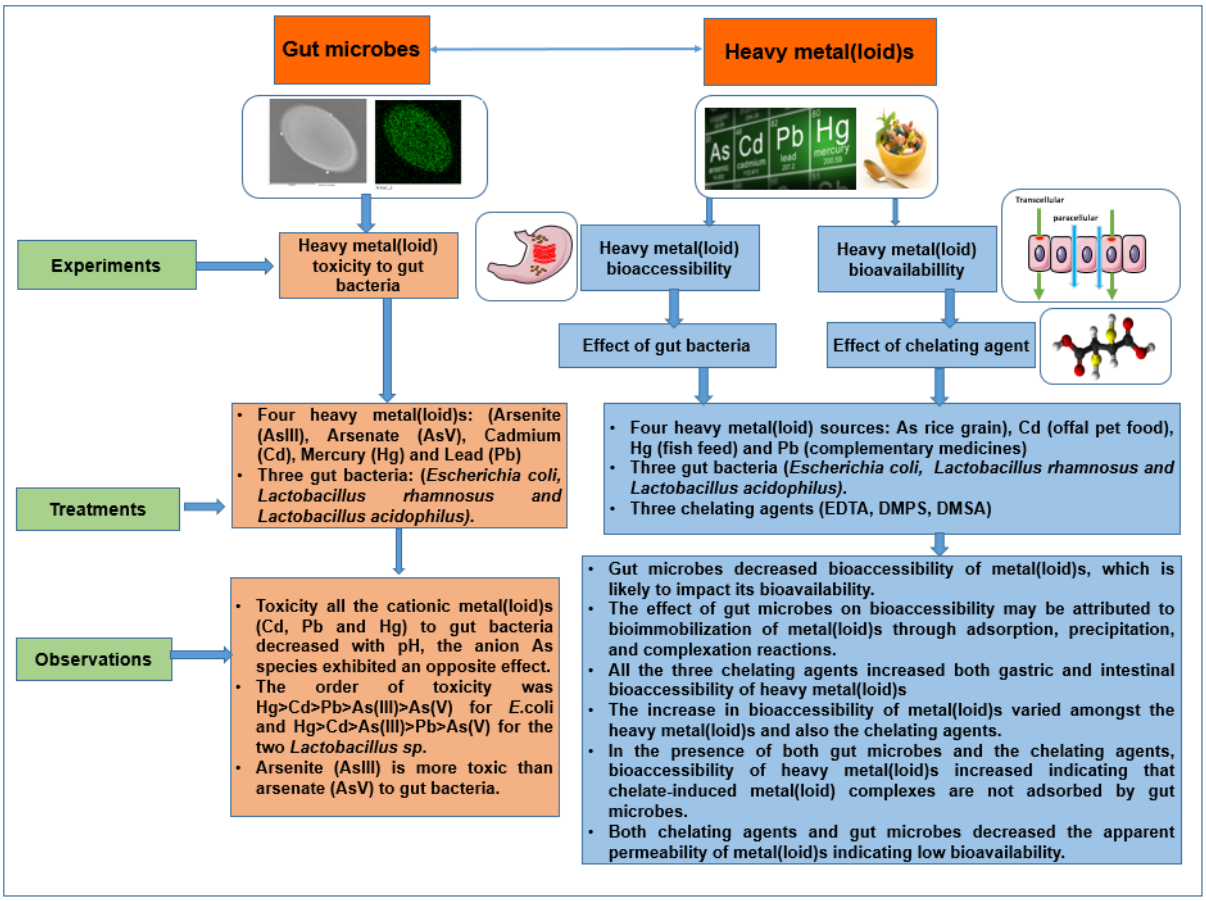


Figure 8.1 The overall concept of the study

The toxicity of heavy metal(loid)s varied amongst the gut bacteria, which was attributed to the difference in the adaptability of these bacterial species to the pH conditions in the gut and the adsorption of these metal(loid)s by gut bacteria. While As is an anion, other three metals are cations and hence the binding capacity of these heavy metal(loid)s to the bacterial cell wall varied depending on the charge.

8.4 Bioaccessibility of heavy metal(loid)s

Bioaccessibility of contaminants, including heavy metal(loid)s measures the solubility of these contaminants, thereby determining their bioavailability and toxicity to biota including microorganisms and human being. The bioaccessibility of As (rice grain), Cd (offal pet food), Hg (fish feed) and Pb (complementary medicines) was examined by measuring gastro-intestinal bioaccessibility test (physiologically based extraction test). In general, the gastric bioaccessibility of metal(loid)s is higher than intestinal bioaccessibility, which is attributed to the difference in the pH between these two extractants. The pH of gastric extract is much less (1.5) than that of intestinal extract (5.8). The solubility and bioaccessibility of metal(loid)s such as Cd, Pb and Hg decreases with increasing pH. Majority of the metal(loid)s extracted in gastric and intestinal extracts was present as metal(loid) complexes. The distribution of metal(loid)s in the gastric and intestinal extracts will have implications on their bioavailability as measured by intestinal permeability.

8.4.1 Effect of gut microbes

Gut microbes play an important role in the absorption of nutrients and heavy metal(loid)s in human intestine through their effect on bioaccessibility. This study demonstrated that gut microbes decreased bioaccessibility of metal(loid)s, which is likely to impact their bioavailability, intestinal absorption, and toxicity. The effect of gut microbes on bioaccessibility may be attributed to ‘bioimmobilization’ of metal(loid)s through adsorption, precipitation, and complexation reactions (Figure 5.6). Gut microbially-induced bioimmobilization of metal(loid)s is determined by the surface charge characteristics of these gut microbes and the pH of their residing medium. For example, the intestinal phase pH is higher (pH 5.8) than that of gastric pH (pH 1.5), indicating that immobilization of heavy metal(loid)s through precipitation is more common in the intestinal phase than gastric phase. It is, therefore, imperative to undertake bioaccessibility measurements in the presence of gut microbes, especially for orally-ingested contaminants. It is important to point out that the human gut hosts a large number of microbial species including bacteria, fungi, and archaea. In this study, the effect of only selected bacterial species on bioaccessibility was examined. Future studies should focus on the effect of composite gut microbial consortia on bioaccessibility and subsequent bioavailability of toxic metal(loid)s.

8.4.2 Effect of chelating agents

The results indicated that all the three chelating agents (EDA, DMSA and DMPS) increased both gastric and intestinal bioaccessibility of As, Cd, Hg and Pb. The increase in bioaccessibility of metal(loid)s varied amongst the heavy metal(loid) species and also the chelating agents. There was a significant relationship between percentage complexed metal(loid)s and absolute gastric bioaccessibility (Figure 6.3). This indicates that the increase in the bioaccessibility caused by chelating agents is attributed to the complexation/chelation of metal(loid)s by the chelating agents and the subsequent increase in the solubilisation of these metal(loid)s from the respective metal(loid) sources.

The effect of chelating agents in solubilizing heavy metal(loid)s generally depends on the stability of the chelates in the intestine and their solubility in water or lipids. The chelating agents also impact the gut microbes, thereby affecting the bioaccessibility and intestinal permeability of heavy metal(loid)s. In the presence of both gut microbes and the chelating agents, there was a net increase in the bioaccessibility of heavy metal(loid)s indicating that chelated metal(loid) species are not readily adsorbed by gut bacteria. In the absence of chelating agent, the gut bacteria-induced decrease in bioaccessibility is attributed to the adsorption and precipitation reactions. These observations indicate that complexation of heavy metal(loid)s by chelating agents and the adsorption of free ionic metal(loid) species by gut microbes control the bioaccessibility of heavy metal(loid)s in human gut.

8.5 Bioavailability of heavy metal(loid)s

The ability of a metal(loid) to pass through the gastrointestinal barrier is a key property to consider when examining the bioavailability and toxicity of heavy metal(loid)s. The mechanisms of metal(loid) permeation through biological barriers include passive diffusion (or paracellular) and active (or transcellular) transport pathways. In passive, paracellular absorption, the metal(loid) ions diffuses through tight junctions into the basolateral spaces around enterocytes, and hence into blood. Active, transcellular absorption involves import of metal(loid)s into the enterocyte, transport across the cell, and export into extracellular fluid and blood. The rate-limiting step in transcellular metal(loid) absorption is transport across the epithelial cell, which is greatly enhanced by the carrier proteins. Intestinal permeability is used an index of bioavailability of heavy metal(loid)s. In this study, Caco-2 cell technique was used to measure the bioavailability of heavy metal(loid)s. The intestinal permeability of As, Cd, Hg

and Pb in intestinal extract as impacted by gut microbes and chelating agents was monitored using Caco-2 test (Chapter 7).

8.5.1 Effect of gut microbes

The ability of gut bacteria to adhere to mucus and/or intestinal epithelial cells is one of the major mechanisms protecting the host from contaminant invasion and adhesion. *L. acidophilus and E. coli* were selected to compare their influence on the metal(loid) transport across the Caco-2 cell monolayer. Treatment with these gut bacteria significantly reduced the permeability of metal(loid)s in Caco-2 cells. The P_{app} (apparent permeability coefficient, which evaluates the velocity with which a solute crosses the cell monolayer) values were markedly reduced in the presence of bacteria for all the metal(loid)s indicating low intestinal absorption. Differences in P_{app} values amongst the metal(loid)s in the presence of bacteria were noticed. In the presence of *L. acidophilus and E. coli*, the transport of metal(loid)s to the basolateral compartment decreased from 60.0% to 37.6% and 50.1% for As, from 13.6% to 6.89% and 8.23% for Cd, from 42.6% to 26.5% and 34.7% for Hg, and from 2.74% to 1.25% and 1.41% for Pb, respectively. The results may be attributed to a direct protection of the intestinal barrier against the metal(loid)s or indirect intestinal metal(loid) sequestration by the probiotic bacteria.

8.5.2 Effect of chelating agents

Both synthetic and natural chelating agents lower the intestinal absorption of metal(loid)s. EDTA and DMPS were selected as the chelating agent to study their impact on metal(loid) permeability using Caco-2 cells. The results showed a marked reduction in the metal(loid) permeability in the presence of chelating agents. The P_{app} value was lower in the presence of chelating agents indicating low intestinal absorption. The decrease in bioavailability of heavy metal(loid)s differed amongst the metal(loid)s. In the presence of EDTA and DMPS, the transport of metal(loid)s to the basolateral compartment decreased from 60.0% to 47.3% and 38.9% for As, from 13.6% to 11.7% and 9.8% for Cd, from 42.6% to 34.1% and 31.1% for Hg, and from 2.74% to 2.04% and 1.71% for Pb, respectively. The chelating agents decreased the concentration of ionic free metal(loid)s. The decrease in the intestinal permeability in the presence of chelate input indicates that mainly ionic free metal(loid)s are transported across the intestinal epithelial cells.

8.6 Application of this research work

The outcomes derived from this research work will have implications for both fundamental and applied research in the area of gut microbes-heavy metal(loid) interactions. In relation to fundamental research, the effect of metal(loid) toxicity on gut microbes will lead to the exploration of molecular physiological mechanisms involved in inducing toxicity. For example, the study has indicated that the tested metal(loid)s (As, Cd, Pb and Hg) varied in their toxicity to gut microbes as measured by LD_{50} value. It is important to examine whether this difference is attributed to the degree of interaction between the metal(loid)s and gut microbes and/or the physiological response by the gut microbes to these metal(loid)s. The study also indicated that As(III) was more toxic than As(V) species. Two reasons have been attributed to the difference in toxicity between As species. Firstly, As(V) is strongly adsorbed compared to As(III), resulting in the less bioaccessibility of the former species. Secondly, there is a difference in the uptake of As species within the cells. Arsenate (AsV)) species enters the cell through phosphate transporters. With high serum concentrations of phosphate (~ 34,000 µg/L), As(V) is likely to have very little cellular uptake due to competition of phosphate. Arsenite (AsIII) enters the cell via cell diffusion or through aquaporin transporters. Since As(III) can gain entry into the cell by diffusion, this species potentially is much more toxic than As(V). However, the physiological responses by microbes to toxicity by these As species and other heavy metal(loid)s need to be examined using advanced molecular approaches such as genomics, proteomics, transcriptomics and metabolomics (Grice and Segre, 2012; Cortassa et al., 2015; Malla et al., 2018)

In relation to applied research, there are several potential avenues for applying the major outcomes of this research work. Some of these applications include:

- Gut bacteria have been shown to reduce the bioaccessibility and bioavailability of heavy metal(loid)s to their host system. Thus, probiotics containing some of the selected gut bacteria can be developed, which can be used to reduce heavy metal(loid) toxicity to human.
- Chelates can be used in combination with gut bacteria to reduce both the bioaccessibility and bioavailability of metal(loid)s to both gut microbes and the host.
- The data will help us to develop a much-improved model of contaminant bioavailability, which accounts for the presence of gut bacteria.
- More broadly, this study will also help us to understand the extent to which specific contaminants threaten human health. This in turn will allow better risk assessments

for prioritising the limited resources for the management of heavy metal(loid) contamination.

8.7 Future research directions

As indicated in the review of literature, the human gut microbiota are a composite structure of up to 1000 distinct bacterial species that reside in the human digestive tract. Fungi, protozoa and archaea are found in lesser numbers and constitute the rest of the gut microbiota. In this study, only 3 gut bacteria were examined for selected heavy metal(loid) toxicity and their effects on both bioaccessibility and bioavailability of these heavy metal(loid)s.

The research reported in this thesis suggests a number of areas that need further study (Phillips, 2009; Diaz-Bone and Van de Wiele, 2010; Pinto et al., 2014; Salim et al., 2014; Lu et al., 2015; Wang et al., 2015; Claus et al., 2016; Ghaisas et al., 2016; Firrman et al., 2018). Some of the future research directions include:

- Metal(loid) toxicity using composite gut microbial consortia in the presence of mixed metal(loid) and other contaminants.
- Bioaccessibility and subsequent bioavailability of toxic metal(loid)s as impacted by composite gut microbial consortia.
- Application of Simulator of the Human Intestinal Microbial Ecosystem (SHIME) system that mimics digestive processes in the gut in the examination of both metal(loid) toxicity and their bioaccessibility.
- Modulation of metal(loid) toxicity and bioavailability to human as impacted by probiotics intakes.
- The adhesion of gut microbes to intestinal epithelial cells, thereby providing protection against ‘leaky gut’ syndrome.
- The role of gut microbe-contaminant interaction in relation to leaky gut syndrome as monitored by intestinal permeability tests.
- Long-term impact of chronic intake of heavy metal(loid)s (as in the case of As intake from drinking water sources and Pb intake from complementary medicines) on gut microbes’ genomic and functional diversity.
- In this study, the caco-2 cell technique was used to monitor the intestinal permeability of metal(loid)s from reference compounds as a measure of bioavailability. It is important to measure the intestinal permeability of feed-derived metal(loid) sources using this technique.

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APPENDIX

Appendix 3.1 Protocol for Liquid Lysogeny broth (LB) preparation:

Lysogeny broth (LB) (also known as Luria broth or Luria-Bertani broth) is a nutritionally rich medium primarily used for the growth of bacteria.

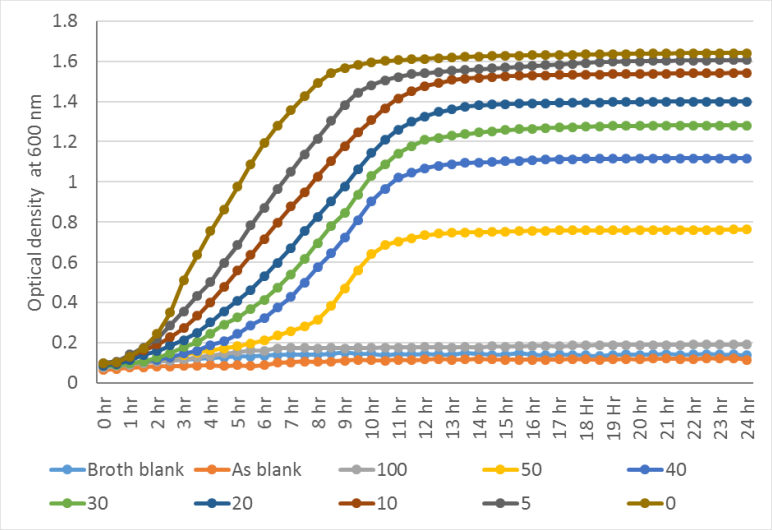
1. Prepare liquid LB. For example, to make 400 mL of LB, weigh out the following into a 500 mL glass bottle:
    - 4 g NaCl
    - 4 g Tryptone
    - 2 g Yeast Extract
    - and dH_2O to 400 mL

    Loosely close the cap on the bottle (do NOT close all the way or the bottle may explode during autoclaving) and then loosely cover the entire top of the bottle with aluminum foil. Autoclave and allow to cool to room temperature. Now screw on the top of the bottle and store the LB at room temperature.
2. When ready to grow bacterial culture, add liquid LB to a tube or flask and add the appropriate antibiotic to the correct concentration.
3. Using a sterile pipette tip, select a single colony from the LB agar plate.
4. Drop the tip into the liquid LB + antibiotic and swirl.
5. Loosely cover the culture with sterile aluminum foil or a cap that is not air tight.
6. Incubate bacterial culture at 37°C for 12-18 hr in a shaking incubator.
7. After incubation, check for growth, which is characterized by a cloudy haze in the media.

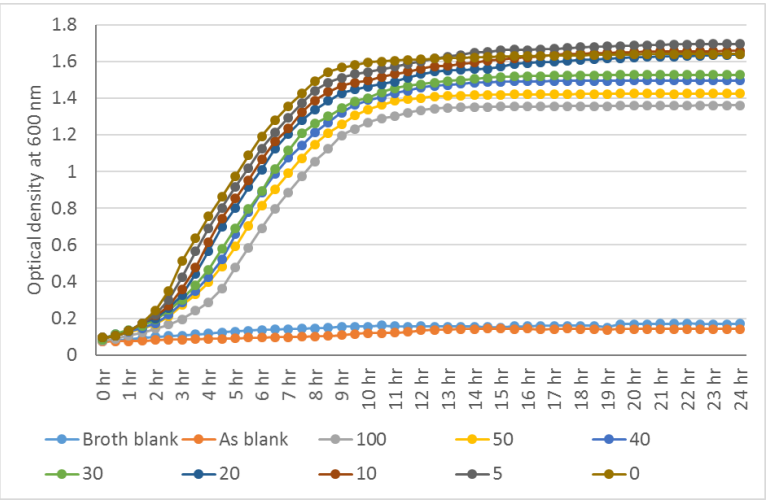
    Note: *A good negative control is LB media + antibiotic without any bacteria inoculated. We should see no growth in this culture after overnight incubation.*
8. For long-term storage of the bacteria, we can proceed with Creating a Glycerol Stock.

Appendix 3.2 Growth response curve of *E. coli* as affected by increasing concentrations of As(III) (a) and As (V) (b) input

a. As(III)

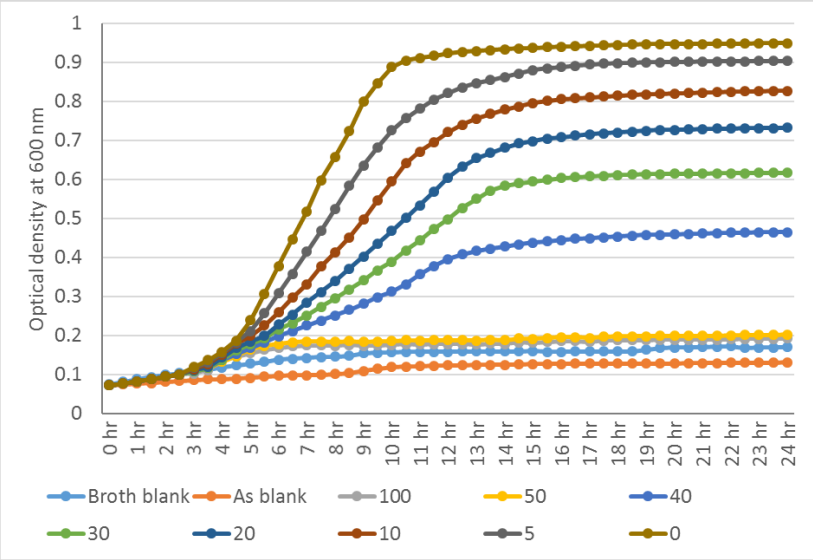


b. As(V)

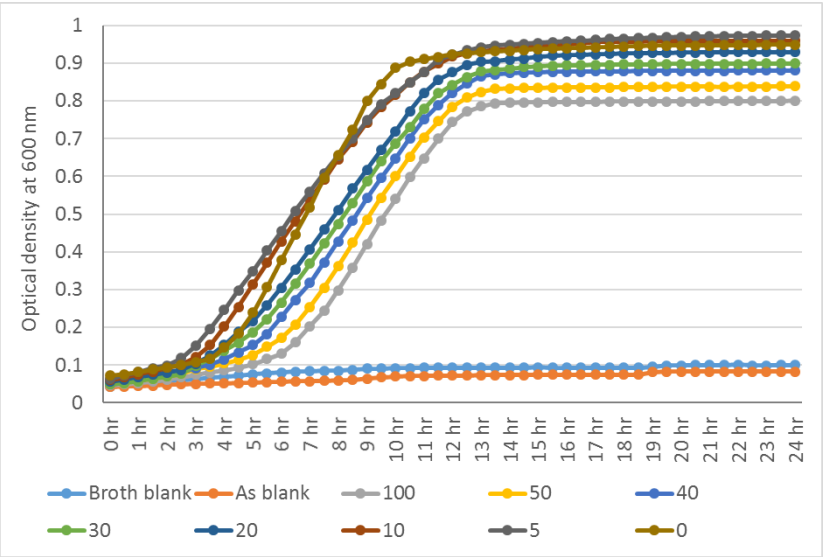


Appendix 3.3 Growth response curve of *Lactobacillus rhamnosus* as affected by increasing concentrations of As(III) (a) and As (V) (b) input

a. As(III)

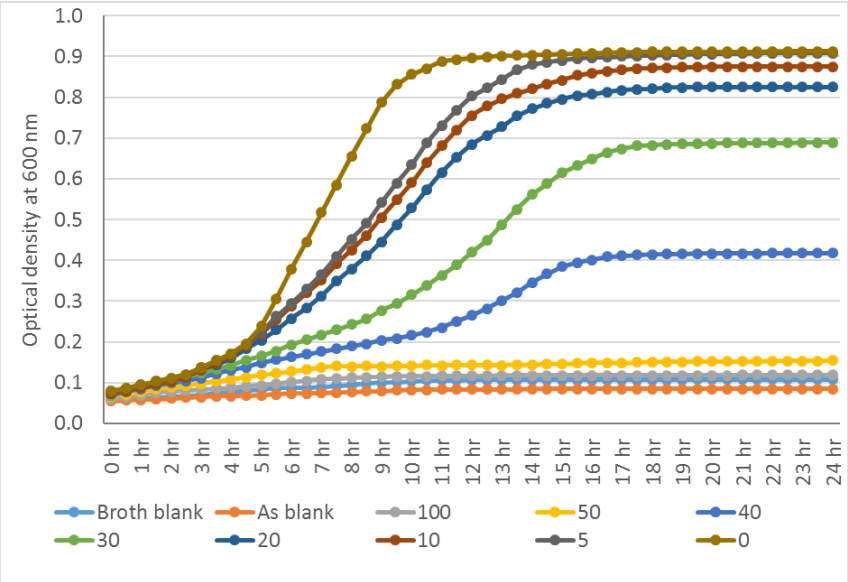


b. As(V)

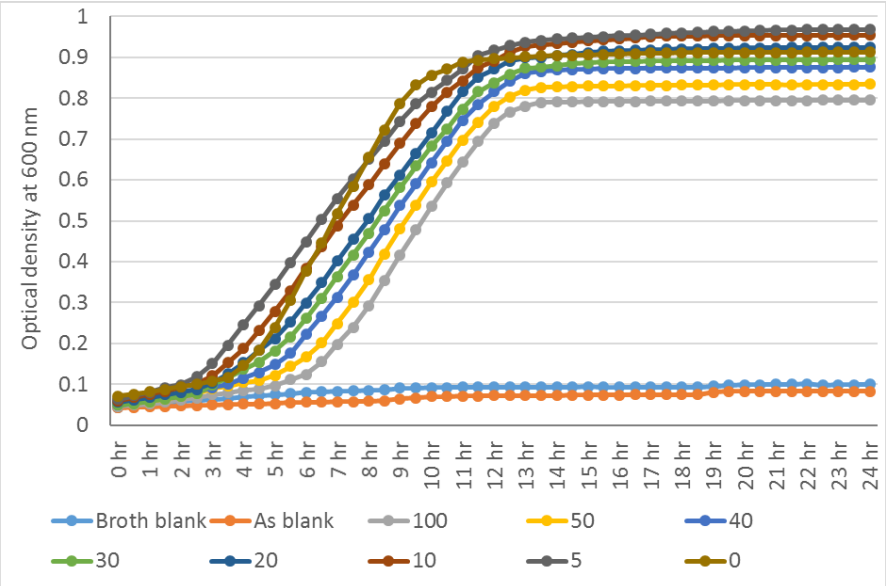


Appendix 3.4 Growth response curve of *Lactobacillus acidophilus* as affected by increasing concentrations of As(III) (a) and As (V) (b) input

a. As(III)

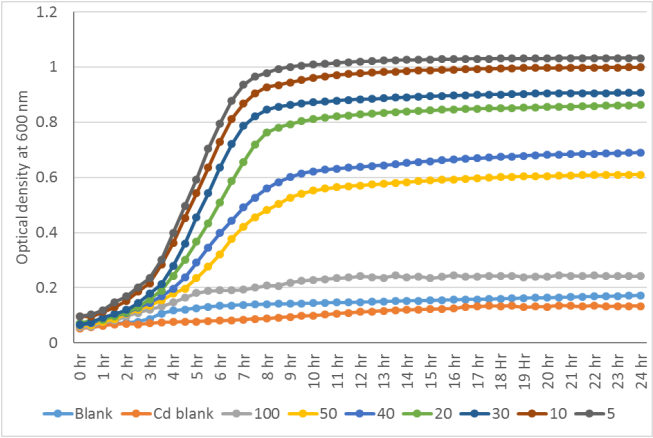


b. As(V)

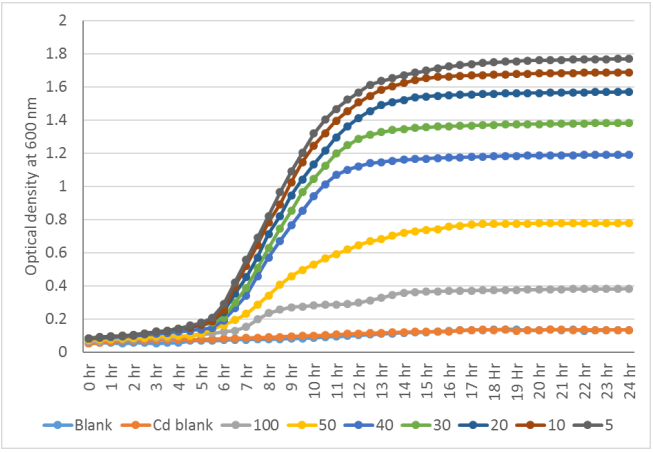


Appendix 3.5 Growth response curve of *Esherichia coli* (a), *Lactobacillus rhamnosus* (b), and *Lactobacillus acidophilus* (c) as affected by increasing concentration of Cd input

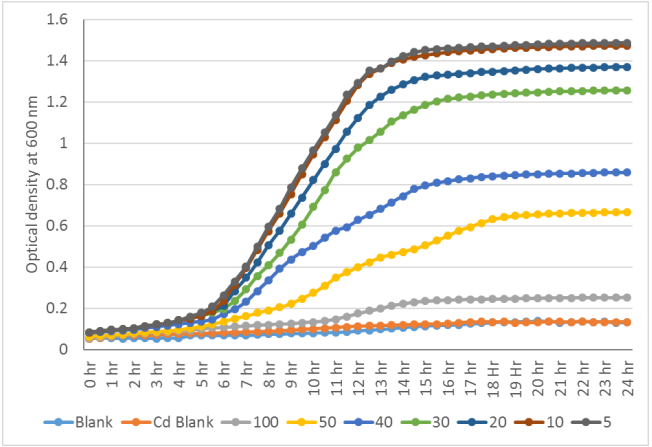
a. Esherichia coli



b. Lactobacillus rhamnosus

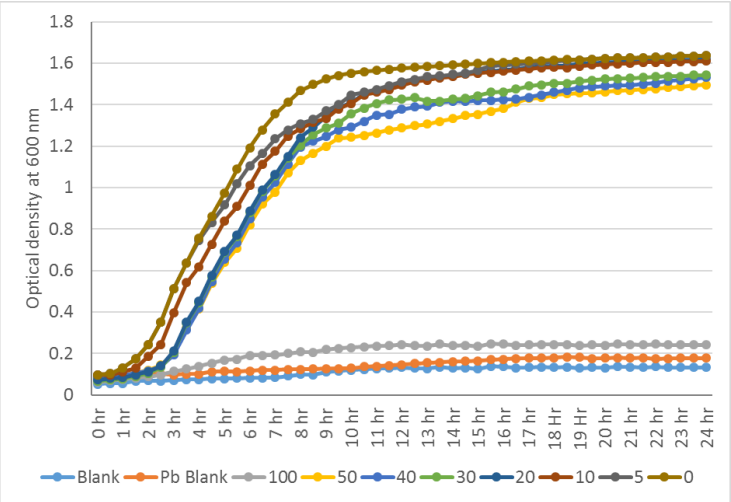


c. Lactobacillus acidophilus

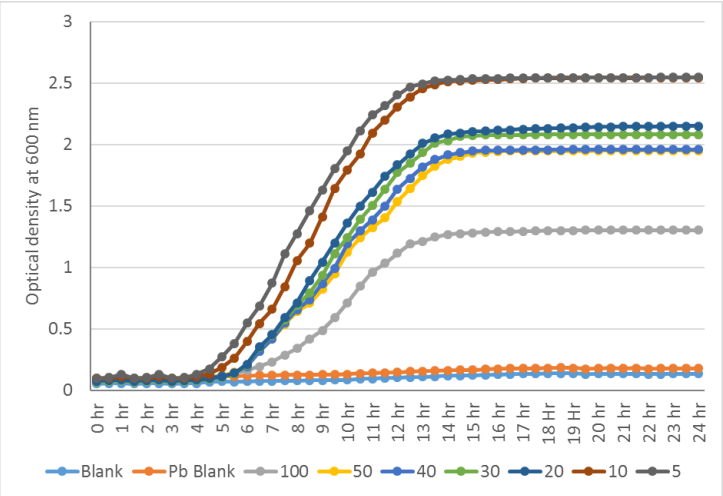


Appendix 3.6 Growth response curve of *Esherichia coli* (a), *Lactobacillus rhamnosus* (b), and *Lactobacillus acidophilus* (c) as affected by increasing concentration of Pb input

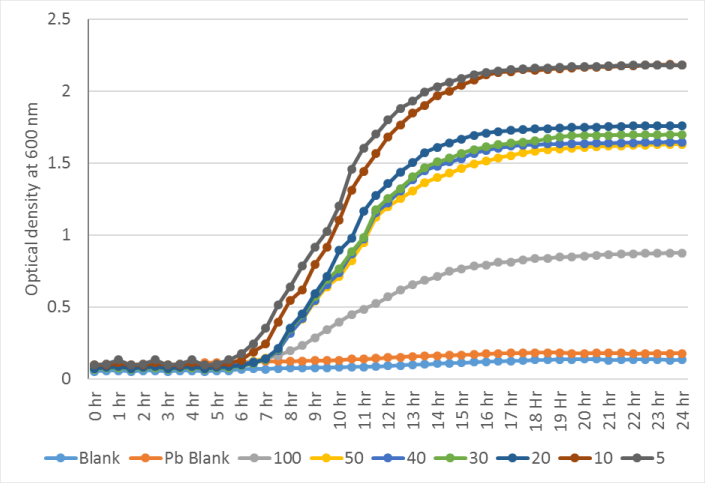
a. Esherichia coli



b. Lactobacillus rhamnosus



c. Lactobacillus acidophilus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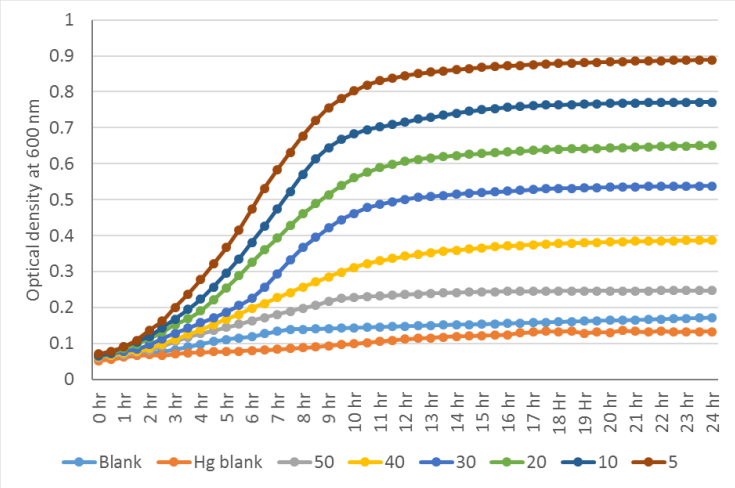
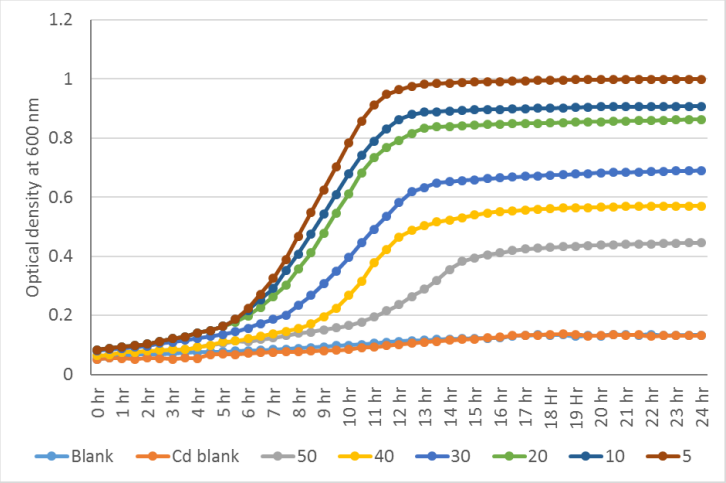
Appendix 3.7 Growth response curve of *Esherichia coli* (a), *Lactobacillus rhamnosus* (b), and *Lactobacillus acidophilus* (c) as affected by increasing concentration of Hg input

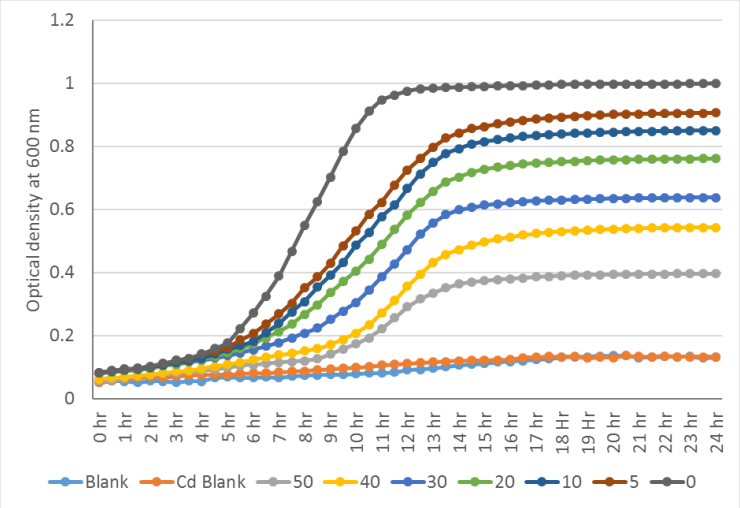
a. Esherichia coli



b. Lactobacillus rhamnosus



c.Lactobacillus acidophilus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