Potential Health Impact of Pollutants in Potatoes, Grown with Wastewater, in a Simulated Human Gut Digestion Model

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ABSTRACT

Wastewater irrigation is undoubtedly an important strategy to cope with the global water scarcity crisis. Despite being an alternate source of water, it is also rich in pollutants, both organic (hormones and pharmaceuticals) and inorganic (heavy metals). These contaminants can make their way into the food chain via plant uptake and leading to biomagnification. Some of the heavy metals such as lead (Pb), cadmium (Cd), arsenic (Ar), mercury (Hg), etc. inside the human body, have no biological health promoting significance but can cause adverse effects. Their presence in biological organisms have been associated with disrupted immune system, oxidative stress, DNA damage, cardiovascular diseases, organ dysfunctionality, and carcinogenesis. Recent studies have found gut microbiota dysbiosis caused by heavy metal exposure. Considering the health risks associated with heavy metals, the present study was conducted on wastewater irrigated potatoes. Four different treatments had varying concentrations of heavy metals by virtue of the soil amendments applied and one treatment was freshwater irrigated to represent sample without contamination. The study consisted of fermentation of one daily serving of cooked potatoes in batch reactors using human fecal microbiota. The samples were taken from the fermenters at 0, 6, 12 and 24 h of incubation. The microbial processes were assessed by measuring short chain fatty acid (SCFA) concentrations, antioxidant capacity, and total phenolics. Lactobacilli plate count using MRS agar was also conducted to evaluate the effect on beneficial bacteria. The SCFA concentration trends were similar in all the treatments. The antioxidant capacities and total phenolics determined during the experiment/fermentation process were comparable to the pre-digestion values for all the treatments. The Lactobacilli population increased with time in all the reactors. It was concluded that the polyphenol antioxidants of potatoes could have neutralized the possible adverse effects of heavy metals on gut microbiota in the single meal exposure.

RÉSUMÉ

Sans doute une importante stratégie pour s'adresser à l'important risque globale de pénurie d'eau, l'irrigation au moyen d'eaux usées représente une source alternative d'eau. Cependant ces eaux, sont riches en polluants, à la fois organiques (hormones et produits pharmaceutiques) et inorganiques (métaux lourds). Ces contaminants peuvent, par l'entremise de leur absorption par les plantes, entrer dans la chaîne alimentaire et s'y bio-amplifier. Certains métaux lourds, tel le Pb, Cd, Ar, Hg, etc. n'ont aucuns bienfaits pour la santé humaine, mais peuvent avoir des effets nocifs. L'on associe leur présence dans les organismes biologiques supérieurs à des perturbations du système immunitaire, au stress oxydatif, à l'endommagement de l'ADN, aux maladies cardio-vasculaires, à la défaillance d'organes, et à la carcinogenèse. De récentes études ont noté une dysbiose de la microflore intestinale liée à l'exposition aux métaux lourds. En vue des risques de santé associés aux métaux lourds, la présente étude entrepris une culture des pommes de terre avec une irrigation au moyen d'eaux usées. Quatre différents traitements expérimentaux, à savoir trois niveaux de contamination en métaux lourds obtenus par différents niveaux d'amendement du sol de culture, et un sol sans contamination ayant reçu de l'eau fraîche (témoin) servirent à cultiver des pommes de terre. Récoltés, ces pommes de terre furent cuites, puis une portion journalière provenant de chacun des traitements fut fermentée par un microbiote fécal humain dans des réacteurs en lots. Des échantillons furent prélevés à différents moments des 24 heures d'incubation. L'ampleur des processus microbiens fut évaluée en mesurant les teneurs en acides gras à chaîne courte (AGCC), le pouvoir antioxydant, et le contenu phénolique total. Un dénombrement de lactobacilles sur plaque d'agar MRS permit d'évaluer l'effet sur les bactéries bénéfiques. Les teneurs en AGCC indiquèrent une tendance semblable pour tous les traitements. De plus, le pouvoir antioxydant et le contenu phénolique total des pommes de terre digérés demeura semblable à ceux des pommes de terre avant la digestion. La population de lactobacilles augmenta avec le temps dans tous les réacteurs. En

conclusion, suite à une exposition unique, les antioxydants phénoliques des pommes de terre ont peut-être neutralisé les effets néfastes des métaux lourds sur le microbiome intestinal.

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CONTRIBUTION OF AUTHORS

Harmanjot Kaur (Candidate): conducted the experiments, performed the tests on samples and analysed the data. The candidate prepared the thesis manuscript based on results derived with the help of co-authors.

Dr. Shiv O. Prasher (Supervisor, Distinguished James McGill Professor, Department of Bioresource Engineering, McGill University): Supervised the candidate for the study, provided feedback at every step and edited the thesis.

Dr. Stan Kubow (Committee member, Associate Professor, School of Human Nutrition, McGill University): Provided expert advice at various stages of research, helped derive the results and also edited the thesis.

Mohd. Baasir Gaisawat (PhD Student, School of Human Nutrition, McGill University): Helped in study design, experimentation and data handling.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AOC	Antioxidant capacity
BC	Biochar
BC-SAP	Mixture of Biochar and Superabsorbent Polymers
DPPH	2,2-diphenyl-1-picrylhydrazyl
FC	Folin Ciocalteau
FID	Flame Ionisation Detector
FRAP	Ferric Reducing Antioxidant Power
FW	Freshwater
GC	Gas Chromatography
h	Hour
mM	Milimole
ROS	Reactive oxygen species
SAP	Superabsorbent Polymers
SCFA	Short Chain Fatty Acids
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
TP	Total phenolics
TSCFA	Total Short Chain Fatty Acids
UV	Ultra Violet light
WW	Wastewater

CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1 Introduction

Fresh water crisis is seen as an alarming global risk for the decade (Koncagül et al., 2017). Freshwater resources are declining worldwide, therefore wastewater is becoming a potential source of irrigation water to cope with increasing demand for food. Wastewater irrigation has both benefits and risks associated with it. Wastewater use can increase food crop production and improve soil properties by adding organic matter, but it also add to soil various pollutants (biological and chemical) released from the industries and domestic areas. This can lead to deterioration of soil health and damage to the environment. There is a great danger of pollutants translocation to food chain. Research has indicated that pollutants are hazardous to human health. Therefore innovative techniques are needed to avert the danger of pollutant entering food chain from wastewater when used for irrigation. But an untreated wastewater is used for irrigation in many developing economies (Jaramillo and Restrepo, 2017), which is posing a risk of health hazard (Chary et al., 2008; Hu et al., 2013; Khan et al., 2008).

Metals having densities higher than 5 g cm^{-3} are referred to as heavy metals. Heavy metals are the commonly found pollutants in wastewater. Most of the organic pollutants could dissipate over time, but heavy metals are persistent, and therefore build-up in the soil and plants irrigated with wastewater. While all the heavy metals are lethal above certain level of exposure, but heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), arsenic (Ar) are toxic even at very low concentrations as they have no function in biological systems (El-Kady and Abdel-Wahhab, 2018). Pb pollution is a result of widespread use in leaded gasoline, smelting, use in paints, pipes and batteries, and making its way to human food chain; it is poisonous to human body. Pb poisoning in humans can be detected by elevated blood lead levels (Wani et al., 2015). Pb accumulation usually occurs in the bones where it can replace Ca^{2+} ions and has been

associated with the Parkinson's disease (PD) (Weisskopf et al., 2010). It has been found to interfere with neurochemicals and causes neurocognitive deficit in children by chronic exposure (Counter et al., 2008). Chronic exposure to mice has revealed that Pb induced metabolic disorders and gut dysbiosis (Xia et al., 2018) and also related to genotoxic activities in the intestine (Breton, Le Clère, et al., 2013).

Cd is widely used in manufacturing products such as metal plating, pigments and plastics. Prolonged Cd ingestion causes tubular and glomerular dysfunction in kidney, which is called Itai-itai disease (Baba et al., 2013). Cd also gets absorbed into the lungs via tobacco smoking or dust inhalation which causes lungs toxicity. Kidneys are the most affected organ and Cd exposure causes renal dysfunction (Huang et al., 2017). A study indicated that chronic exposure of Cd and chromium (Cr) to mice induced cell apoptosis (Jin et al., 2016). Dietary Cd risks include intestinal damage and inflammation caused by sub-chronic exposure in mice (Ninkov et al., 2015) and also perturbs the diversity of gut microflora and metabolism (Zhang et al., 2015).

Cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn) are the micronutrients essential for the organisms in trace amounts. Cu mostly exists as bound to certain enzymes or proteins and its transport is tightly regulated, reducing the probability of finding free Cu in the body. Excess Cu in body is controlled by either reduced absorption or excretion through bile (Gaetke et al., 2014). Wilson's disease is a rare disease caused by Cu overload (Scheiber et al., 2017). Fe gets accumulated in the heart and liver causing dysfunctionality as the body lacks proper mechanism to control excess Fe. Hence, Fe overload causes haematological disorders in the human body (Fibach and Rachmilewitz, 2017).

Potatoes are a source of carbohydrates and also contain micronutrients such as potassium (K), Mg, Fe, folate, polyphenols and vitamin C (Lutaladio and Castaldi, 2009).

Polyphenols are the antioxidants, which reduce the reactive oxygen species and prevent the oxidation of body tissues (Brown, 2005). About 90-95% of the polyphenols are undigested and reach the large intestine (D'Archivio et al., 2007) where they are converted to polyphenol metabolites by the gut microbes and increase the colonic antioxidant level (Sadeghi Ekbatan et al., 2016). Polyphenols also improve the gut ecology along with other health-promoting benefits (Cardona et al., 2013). Short chain fatty acids (SCFA), the end products of various metabolic processes of microbial fermentation inside the colon (Hijova and Chmelarova, 2007), are also induced by the polyphenols (Parkar et al., 2013) as well as potato-based resistant starch (Kleessen et al., 1997). The SCFA also protect against colon cancer and several gastrointestinal diseases (Roy et al., 2006).

Therefore, a batch fermentation of the contaminated potatoes was conducted, simulating the colonic region of human gastrointestinal tract; batch fermentation is a simple way to conduct short-term studies and fits well with the limited amount of sample available for this study.

1.2 Objectives

This study was conducted with the following objectives:

i. To study the potential health risks of acute exposure of heavy metal contaminated potatoes on gut microbial processes by determining production of SCFA and colonic antioxidant capacity; and
ii. To assess changes in the abundance of *Lactobacillus spp*, a beneficial bacteria, in the gut with consumption of heavy metal contaminated potatoes.

CHAPTER 2
LITERATURE REVIEW

2.1 Wastewater Irrigation

Water crisis has been assessed as a major global risk over the past several years by the World Economic Forum (WEF, 2016), and it has been determined as the global risk of highest concern for next decade (Koncagül et al., 2017) . Given the increasing population, water demand is predicted to increase significantly over the coming decade for agricultural sector which is currently responsible for 70% of water abstractions worldwide (Koncagül et al., 2017).With industrial development and population growth, globally, wastewater discharge has reached 400 billion m^3/year. There is urgent need to develop strategies for safe disposal of large volumes of wastewater. Recycling of wastewater for irrigation could serve dual purpose of safe disposal of wastewater and increase in food production (Zhang and Shen, 2017). Wastewater irrigation practice spans over centuries. Historical evidences reflect the use of wastewater for irrigation as early as in 3500 BC (Zhang and Shen, 2017). Wastewater irrigation farms were formed by Romans in Germany in 1531 and in Scotland in 1650 (Zhang and Shen, 2017). Wastewater reuse in agriculture worldwide has increased rapidly in recent years. Israel uses more than 80% treated water for agriculture (Zhang and Shen, 2017). Tunisia in North Africa has a vision of irrigating 20,000 – 30,000 ha of land with about 290 million m^3 of treated wastewater by 2020 (Hamilton et al., 2007). In Asia, Japan is known for urban wastewater reuse; most of the wastewater used in china is untreated and of poor quality (Hamilton et al., 2007). Wastewater reuse is gaining attention in Central and South America despite the abundant fresh water resources. In North America, California is the undisputed pioneer in wastewater irrigation dating back to 1890. Mexico also irrigates 350,000 ha of farmland with wastewater, only 11% of which is treated (Hamilton et al., 2007).

The underlying key drivers for use of wastewater for irrigation include rapidly increasing water stress and food demand, increased wastewater discharge, supply of nutrient

and organic matter, and its reliable and consistent supply at low cost as compared to seasonal variations of costly freshwater supply (Ashraf et al., 2017). Although nutrients and organic matter present in wastewater can decrease fertiliser cost and boost the plant growth, the parasitic worms, organic contaminants and heavy metals can pose risk to human health and environment (Zhang and Shen, 2017). Increase in awareness over the years about the health risks of untreated wastewater has led to development of guidelines and standards on wastewater reuse in agriculture. The technological advancements has reduced untreated wastewater irrigation in developed countries but the practice still persists in many developing nations (Keraita, 2008).

Wastewater can be of different qualities based on its source, treatment level (or completely untreated effluents) and method for discharge to the water bodies. Domestic wastewater from municipalities or urban wastewater contains large amount of microbial contamination, including bacteria, viruses, protozoans and parasitic helminthic worms (Hamilton et al., 2007). Vegetables grown with pathogenic wastewater can cause diseases, as the pathogens stick to the surface of vegetables and survive for weeks (Ashraf et al., 2017). Wastewater originated from industrial areas contains many heavy metals. Of these, heavy metals such as Pb, Hg, Cd are taken up by crops and eventually ends up in human bodies and pose health risk (Ashraf et al., 2017).

One of the key components of human exposure to metals through food chain is soil-to-plant transfer. Crops growing in soils contaminated by untreated wastewater irrigation translocate the heavy metals from the soil and transfer to humans through food. Many studies have reported metal contamination in market vegetables grown with wastewater or in contaminated sites. Khan et al. (2008) studied the heavy metal build-up in soils and plants due to long-term wastewater irrigation in Beijing, China. The results showed a significant enrichment of soil with Cr, Cu, Ni, Pb and Zn while it was heavily enriched with Cd. The plants

grown in the contaminated soil had accumulated the same heavy metals; the amount exceeded the permissible limits. Another study in India also reported heavy metals in vegetables and cereal crops grown with treated and untreated wastewater at levels above permissible limits (Singh et al., 2010).

2.2 Health Effects of Heavy Metals

Metals having specific gravity between 3.5 to 7 g/cm^3 are considered as heavy metals (El-Kady and Abdel-Wahhab, 2018). While some metals are essential for the biological systems but exposure above certain level for all metals is considered toxic. Metals such as Cu, Zn, Fe, cobalt (Co) etc. are essential for human health, for example cobalt is a component of vitamin B_{12} and important for red blood cells. However, metals such as Pb, Cd, mercury (Hg) and uranium (U) are toxic even at low doses and have no biological role (El-Kady and Abdel-Wahhab, 2018). These heavy metals are the inorganic pollutants in the environment and are not biodegradable leading to accumulation over time. Chronic exposure to trace metals leads to accumulation of metals in human body organs and cause toxicity such as hepatonephrotoxicity and neurotoxicity (El-Kady and Abdel-Wahhab, 2018).

Pb is a toxic metal used extensively as lead oxide (PbO) in glazing pottery, lead acetate for cotton dyeing, in pesticide and paint productions and in form of leaf nitrate in matches, textile printing and in rodenticides, etc. (El-Kady and Abdel-Wahhab, 2018). Prolonged exposure of Pb in humans leads to accumulation in bones, teeth, blood and many other tissues such as brain, spleen, kidneys, liver and lungs. Its removal from body through faeces, hair nails and sweat is very slow (Wani et al., 2015). Pb exerts its toxic effect through its ability to replace calcium in the bones since Pb^{2+} ion has greater affinity at calcium binding sites than Ca^{2+} ion itself (Tchounwou et al., 2012). Bone-lead is used as a biomarker for evaluating cumulative Pb exposure; a study found bone-lead to have association with occurrence of Parkinson's disease

(PD) (Weisskopf et al., 2010). Pb is found to be associated with chronic renal disease and prolonged exposure can lead to coronary heart disease and stroke (Wani et al., 2015). Pb also interferes with various enzyme mechanisms reducing their activities. It is also considered as probable human carcinogen; and has induced renal tumors in rats and mice in clinical studies (Tchounwou et al., 2012). Pb toxicity also affects the reproductive system in both males and females (Wani et al., 2015).

Cd is a carcinogenic metal used in electroplating metals like Fe, used in nickel-cadmium batteries and also as stabiliser for PVC (El-Kady and Abdel-Wahhab, 2018; Mudgal et al., 2010). Kidney Tubular injury and dysfunction is the most reported Cd toxicity effect. It is reflected by an increase in urinary excretion of N-acetyl-b-D-glucosaminidase (NAG), lysozyme, total protein, albumin, b2-microglubin (b2-MG), a1-microglobulin (a1-MG), and kidney injury molecule-1 (KIM-1) (Satarug et al., 2017). It is considered as biomarker, although a few studies have disputed the correlation with these biomarkers and have associated tubular dysfunction with increased hypertension. Recently, an experimental model has found evidence of intestinal fibrosis induced by low-dose Cd (Satarug et al., 2017). Cd exposure have repeatedly been associated with lung cancer in many findings (Tchounwou et al., 2012), and has also been associated with learning disabilities in children (Satarug et al., 2017).

Cr exists in three forms; elemental, trivalent (Cr^{3+}) and hexavalent (Cr^{6+}). Elemental Cr is used in stainless steel manufacturing and alloys while leather tanning industry uses Cr^{3+} and Cr^{6+} is used in corrosion protection and production of textile dyes (El-Kady and Abdel-Wahhab, 2018). Cr is nutritionally important but in low doses. Cr^{6+} is considered to be much more toxic compared to Cr^{3+}, which can be attributed to the ease with which Cr^{6+} pass through cell membranes. It can be easily absorbed in lungs and gastrointestinal tract (Tchounwou et al., 2012). In animal studies, Cr^{6+} is found to cause renal damage in rats and also induced hepatic mitochondrial and microsomal lipid peroxidation. Recent studies have demonstrated

biochemical, genotoxic, and histopathologic effects in liver and kidney of goldfish by Cr^{6+} (Tchounwou et al., 2012). Human investigations have reported respiratory cancer by Cr^{6+} exposure (Tchounwou et al., 2012).

2.3 Potatoes

Potato is a high-yielding carbohydrate-rich crop with carbohydrates forming 75% of the dry matter (Camire et al., 2009). It has about 80% water content and 20% dry matter. Potatoes are a source of vitamins such as B1, B3, B6 and C as well as minerals like Fe, phosphorous (P), potassium (K) and Mg (Lutaladio and Castaldi, 2009). In recent years, more attention is given to the antioxidants in potatoes due to their health benefits. Polyphenols such as phenolic acids and flavonoids are found in significant amounts in potato tubers, which contribute towards the antioxidant capacity of potatoes. Other major phenolics found in potatoes are catechin, caffeic acid, ferulic acid, gallic acid and malvadin (Camire et al., 2009).

In a study by Blessington et al. (2010), three genotypes Innovator, Russet Burbank and Santana were analysed for phenolic compounds. The phenolic acids such as chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid and *t*-cinnamic acid, accounted for 67% of the total polyphenols, of which, chlorogenic acid was the most abundant. The remaining 33% was found to be flavonoids (rutin, myricetin, (-) epicatechin and quercetin dehydrate).

Various cooking methods such as boiling, baking, microwaving can reduce the polyphenol, carotenoid and antioxidant activity of potato tubers (Blessington et al., 2010; Perla et al., 2012). The storage conditions such as at 4^{o}C for four months lead to increase in antioxidant activity (Blessington et al., 2010). Various other factors such as injury during storage also affect the antioxidant activity of the potatoes.

2.4 Polyphenols and their Antioxidant Capacity

2.4.1 Classification of Polyphenols

Polyphenols have a molecular structure with several hydroxyl groups on aromatic rings (D'Archivio et al., 2007); they are the major groups of phytomicronutrients found in abundance in our diet (Crozier et al., 2009). Several thousand polyphenol molecules are found in plants and hundreds of them in edible plant parts (Manach et al., 2004). They are found in fruits, vegetables, cereals, olive, dry legumes, chocolate and beverages such as tea, coffee and wine (D'Archivio et al., 2007). They are classified into different groups: phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004).

Phenolic acids are further classified into derivatives of benzoic acid and cinnamic acid, of which caffeic acid is the most commonly found one and also in form of ester, i.e., chlorogenic acid, in fruits, vegetables and coffee. Cereals are the main source of ferulic acid. Flavonoids are the most widespread among the polyphenols. The main dietary flavonoids can be grouped into flavones, flavonols, isoflavones, anthocyanins, proanthocyanidins and flavanones (Scalbert and Williamson, 2000). Stilbenes are almost negligible in our diet under normal nutritional intakes. One of these, resveratrol is present in wine in very low quantity (Manach et al., 2004). Linseed is found to be the prolific source of lignans, containing up to 3.7 g/kg dry weight of secoisolariciresinol (D'Archivio et al., 2007). Enterodiol and enterolactone are the intestinal microbial metabolites of lignans (Manach et al., 2004). But a very low amount of lignans in our diet does not account for the enterodiol and enterolactone concentrations found in urine and tissues (D'Archivio et al., 2007). The other plant origins of lignans are yet to be established (Heinonen et al., 2001).

2.4.2 Antioxidant Capacity of Polyphenols

Highly reactive molecules in the form of free radicals (reactive oxygen and nitrogen species) are produced by different biological reactions (Fernandez-Panchon et al., 2008). The oxidation

of body tissues occur when these free radicals predominate the defence mechanism leading to chronic degenerative diseases like coronary heart diseases and cancer (Fernandez-Panchon et al., 2008). Polyphenolic compounds in our food have recently been found to have antioxidant and free-radical scavenging capacity (Bravo, 1998). Polyphenol structural chemistry predicts their reducing potential as hydrogen or electron donating agent (Rice-Evans et al., 1997). The fermentation of a mixture of pure dietary polyphenols (chlorogenic acid, caffeic acid, ferulic acid and rutin) in a continuous gastrointestinal digestion model resulted in formation of microbial phenolic metabolites in all the colonic compartments. Anthocyanin-rich purple fleshed sweet potato increased antioxidant capacity in all the colonic vessels along with the formation of anthocyanin metabolites in the colonic chambers of the gastrointestinal digestion model (Kubow et al., 2016).

2.4.3 Polyphenol - Microbiota Interaction

Bioavailability is defined as the amount of substance digested, metabolised and absorbed across the intestinal barrier into the blood streams. Polyphenols in their dietary form are poorly absorbed. Polyphenols are very high molecular weight compounds and considered as xenobiotics by human body. Polyphenols are poorly absorbed in their native form, therefore 90-95% of polyphenols reach the colon undigested, while 5-10% absorbed by small intestine as aglycones (Cardona et al., 2013; D'Archivio et al., 2007). Glycosylation and esterification also provide structural complexity making it difficult for polyphenol absorption. Polyphenols in the form of esters, glycosides, or polymers are broken down by gut microbes into simpler bioactive metabolites that are more readily absorbed and can undergo further biotransformation into different forms before reaching the blood or tissues (D'Archivio et al., 2007).

The role of intestinal microbiota in polyphenol biotransformation is also evident from the studies indicating absence of metabolites in germ free or antibiotic treated animals (Selma

et al., 2009). It is also known that different species of microbes are responsible for metabolism of different polyphenols. Phenolics such as isoflvones, flavonols, flavones and flavan-3-ols are metabolised mainly by the microbes from *Clostridium* and *Eubacterium* genera (Cardona et al., 2013). Hydroxycinnamates, such as chlorogenic acid, found in abundance in potatoes, are metabolised by gut bacterial species belonging to *Lactobacillus* and *Bifidobacterium* genera (Couteau et al., 2001). Sadeghi Ekbatan et al. (2016) conducted a study using pure phenolic acids (chlorogenic acid, caffeic acid and ferulic acid) and flavonoid (rutin) in *in vitro* multistage gastrointestinal model. The phenolic metabolites such as phenylpropionic, benzoic, phenylacetic and cinnamic acids were identified only in the colonic compartments due to the biotransformation of the polyphenols by colonic microbiota. The phenolic metabolite profile was different in each of the ascending, transverse and descending colon compartments, and it also lead to the formation of SCFA and increase in antioxidant capacity.

Several studies, both *in-vitro* and *in-vivo*, have reported bacteria-polyphenol interactions. The microbial metabolites formed varied with inter-individual microbiota variations in *in-vitro* fermentation of red wine extract (Sanchez-Patan et al., 2012). Grape and chokeberry wine polyphenol-rich extracts lead to the inhibition of pathogenic bacteria *Enterobacteiaceae* along with the formation of phenolic metabolites (Gumienna et al., 2011).

2.4.4 Health Benefits of Polyphenols

Studies have found positive relation between diet and colon cancer as consuming foods rich in fibre was considered to reduce colon cancer risk. Recently, many studies have indicated the protective effect of consumption of fruits, tea, soy and vegetables against colon cancer as these foods are found to be rich in phytochemicals such as phenolics (Macdonald and Wagner, 2012). The stilbene compound, resveratrol, found in red wine has particularly gained attention as studies conducted on mice indicated its anti-inflammatory and anti-carcinogenic effects

(Cardona et al., 2013). A study on colon carcinogenesis induced in rats by azoxymethane or dimethylhydrazine reported a positive inhibition of tumors by red wine polyphenols. The wine polyphenols also changed the gut microbial ecology with *Bacteroides, Lactobacillus* and *Bifidobacterium spp.* as dominant strains from *Bacteroides*, *Clostridium* and *Propionibacterium spp.* in control-fed rats (Dolara et al., 2005). A study reported a significant increase in number of *Lactobacillus spp.* and *Bifidobacterium spp.* after increasing flavanol dose for 4 weeks in healthy volunteers, which also led to decreased plasma C-reactive protein concentrations in blood (Tzounis et al., 2011). Polyphenols in green tea, fruits and vinegar wine are also hypothesized to be related to body weight lowering properties. Obesity is related to a decrease in the relative proportions of *Bacteroidetes* to *Firmicutes,* while phenolic metabolites may alter positively the balance between these groups (Rastmanesh, 2011).

2.4.5 Polyphenolic Content in Plants under Stress

Plants are exposed to various environmental threats throughout their life cycle in the form of drought, extreme temperatures, nutrient deficiency, salt, toxicity, pests and diseases. But plants, being sessile, have developed their own defence mechanisms in response to the external constraints. Some of these mechanisms include formation of secondary plant metabolites such as polyphenols. The polyphenols act as antioxidants against the reactive oxygen species produced during biotic and abiotic stresses.

Some heavy metals such as Fe, Cu, Zn, Co or Ni are the micronutrients essential for plant growth and development. While heavy metals like Cd, Pb, Hg, Ar etc. are non-essential and toxic to plants (Gasic & Korban, 2006).

Reactive oxygen species (ROS) such as $\cdot O_2^-$, ·OH, H_2O_2 are produced under heavy metal-induced stress and are very reactive. The most reactive ·OH radicals are formed either

by *Fenton reaction* or the metal-independent *Haber-Weiss reaction* as given below (Mithofer et al., 2004).

Fenton reaction:

$$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow \cdot OH + OH^{-} + Fe^{3+}/Cu^{2+}$$

$$\cdot O^{2-} + Fe^{3+}/Cu^{2+} \rightarrow Fe^{2+}/Cu^{+} + O_2$$

Haber-Weiss reaction:

$$H_2O_2 + \cdot O_2^{-} \rightarrow \cdot OH + OH^{-} + O_2$$

Heavy metal stress due to its accumulation in plants is known to trigger the antioxidant defence mechanisms in the form of increased production of both enzymatic and non-enzymatic antioxidants. Several studies have showed the increase in Total Phenolics (TP), Total Flavonoids (TF) and antioxidant activity in different plants by exposure to heavy metals at different concentrations.

Manquian-Cerda et al. (2016) studied changes in phenolic profiles and antioxidant activity in blueberry (*Vaccinium corymbosum L.*) plantlets grown *in-vitro* under the exposure of Cd in concentrations of 50 and 100 µM. There was an increase in phenolic compounds, especially chlorogenic acid, which was in correlation with an increased FRAP activity. The results also indicated changes in phenolic composition, which showed the presence of vanillic acid and luteolin and absence of gallic, ferulic, and sinapic acids, phloridzin, quercetin, methoxy carnosol, catechin, and caffeic acid hexoside in treated plantlets compared to control. Similar results of increase in total phenols in leaves and roots of young date palm (*Phoenix dactylifera* L.) under Cd-induced stress was reported by (Zouari et al., 2016). Al-mediated stress lead to almost 50% increase of TP in shoots of *Rumex acetosa* L. strongly increasing the concentration of catechol, catechin and rutin (Tolra et al., 2005).

Musilová et al. (2011) conducted a study at Hontianske and Banska Stiavnica regions in south-western part in middle Slovakia on potato tubers grown in soil with metallic loading. . The soils in these regions were found to be contaminated with Cd, Pb and Zn. The amount of total polyphenols in potatoes increased with increased amount of Cd and Pb. The TP were also highly positively correlated with antioxidant activity.

Studies also indicated the increase in TP, TF and antioxidant capacity of certain plants under certain threshold concentration of heavy metals stress. Stress beyond the threshold concentrations lead to decrease in TP, TF and antioxidant capacity. Decrease in TP, TF and antioxidant capacity were reported in *Erica andevalensis* plants (Márquez-García et al., 2012) and in *Camellia sinensis* L. plants (Zagoskina et al., 2007) under high concentrations of Cd. This effect was hypothesized to be caused by an impaired antioxidant response system under increased metal stress. Kováčik et al. (2008) also reported an increase in most of the phenolic compounds in *Matricaria chamomilla* under Cu-induced stress up to 60 µM, whereas at 120 µM the compounds decreased to values equal to control or lower.

The antioxidant defence mechanism against heavy metal stress is governed by type of metal, time and concentration of exposure. Mongkhonsin, et al.Mongkhonsin et al. (2016) studied the response of phenolic compounds to Zn and/or Cd toxicity in *Gynura pseudochina* (L.) DC. plant extracts. Zn and Cd exposure lead to increase in TPC, TFC and antioxidant capacity, while a higher increase was found with dual Zn and Cd exposure. This indicated an alleviation of Cd toxicity by Zn. Another study conducted by Manquian-Cerda et al. (2018) showed a 2-3 times higher increase in three phenolic acids (chlorogenic, ellagic, gallic) in blueberry *(Vaccinium corymbosum* L.) under combined Al + Cd stress than Al alone. Öncel et al. (2000) reported an increase in total soluble phenolics in two different varieties of wheat seedlings. This increase due to Cd was heightened by temperature whereas no such increase was found with Pb application.

2.4.6 Measurement of Antioxidant Activity and Total Phenolics

Different assays are used for measurement of antioxidant capacity (AOC) but no individual assay can provide total antioxidant capacity for all types of oxidants. This uncertainty is because of many different sources of radicals (or oxidants) and antioxidants, and different mechanism underlying their reactions. The First International Congress on Antioxidant methods has also recommended the use and comparison of multiple assays to determine AOC for foods and dietary supplements (Prior et al., 2005).

2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is a decoloration assay and spectrophotometer is used to measure the absorbance of $ABTS^{\cdot+}$ radical. The deep blue/green color is lost as the antioxidants in the sample react with $ABTS^{\cdot+}$ radical (Re et al., 1999). The absorbance maxima is found at several wavelengths, 415, 645, 734 and 815 nm, while 415 nm and 734 nm are most commonly used wavelengths.

The $ABTS^{\cdot+}$ radical was originally formed by the reaction of metmyoglobin and H_2O_2 generated ferrylmyoglobin with ABTS. But there was a limitation of overestimation of antioxidant capacity as the antioxidants also reacted with ferrylmyoglobin. After several modifications in the assay, the method currently used was developed, which involves the production of $ABTS^{\cdot+}$ radical by direct reaction between potassium persulfate and ABTS (Prior et al., 2005). The extent of decolorization is measured as a function of time and absorbance, measured 4-6 min after adding sample or standard (Trolox). The antioxidant capacity is measured as Trolox equivalent (Prior et al., 2005; Re et al., 1999).

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is an easy and valid antioxidant assay. It is based on the measurement of the reducing ability of antioxidants in a sample (Sánchez-Moreno, 2002). The DPPH solution containing stable free ·DPPH radicals is deep purple in color and loses its color after reduction by the test compounds. The spectrophotometer technique

measures the decrease in absorbance at 515-528 nm. It is convenient and most commonly used decoloration assay (Prior et al., 2005; Sánchez-Moreno, 2002). The absorbance is then expressed as Trolox Equivalents after comparing it with the standard Trolox curve. However, this assay gives higher AOC (Antioxidant capacity) for the molecules small in size and it is easily accessible to the radical. Moreover, some inaccuracies in AOC may also result from side reactions such as H-atom transfer leading to loss of DPPH color (Prior et al., 2005).

The Folin-Ciocalteau (F-C) assay was originally developed to measure tyrosine (Prior et al., 2005). A colored product was formed at 745-750 nm due to oxidation of phenolics by molybdotungstate. But this reaction was slow and lacked specificity. Later, the method was improved in which a molybdotungstophosphoric hetropolyanion reagent oxidises phenolics and obtain blue colored product at 765 nm. Gallic acid equivalent was the most commonly used measure. However, researchers have also used other equivalents, catechin and ferulic, caffeic, vanillic and chlorogenic acids (Prior et al., 2005). Although this method is very simple and useful, the F-C measurements can be biased by additional reactions with sugars, aromatic amines, organic acids and also by nonphenolic organic substances (Prior et al., 2005).

Ferric Reducing Antioxidant Power (FRAP) assay is a simple, fast and robust method which measures the total reducing power of the antioxidants in sample. The assay can be performed using microplate reader (Prior et al., 2005). Originally, this assay was used to measure the reducing ability of plasma, but later adapted for botanical samples too (Benzie and Strain, 1996). The assay is performed at low pH where an intense blue color is formed as Ferric-trypyridyltriazine (Fe^{III}-TPTZ) is reduced to Ferrous (Fe^{II}) form by the sample or standard. The absorbance maxima of the blue color formed is measured at 593 nm (Benzie and Strain, 1996; Prior et al., 2005). The antioxidant capacity is measured as ferrous sulphate equivalents.

2.5 Microbiota

2.5.1 Human Gut Microbiota

The human gastrointestinal tract is colonised by 300-500 different species of bacteria (Guarner and Malagelada, 2003). The microbial concentration is less in stomach due to low pH and gradually increases towards the colon, with concentrations up to 10^{11} or 10^{12} cells/g of colon content (Guarner and Malagelada, 2003). The gut microbiota is very complex, dynamic and vary from one individual to another. The development of microbiota depends on many factors like birth conditions, age, diet, lifestyle, geographic location and use of antibiotics, prebiotics, probiotics, etc. (Murgas Torrazza and Neu, 2011; Yatsunenko et al., 2012). The microbiota plays a very crucial role in regulating health and disease in host's body. It has metabolic, trophic and protective functions (Guarner and Malagelada, 2003). It ferments the non-digestible carbohydrates including cellulose, xylans, resistant starch and inulin. This metabolic process produces energy for microbial growth and SCFA (Tremaroli and Backhed, 2012). These SCFA play a very pivotal role in epithelial cell proliferation and differentiation (Frankel et al., 1994). Gut microbiota protects the host by creating a resistance for invading pathogens by production of antimicrobial substances and competing for attachment sites and nutrients (Sekirov et al., 2010).

2.5.2 Effects of Heavy Metals on Gut Microbial Health

The tremendous amount of microbiota nurturing inside our body is very critical for our health and very sensitive to environmental pollutants. Although gut microbiota is not directly exposed to environment but pollutants make their way to gut through different pathways such as food and water (Jin et al., 2017). Metals such as Cd, Pb, Cr, Ar, etc. are found to generate reactive oxygen and nitrogen species which have toxic and carcinogenic effects inside our body (Valko et al., 2006). The absorbed heavy metals in the gastrointestinal tract lead to their accumulation in different parts such as liver, kidneys, bones, body tissues, etc. While the unabsorbed heavy

metals remain in the microbial environment and induce gut microbiota dysbiosis (Breton, Massart, et al., 2013).

Cd pollution is related to various industries such as electroplating, batteries, paint and fertilisers. In a study, in drinking water borne Cd exposure at the rate of 23 to 50 mg kg^{-1} for 45 days produced significant imbalance in the microbial population of mice microbiota (Fazeli et al., 2011). *B. cereus* and *Enterococcus* spp., were found to be the most sensitive while *E. coli* and *Klebsiella* spp., were the most resistant to toxic effect of Cd. In another report, Cd at the rate of 20 – 100 mg kg^{-1} inhibited the overall growth rate of intestinal microbiota and impaired the gut barrier in mice. Some beneficial bacteria; *Bifidobacterium* and *Lactobacillus* suffered more due to Cd stress. There was significant decrease in SCFA production (Liu et al., 2014).

Pb pollution in air originate from use of tetraethyl lead in gasoline while its presence in soil and dust is by the use of Pb based paints and industrial activities. Pb enters human bodies via ingestion, inhalation and dermal absorption (Jin et al., 2017). A study was conducted on mice with perinatal Pb exposure of 32 mg/L of lead in drinking water for 40 weeks. An increase in body weight of males was observed and also a shift in gut microbiota was observed in adult offspring (both male and female). A significant change in ratio of Bacteroidetes and Firmicutes was observed to be associated with Pb exposure (Wu et al., 2016). Another report demonstrated the role of gut microbiota in limiting the heavy metal burden upon exposure to Pb and Cd. The results showed significantly greater accumulation of heavy metals in liver, kidneys, blood and tissues of germ-free mice as compared to specific pathogen free (SPF) mice (Breton, Le Clère, et al., 2013).

2.6 Simulated Human Gut Digestion Models

The microbial communities associated with gastrointestinal tract have gained enormous attention over the past few decades. Given its vast microbial diversity and associated functions influencing the host health, the gut microbiota is considered as an organ within an organ (Venema and van den Abbeele, 2013). In order to study the gut microbiota, human trials are limited to fecal samples and cannot be used for toxicological studies (Rumney and Rowland, 1992; Venema and van den Abbeele, 2013). Animals such as rodents and pigs have been used as *in-vivo* models to investigate ecological mechanisms and microbial metabolism inside gut. But there are some questions about differences in gut microbial composition and metabolism in these animals versus those in humans (Rumney and Rowland, 1992). Another *in-vivo* model developed to overcome the microbial composition differences among humans and animals include colonising germ-free rodents with human fecal microbiota. Experimental studies on this model have shown that the human microbiota in mice remain unchanged for at least 5 weeks (Rumney and Rowland, 1992).

Over the last few decades, *in-vitro* systems that mimic the microbiota and its processes in the lumen have come into existence. They are an innovative technological procedure to conduct investigations on microbial species and functions and range from very simple static models to sophisticated multi-chamber models. Batch fermentation models consists of closed reactors containing fecal suspensions in a medium maintained under anaerobic conditions (Macfarlane and Macfarlane, 2007). These are inexpensive, and can be used for testing a large number of substrates. However, microbial fermentations result in changes in pH and redox potential, making them limited to short-term studies (Payne et al., 2012). Batch fermenters have been used to conduct studies on resistant starch and other dietary components such as inulin-type fructans (Lesmes et al., 2008; Pompei et al., 2008). Continuous fermentation models allow addition of fresh substrate and removal of toxic used substrate from the chemostats (Moon et al., 2016). They can be used as single-stage or multi-stage systems and permit long term

studies. Single-stage continuous model has a single chemostat commonly used to mimic the proximal colon segment of large intestine (Moon et al., 2016; Payne et al., 2012). However, multi-stage models horizontally demonstrate the ascending, transverse and descending colon regions with all their metabolic and microbial differences. The model provides optimal temperature, pH, retention time, flow rate and anaerobic conditions in the fermenters as *in-vivo* human gut microbiota (Payne et al., 2012). SHIME (Simulator of the Human Intestinal Microbial Ecosystem) is an evolved continuous culture model, consisting of five reactors mimicking both the upper and lower digestive tract. The first two reactors simulate the stomach and small intestine digestion and are supplied with required nutritive medium, pancreatic enzymes and bile and also maintained at the pH found *in-vivo.* The total residence time in the colon vessels is 72 h (Venema and van den Abbeele, 2013).

Enteromix is also a continuous culture model comprising of four vessels representing the ascending, transverse, descending and distal regions of human large intestine with pH 5.5, 6.0, 6.5, 7.0 respectively. Fresh media is pumped into first vessel every three hours and then transferred to next vessels in a chain with total fermentation extending up to 48 h (Venema and van den Abbeele, 2013). Recent developments in the digestion models have brought TIM-1 and TIM-2 into existence. TIM-1 model represents small intestine digestion mimicking pH, motility, bile secretions and even the absorption component of the small intestine (Payne et al., 2012). Likewise, TIM-2 is a computer-controlled proximal colon model, in which glass vessels are incorporated with flexible silicon membranes to simulate peristaltic movements and absorption of the microbial metabolites, which otherwise would accumulate in vessels and inhibit microbial activity (Venema and van den Abbeele, 2013).

Despite of all the developments, *in-vitro* digestion models still face many limitations and challenges. The reproducibility and functional stability of the microbiota is often questioned. The exact replication of *in-vivo* microbiota is questionable due to missing epithelial

and immune cells, which are important for host-microbe interaction. Obtaining exact biological replication to conduct repetitive studies is also a limitation of the *in-vitro* gut fermentation modelling (Payne et al., 2012; Venema and van den Abbeele, 2013).

2.7 Short Chain Fatty Acids and Health Benefits

SCFA are organic fatty acids having 1-6 carbon atoms and are produced by the bacterial fermentation of non-digestible food components reaching the colon (Wong et al., 2006). These components include resistant starch, dietary fibre, unabsorbed sugars, raffinose, starchyose, polydextrose and modified cellulose (Wong et al., 2006). Mucus, sloughed cells and gastrointestinal secretions also promote SCFA production (Cook and Shellin, 1998). Three major SCFA formed are acetate, propionate and butyrate, whereas other SCFA such as valerate, hexanoate, isobutyrate and isovalerate form only 5-10% of total SCFA (Cook and Shellin, 1998). Various human studies have found the molar ratio of acetate:propionate:butyrate as 60:20:18 (Roy et al., 2006). The production of SCFA is governed by number and type of microflora, substrate availability and gut transit time (Wong et al., 2006). Most of the SCFA production occurs in cecum and proximal colon with highest substrate availability; whereas carbohydrate and water is depleted as it reaches the distal colon. These regional differences in SCFA concentration in the colon is also implicated with the occurrence of most colonic diseases such as cancer in the distal region (Wong et al., 2006). After production, SCFA are absorbed by the colonocytes, therefore only 5-10% are left in the fecal matter (Fluitman et al., 2017). SCFA are absorbed either by the diffusion or anion exchange process and stimulate sodium and water absorption (Cook and Shellin, 1998). After absorption, the SCFA are metabolised in ceco-colonic epithelium cells, liver cells and muscle cells (Wong et al., 2006). They provide approximately 10% of daily energy requirements in humans (Fluitman et al., 2017).

Acetate is the principal SCFA produced, which is later absorbed and transported to liver, muscles and other peripheral tissues where it is further metabolized (Roy et al., 2006; Wong et al., 2006). Acetate is used as main route to absorb energy from carbohydrates not absorbed in the small intestine (Roy et al., 2006) and as primary substrate for cholesterol synthesis (Wong et al., 2006). It is used to monitor colonic events in human studies as it is the main SCFA found in blood (Hijova and Chmelarova, 2007). Acetate is also involved in synthesis of long SCFA, glutamine, glutamate and betahydroxybutyrate (Cook and Shellin, 1998). Propionate is produced mainly via two pathways; fixation of CO_2 to form succinate or from lactate and acrylate (Wong et al., 2006). Propionate metabolism is less understood in humans while it is the major precursor of gluconeogenesis in ruminants (Hijova and Chmelarova, 2007). Apart from being the primary substrate of gluconeogenesis, it also has an inhibiting effect which can be associated to its metabolic intermediates (Wong et al., 2006). Butyrate is the most used SCFA, over propionate and acetate in a ratio 90 : 30 : 50 (Cook and Shellin, 1998). It is the major energy source for epithelial cells and also regulates cell differentiation and proliferation. Most of the butyrate around 70-90% is metabolized by the colonocyte (Hijova and Chmelarova, 2007; Wong et al., 2006). Butyrate protects the gut epithelial cells from injury by enhanced expression of heat shock proteins (Roy et al., 2006) and is also known to stimulate immunogenicity of cancer cells (Hijova and Chmelarova, 2007).

2.7.1 Effect of Polyphenols on Short Chain Fatty Acids

In-vitro batch culture fermentation of common dietary polyphenols (rutin, quercetin, chlorogenic acid and caffeic acid) enhanced the production of major SCFA (acetate, propionate and butyrate) after 48 h incubation (Parkar et al., 2013). In a similar study conducted in a multi-reactor gastrointestinal model, using pure polyphenol mixture of chlorogenic acid, caffeic acid, ferulic acid and rutin, fermentation resulted in an increase in total SCFA in all the colonic

compartments while butyric acid increased only in ascending and transverse colon compartment (Sadeghi Ekbatan et al., 2016). SCFA and AOC enhancement was also demonstrated in rats fed with a potato diet for 3 weeks (Robert et al., 2013). Another study on pomegranate by-products composed of gallic acid, ellagic acid and glucose units and pomegranate polyphenol punicalagins resulted in an enhanced SCFA production by all the by-products but not by punicalagins (Bialonska et al., 2010).

2.7.2 Role of SCFA in Disease

Absorption of SCFA in the colon stimulates the absorption of sodium and water. In various studies on diarrheal disorders, addition of resistant starch to oral dehydration solutions, such as rice water, carrot soup was significantly effective in shortening the duration of diarrhea (Roy et al., 2006). Diversion colitis occurs in patients who undergo colectomies and ileostomies, leading to mucosal changes and alterations in the functions of colon resulting from depletion of SCFA. It is a general observation that the problem is resolved after the fecal stream is restored and SCFA production revives (Roy et al., 2006). Studies have found impaired butyrate oxidation in colonocytes in patients with Ulcerative Colitis. Other observations include decrease in faecal SCFA, particularly butyrate, increase in faecal lactate. Nine out of ten patients with distal colitis improved clinically and histologically in a study, after consuming acetate, propionate and butyrate mixed solution for 6 weeks (Cook and Shellin, 1998). Similarly, SCFA in the colon through *in-vitro* models, mouse studies or through clinical observations have showed some direct or indirect benefits against many other diseases such as pouchitis, irritable bowel syndrome and colon cancer. In many of these studies, the role of SCFA has not yet been proved clinically, but there are mounting evidences that SCFA plays a key role in colonic health (Cook and Shellin, 1998; Roy et al., 2006; Wong et al., 2006).

CHAPTER 3

POTENTIAL HEALTH IMPACTS OF HEAVY METALS IN POTATOES

ABSTRACT

Wastewater irrigation is undoubtedly an important strategy to cope with the major global risk of water scarcity. Despite being an alternate source of water, it is also rich in pollutants, both organic (hormones and pharmaceuticals) and inorganic (heavy metals). These contaminants can make their way into the food chain via plant uptake and leading to biomagnification. Some of the heavy metals such as Pb, Cd, Ar, Hg, etc., inside the human body, have no biological health promoting significance but can cause adverse effects. Their presence in biological organisms have been associated with disrupted immune system, oxidative stress, DNA damage, cardiovascular diseases, organ dysfunctionality, and carcinogenesis. Recent studies have found gut microbiota dysbiosis caused by heavy metal exposure. Considering the health risks associated with heavy metals, the present study was conducted on potatoes, grown with wastewater irrigation. Four different treatments had varying concentrations of heavy metals by virtue of the soil amendments applied and one treatment was freshwater irrigated to represent sample without contamination. The study consisted of fermentation of potatoes in batch reactors using human fecal microbiota using a single daily serving of cooked potato. The samples were taken from the fermenters at different time intervals during 24 h of incubation. The microbial processes were assessed by measuring short chain fatty acid (SCFA) concentrations, antioxidant capacity, and total phenolics. Lactobacilli plate count using MRS agar was also conducted to consider the effect on beneficial bacteria. The SCFA concentrations followed a similar trend for all the treatments and antioxidant capacities as well as total phenolics were also comparable with pre-digestion values for all the treatments. The Lactobacilli population increased with time in all the reactors. To conclude, the polyphenol antioxidants of potatoes might have neutralized the possible adverse effects of heavy metals on gut microbiota in the single meal exposure.

3.1 Introduction

Wastewater irrigation is a promising solution to the meet the food demands of increasing population in this phase of global water crisis (Koncagül et al., 2017). It is estimated that more than 20 million ha of land worldwide is irrigated with untreated or partially treated wastewater (Zhang and Shen, 2017). Although wastewater irrigation could provide cost-effective nutrients and organic matter supply to soil for boosting food crop production, the danger of heavy metals' loading in soil and their transfer to human food chain cannot be overlooked. Many developing countries lack the infrastructure needed to treat the wastewater before using it for irrigating crop fields; it is contaminating the soil and groundwater through leaching, and transferring it to food chain. Some cost-effective alternatives such as biochar, derived from readily available biomass, and hydrogels are found to be effective in remediating the contaminated soils or aqueous solutions.

Heavy metals such as Co, Cu, Cr, Fe, Mg, Mn, Mo, Ni, Se and Zn are the essential micronutrients, if consumed in adequate amounts (Tchounwou et al., 2012). While metals such as Pb, Cd, Hg, Ar are toxic even at low concentrations and have no positive biochemical role in the body (El-Kady and Abdel-Wahhab, 2018). The metals entering human body via food are absorbed in various sections of the gastrointestinal tract and accumulate in the body organs causing toxicity (Wani et al., 2015), carcinogenesis (Yuan et al., 2016), oxidative stress (Valko et al., 2006), DNA damage (Tchounwou et al., 2012), and are also found to causes gut microbiota dysbiosis (Jin et al., 2017).

Potato, called "Hidden Treasure" at "The International Year of Potato, 2008" a rich source of carbohydrates and proteins, and also provides essential micronutrients such as vitamin B1, B3, B6 and C, folate, potassium, phosphorous, magnesium, iron and antioxidants (Lutaladio and Castaldi, 2009). The antioxidant capacity in the potatoes is largely due to polyphenolic compounds in it, but its carotenoids and ascorbic acid also play a significant role

(Camire et al., 2009). Consumption of potato has also been associated with cancer risk prevention by an *in-vitro* study whereby the phytochemicals of a speciality potato cultivar (CO112F2-2) exerted proapoptotic effect on prostate cancer cells (Reddivari et al., 2007).

The large intestine in human contains a vast dynamic ecosystem of living bacteria with concentrations of $10^{11} – 10^{12}$ cells/g of luminal content affecting the host homeostasis (Guarner and Malagelada, 2003). *Bacteroidates* and *Firmicutes* are the most abundant phyla among these bacteria (Eckburg et al., 2005). The microbial community performs many vital metabolic, protective and trophic functions (Robles Alonso and Guarner, 2013). It breaks down the complex carbohydrates to form SCFA which are primary energy source for epithelial cells (Wong et al., 2006) and also converts undigested polyphenols into phenolic metabolites (D'Archivio et al., 2007).

In-vitro human digestion models are developed to monitor microbial metabolic processes; monitoring these processes in human and animal trials. Although there are many complex models such as SHIME (Simulator of the Human Intestinal Microbial ecosystem) mimicking the dynamics of different colon regions (Molly et al., 1993), batch fermentation studies are simple and inexpensive. Despite of some limitations such as accumulation of microbial metabolites, they are very convenient and allow testing a large number of sample over a short period of time (Venema and van den Abbeele, 2013).

This study is a part of wastewater use project being conducted at Macdonald Farm of McGill University. Russet Burbank potato tubers were grown in in field lysimeters. Randomly allocated five treatments of amendments and irrigation water combination in triplicate were: (1) WW: wastewater to untreated soil, (2) BC: wastewater to biochar treated soil, (3) SAP: wastewater to SAP treated soil, (4) BC-SAP: wastewater to Biochar + SAP treated soil, and (5) FW: freshwater to untreated soil. The tubers harvested from these treatments were collected to evaluate the potential impact of heavy metals taken up by the tubers on gut microbial

metabolism in terms of SCFA, antioxidant capacity and *Lactobacilli* counts. The triplicate potato samples of each treatment were mixed to form one composite sample. The amounts of pollutants are given in Table 3.1. The study was conducted in batch fermentation reactors operated in the School of Human Nutrition of McGill University. .

3.2 Materials and Methods

3.2.1 Sample Preparation and Storage

The potato tuber samples were stored at 4°C immediately after harvest. Before starting for the study, the tubers were first washed, dried and sorted according to size. Uniform sized potatoes from all the treatments were selected. They were then cut to a similar size range to avoid overcooking. The samples were then put in boiling water and boiled in stainless steel containers for 15 min. Then the potatoes were wiped with kitchen towel, and chopped using standard kitchen knife, mixed and subsequently put in standard urine cups and measured the wet weight. The samples were frozen in liquid nitrogen and stored at -80°C until put in a freeze drier. After freeze drying, dry weight was measured and the lyophilized material was ground using a coffee grinder to form a powder. The powder was put in Falcon tubes to store at -80°C until use. All the processing was done in a lab with UV light filters to avoid degradation of antioxidants.

3.2.2 Measurement of Antioxidant Capacity of Potatoes

Antioxidant capacity of the freeze dried potato samples was measured using ABTS, DPPH and Folin-Ciocalteau for total phenolics. The samples were extracted as follows.

Methanolic Extract Preparation: Freeze dried potato samples of 0.1 g were measured into 1.5 mL Eppendorf tubes. 900 µL of 90% methanol was added and vortexed for 60 s. The samples were then allowed to sonicate in ice water for 30 min and were later vortexed. The samples

were then centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, supernatants were collected into a separate Eppendorf. Then 600 µL of 90% methanol was again added to the pellets, vortexed, centrifuged and the supernatants collected as before.

3.2.2.1 ABTS Antioxidant Capacity Measurement

ABTS antioxidant capacity was measured as µM Trolox Equivalent per 100 g DM according to the method adopted by (Re et al., 1999). Methanol, ethanol, Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) and potassium persulphate were used for preparing the assay. Trolox (500 µM) was prepared as a standard in methanol. ABTS stock was prepared by combining the ABTS and potassium persulfate (2.45 mM) solutions in deionised water and incubating them in dark for 12 h at room temperature. The ABTS working solution was prepared by diluting it with ethanol (95%) until the absorbance of 0.7(±0.05) at 734 nm was obtained. The 1.2 mL of ABTS solution was then added to 100 µL of standards and methanolic sample extracts and the absorbance was recorded between 0.1-0.9 at 734 nm. The absorbance was recorded within 10 min after adding ABTS to the methanolic extract. All the standards were prepared in duplicate while the samples as triplicate. Spectrophotometer and disposable plastic cuvettes were used to measure the absorbance.

3.2.2.2 DPPH Antioxidant Capacity

DPPH absorbance was also compared against the Trolox Standard curve and antioxidant capacity measured as µM trolox equivalent per 100 g DM. The method was adapted from (Brand-Williams et al., 1995) with modifications by (Thaipong et al., 2006). DPPH activity was measured as absorbance at 514 nm using spectrophotometer. For this, DPPH stock was prepared in methanol and stored in dark at 4°C. The stock was then diluted to prepare working solution with absorbance 0.7 (±0.05). 100 µL of methanolic extracts of the samples and standard were placed in plastic cuvettes and 1.5 mL of DPPH added followed by incubation

in dark for 1 h. Absorbance of samples and standards was then recorded between 0.1-0.9 at 514 nm and the DPPH activity was measured against the standard curve.

3.2.2.3 Total Phenolics

Total phenolics were measured as chlorogenic acid equivalents. Chlorogenic acid (0.1%) and sodium carbonate (7.5%) standards were prepared. 100 µL of standard and methanolic extract of samples were placed in disposable plastic cuvettes, and 2 mL of deionized water was added. Then 200 µL of F-C reagent (2 N) was added to the mixture and allowed to incubate in dark for 30 min, which was followed by addition of 1 mL of sodium carbonate. Finally, after 1 h incubation, the absorbance was recorded at 765 nm. Total phenolic content was then calculated as mg chlorogenic acid/100 g DM by comparing against the standard curve.

Digestion and Batch Fermentation Design

3.2.3 *In-vitro* Human Enzymatic Digestion

The *in-vitro* digestion method was adapted from (Miranda et al., 2013) with some modifications. Six digestions were done simultaneously, which consisted of five treatments: WW, FW, BC, SAP and BC-SAP, and one blank or control (without potatoes). The amount of freeze-dried potato used for each treatment was based on study conducted by (Kasper et al., 2011). Each treatment dose was rehydrated with 65 mL of water in vessel and α-amylase (450 U per g of freeze dried potato) was added to initiate salivary digestion. The vessels were then put in incubator with shaker for 10 min at 37°C temperature and pH 6.9. To simulate gastric digestion, the pH was adjusted to 2 using 1 N HCl and thereafter pepsin (6500 U per g of potato) was added. After 1 h of incubation (37°C, pH 2), pH was increased to 7 using 1 N NaOH and then pancreatin (1.8 mg per g of potato) and bile extract (11 mg per g of potato) were added to mimic the small intestinal digestion. The pH was again adjusted to 7 using 0.1 N NaOH and finally incubated at 37°C for 2 h.

3.2.4 Fecal Sample Preparation

Fecal samples were collected on the day of experiment from a male volunteer who did not take antibiotics in last 6 months before the experiment. The fecal samples were passed through a sterile sieve and diluted to 1:10 (w/v) using 0.1 M phosphate buffered saline (pH 7.4). Fecal slurry was homogenised by constantly stirring with sterile spoon. A 10 mL of prepared dilution was added to each of the six batch fermentation reactors for a 24 h incubation.

3.2.5 Batch Culture Fermentation

The model consisted of double glass jacketed containers with circulating hot water. Batch reactor vessels were made of glass and had six different ports (sampling or injection port, acid-base injection ports, gas inlet-outlet ports, pH meter port) in its cap. Each reactor was connected to a peristaltic pump which was further connected to a pH circuit and Raspberry pi model to maintain the pH. The model was used to mimic the conditions of the human colon, therefore pH of 6.8 ± 0.5 was maintained in the vessels by adding 1 N HCl or NaOH.

Batch culture fermentation method was adapted with modifications from (Tzounis et al., 2008). The basal nutrient medium consisting of peptone water (9 g/L), yeast extract (9 g/L), NaCl (0.45 g/L), K_2HPO_4 (0.18 g/L), KH_2PO_4 (0.18 g/L), $NaHCO_3$ (9 g/L), $MgSO_4.7H_2O$ (0.045 g/L), $CaCl_2.6H_2O$ (0.045 g/L), Tween 80 (9 ml/L), hemin (225 mg/L), Vitamin K_1 (45 µL/L), L-cysteine (2.25 g/L), bile salts (2.25 g/L), resazurin (4.5 mg/L) and distilled water was prepared fresh on the day of experiment. Six batch reactors were run at a time containing 35 mL of sterilized water, 30 mL of the basal nutrient medium and 15 mL of the prepared fecal slurry added to each reactor adjusting appropriate pH. The microbial population in the reactors was allowed to grow and stabilise for 24 h. After 24 h, the digested potato meals from the small intestine vessels were added to the reactors and samples taken at time 0 h. The fermentation

continued for 24 h taking samples at 6, 12, and 24 h of potato meal digestion. The experiment was run in triplicate (six treatments x 3 replicates = 18/6 per run =3 runs).

3.2.6 *Lactobacillus* Plating

Serial dilutions (10^{-1} to 10^{-8}) were prepared within 1 h of taking samples at time 0 h and 24 h of batch fermentation. A 0.1 mL of 10^{-4} to 10^{-8} dilution was plated in triplicate on a selective medium of MRS Agar. The plates were then incubated at 37°C in sealed jars made anaerobic using Oxoid AneroGen 2.5 L Sachet. The plates were then counted for the *Lactobacillus* growth after 72 h of incubation.

3.2.7 SCFA Analysis

SCFA were analysed based on modified method (Zhao et al., 2006). Aliquots of 1 mL of the samples from the batch reactors were centrifuged at 3500 rpm for 15 min and filtered using sterile syringe filters (25 mm diameter and 0.2 µm pore size). The samples were then diluted with sterile double distilled water (1:5) and 1 µL was injected into an Agilent 7890 series gas chromatograph (GC) system equipped with flame ionisation detector. Capillary column used was 30 m long with 250 µm internal diameter and 0.25 µm film thickness. The flow rate of helium as carrier gas was maintained at 0.8 mL/min. Split ratio for the injection was 10:1. The inlet and detector temperatures were set at 250°C and 260°C, respectively. The oven temperature was held at 100°C initially, for 10 min and later increased by 8°C/min from 100°C to 220°C and finally was held for 10 min. The SCFA were then identified based on known retention times and quantified using the standard curves prepared using pure SCFA mixture. The concentration was measured as mM.

3.2.8 Antioxidant Capacity of Gut Samples

Antioxidant capacity of samples taken from the batch fermentation at 0, 6, 12 and 24 h was measured using FRAP and Folin-Ciocalteau assays. The samples were centrifuged and filtered before measuring the antioxidant capacity.

3.2.8.1 FRAP Antioxidant Activity

FRAP measured the total antioxidant potential of the digested samples from the batch fermenters based on the modified method of (Benzie and Strain, 1996). Reagents were freshly prepared: 300 mM acetate buffer (pH 3.6) in distilled water, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in HCl (40 mM), and 20 mM $FeCl_3.6H_2O$ in distilled water. A working solution was prepared by mixing the acetate buffer, TPTZ and $FeCl_3.6H_2O$ in a 10:1:1 ratio. A solution of 1 mM of $FeSO_4.7H_2O$ in distilled water was prepared to make the standard curve. 10 µL of samples and standards were mixed with 30 µL of water in microtiter plates. Then, 200 µL of the working FRAP solution was added and mixed. The absorbance was measured at 593 nm using microplate reader after 30 min incubation at room temperature in dark. All the samples and standards were measured in triplicate.

3.2.8.2 Total Phenolics of Gut Samples

Filtered samples, 100 µL each, were placed in plastic cuvettes. 2 mL of deionised water followed by 200 µL of Folin-Ciocalteau Reagent (2N) was added and incubated for 30 min in the dark. Then, 1 mL of sodium carbonate was added and again incubated for 1 h. The absorbance of samples was recorded at 765 nm. The values were then calculated as mg chlorogenic acid/100 g DM.

3.3 Statistical Analysis

The data of antioxidant capacity (ABTS, DPPH, FC) of potato tubers (before digestion) was statistically analyzed using one way ANOVA procedure followed by LS Mean comparison

using Tukey's HSD test. Pearson's correlation coefficient was determined using CORR procedure among different antioxidant assays. MIXED model procedure with repeated measures model was applied for the *Lactobacillus*, SCFA and antioxidant capacity data of fermented samples. The differences were then compared using Tukey's test. Correlation between FRAP and TP of the digested samples was also determined using CORR procedure. All the statistical tests were performed using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Antioxidant Capacity before Digestion

The pre-digestion ABTS， DPPH and F-C values were shown in Table 3.2. The measured ABTS values (mM Trolox equivalent/100 g DM) were significantly ($p<0.05$) higher for WW treatment and lowest for BC treatment. While the values were not significantly different for FW, SAP and BC-SAP (Table 3.2). The DPPH values (mM Trolox equivalent/100 g DM) were also highest for WW and lowest for BC treatments. BC-SAP values were not significantly ($p<0.05$) different from BC and SAP whereas FW DPPH values were second highest after WW.

The FC values (mg chlorogenic acid/100 g DM) again followed a similar trend as ABTS and DPPH, with significantly ($p<0.05$) higher values for WW and lower for BC treatment. The FW value were not significantly different from BC. The correlation between ABTS, DPPH and FC test gave positive results. ABTS was significantly correlated to DPPH ($r=0.83$, $p<0.0001$) and FC ($r=0.91$, $p<0.0001$). While DPPH and FC have correlation coefficient as $r=0.75$, $p<0.0001$.

3.4.2 *Lactobacillus* cfu

Table 3.3 shows the mean ± SE values of the Lactobacilli plate count. The Lactobacilli population increased over the period of 24 h and the increase was significant ($p<0.05$) for all the treatment groups and the controls with no significant difference within the treatments or control.

3.4.3 Antioxidant Activity

The antioxidant activity increase with digestion of the potatoes in treatment reactors。 The trends in post digestion FRAP activity and TP are shown in figures 3.1 and 3.2 respectively。 The fixed treatment effects showed a significantly ($p<0.05$) higher FRAP activity for WW and FW versus control. Antioxidant capacity for WW reactor was significantly ($p<0.05$) different from BC, SAP and BC-SAP with WW and FW reactor showing significantly highest FRAP activity among all treatments. There was an overall increase in the FRAP activity for all treatments at 6 h compared to baseline (0 h). Total phenolics (TP) were also significantly ($p<0.05$) higher in WW treated samples versus all the other treatments and the control whereas TP in FW, BC, SAP and BC-SAP reactors was significantly higher than the control but not different from each other. There was also a time-dependent change in different reactors over a period of 24 h. TP in the reactors increased significantly ($p<0.05$) at 6 h versus baseline (0 h) for all the treatments. The increase in TP was significant in WW reactor even at 12 h compared to all the treatments and the control. The TP concentrations in the reactors had significant ($p<0.0001$) positive correlation with FRAP activity, with a Pearson correlation coefficient of 0.667.

3.4.4 Short Chain Fatty Acids

Figure 3.3 shows the trend in production of Total Short chain fatty acids （TSCFA） production over a period of 24 h。The TSCFA concentration increased with the fermentation of potatoes in all the treatment reactors but decreased in control reactor。 The production was highest at 6 h of incubation and then decreased at 12 h and 24 h for all the treatments. The concentrations were significantly ($p<0.05$) higher for all the treatments compared to the control but no significant difference in concentrations within treatments. SCFA were significantly ($p<0.05$) higher at 6, 12 and 24 h versus the baseline (0 h). Acetic, propionic and butyric acid also followed a similar trend of increase in production till 6 h and then decrease over time of incubation as shown in Figure 3.4-3.6. Acetic, propionic and butyric acid production was significantly ($p<0.05$) higher at 6 and 12 h as compared to 0 h. The proportion of acetic acid was significantly higher than propionic and butyric acid throughout the 24 h of digestion.

3.5 Discussion

The AOC of potato tubers from WW show a wide range of variation compared to FW, BC, SAP and BC-SAP. The values were significantly higher for WW for all three antioxidant capacity tests of ABTS, DPPH and FC. The AOC values for SAP and BC-SAP were similar to FW for ABTS and DPPH and lowest for BC for all the three tests. The higher TP and AOC in WW treatment was corroborated by previous work by Musilová et al. (2011) showing an increase in TP and antioxidant capacity in the potato tubers grown in Cd and Pb contaminated sites.

The WW potatoes were exposed to heavy metal stress throughout their growth period while BC, SAP and BC-SAP potatoes were given a soil treatment thereby decreasing the heavy metal stress. This result is evident from the amount of heavy metals taken up by the plants and the higher amount present in WW potatoes (Table 3.1). Several other studies have supported

the accumulation of phenolic compounds in plants exposed to heavy metal stress. Kováčik et al. (2008); Manquian-Cerda et al. (2016); Zouari et al. (2016) reported an increase in TP and antioxidant capacity in *Matricaria chamomilla* under Cu stress, blueberries and young date palm under Cd stress respectively. The difference in antioxidant capacities in BC, SAP and BC-SAP treatments could be supported by the fact that the three treatments have affinity for different heavy metals, which could have led to differences in accumulation of heavy metals in tubers for the three treatments. Previous studies report the increase in TP and AOC is dependent on type of metal, concentration and time of exposure to the metal stress. *Gynura pseudochina* (L.) DC. plant extracts showed a higher increase in TP and AOC under combined stress of Zn and Cd than Zn or Cd alone (Mongkhonsin et al., 2016). Similar results were reported by (Manquian-Cerda et al., 2018) in blueberry plants under Al + Cd exposure.

An increase in the phenolic content in plants has also been reported due to other abiotic stresses. Drought-induced phenolics were observed in apple (Bolat et al., 2014) and cowpea (El-Enany et al., 2013) plants, while similar effects were reported in tomato and watermelons (Rivero et al., 2001) due to temperature stress.

Gut microbiota limits the accumulation of xenobiotics in blood and body organs (Breton, Daniel, et al., 2013) but studies have reported gut dysbiosis by the exposure of environmental pollutants throughout the digestive tract. For example, a previous study reported a significant decrease in ratio of *Firmicutes* and *Bacteroidetes* after 2 and 3 weeks of Cd exposure to mice in drinking water and beneficial bacteria such as *Lactobacillus spp.* showed a significant decrease after 3-weeks of exposure at concentration of 20 mg/kg (Liu et al., 2014). Another study by (Fazeli et al., 2011) also found a decrease in *Lactobacillus spp.* cfu after feeding 37 mg/kg Cd to mice in drinking water for 45 days. *Lactobacillus spp.* is a beneficial bacteria capable of promoting gastrointestinal health by the production of inhibitory substances that compete for nutrients with pathogens, adhering to epithelial cells, and by stimulating

immunity (Rolfe, 2000). Some studies have also reported the potential of some strains of Lactobacilli in binding and sequestering heavy metals (Bhakta et al., 2012; Monachese et al., 2012).

In the present study, the incubation of samples for 24 h significantly increased the population of *Lactobacillus spp.* compared to the baseline but the increase was not significantly different within the treatments or control. This result could be partly due to a lesser amount of heavy metals in the potatoes and a very short time of exposure as compared to previously reported results and could also be partly due to the prebiotic effect of polyphenols present in the potatoes.

Polyphenols and microbiota share a two-way relationship. Colonic microbiota plays a very important role in breaking down high molecular weight polyphenols into absorbable phenolic metabolites (Scalbert et al., 2002) while the phenolic compounds alter the gut microbiota composition by inhibiting the growth of pathogenic bacteria and allowing the growth of beneficial bacteria and so exerting a prebiotic-like effect (Cardona et al., 2013). Various studies have reported the increase in number of *Lactobacillus spp.* by the intake of polyphenol-rich foods. For example, cocoa-derived flavanols stimulated the growth of *Lactobacillus spp.* and *Bifidobacterium spp.* in healthy volunteers (Tzounis et al., 2011), grape pomace polyphenols induced *Lactobacillus acidophilus* growth in liquid culture media (Hervert-Hernandez et al., 2009). Chlorogenic acid, which is found in abundance in potatoes along with the other polyphenols (Ezekiel et al., 2013), has shown many anti-carcinogenic properties like inhibition of A549 lung cancer cells (Feng et al., 2005) and binding the carcinogen benzo(a) pyrene (Friedman, 1997) due to its high antioxidant activity (Ezekiel et al., 2013).

The AOC capacity measured using FRAP over a period of 24 h show an increase at time 6 h compared to 0 h, which can be due to the gut microbial metabolism of the parent

polyphenols in the potatoes into antioxidant phenolic metabolites. *In-vitro* gastrointestinal studies conducted on pure polyphenols (Sadeghi Ekbatan et al., 2016) and anthocyanins in purple fleshed sweet potato (Kubow et al., 2016) have shown the biotransformation of polyphenols into simpler metabolites and their correlation with the increase in AOC in colon compartments. The presence of microbial derived antioxidant phenolic metabolites is also indicated by an increase in the amount of TP at 6 h. This increase in TP was similar to that observed with FRAP activity and had a positive correlation (r=0.667). The metabolism of chlorogenic acid was also found in a study on the human subjects with colon while only a traces of metabolites were found in urine of subjects without a colon (Olthof et al., 2003). The similarity of the results of present study with the research literature signifies that the gut microbial functionality was not hindered by the heavy metals in the potatoes, which might be related to the beneficial effects of potato polyphenols to overcome the effect of the pollutants. The phenolic metabolites such as phenylacetic acid has shown to have anti-carcinogenic properties (Fernandez-Panchon et al., 2008) and red wine polyphenols have been found to decrease oxidative DNA damage of colonic mucosa and inhibit chemical colon carcinogenesis in rodents (Dolara et al., 2005). The AOC observations after digestion correspond with the AOC values of potatoes before digestion. The WW treated potatoes had the highest AOC values both before and after digestion in the gut model. The AOC values of for ABTS, DPPH and TP of the FW, SAP, BC and BC-SAP treatments also did not generally differ significantly among treatments both prior to and during digestion although the values were slightly lower for BC before digestion.

The increase in AOC was also accompanied by an enhancement in SCFA in the batch reactors with the different treatments. The increase in TSCFA after digestion was statistically similar in all the reactors including the ones containing heavy metals (WW, BC, SAP, BC-SAP), which coincided with the data of AOC and Lactobacilli that also showed an increase

post-digestion. The production of SCFA is linked with improved gut morphology and function (Scheppach, 1994). These compounds act as fuel for the colonic epithelial cells, and may also reduce the risk of gastrointestinal disorders, cancer and cardiovascular diseases (Cook and Shellin, 1998; Wong et al., 2006). Potatoes, being a rich source of complex carbohydrates, are a good source of substrates for the SCFA production. The presence of complex carbohydrates and antioxidant micronutrients in potatoes was related to increased SCFA production and antioxidant status in rats, which improved lipid metabolism and so may limit oxidative stress and reduce the risk of developing degenerative diseases (Robert et al., 2013). Another rat study demonstrated that potato resistant starch stimulates the growth of lactic acid bacteria and SCFA concentration in the large intestine (Kleessen et al., 1997).

The polyphenols in potatoes can also augment the production of SCFA. Pure dietary polyphenols such as; chlorogenic acid, caffeic acid, ferulic acid and rutin enhanced the production of SCFA in all the colonic vessels of multi-reactor gastrointestinal model (Sadeghi Ekbatan et al., 2016). Likewise, rutin, quercetin, chlorogenic acid and caffeic acid stimulated the production of SCFA in an *in-vitro* mix culture model simulating colonic fermentation using human fecal microbiota (Parkar et al., 2013). In the present study, acetate, propionate and butyrate were the major SCFA produced and they all followed a similar trend in increase over time as shown with the TSCFA. The statistically similar concentrations and proportions of SCFA also implicate similar microbial count and activities in all the reactors despite the presence of heavy metals in the four treatments. These findings are in line with previous study conducted on the protective effects of anthocyanins in purple fleshed potatoes against adverse dysbiotic effects of PCBs (Yu, 2017).

Table 3.1 Heavy metals in composite samples of potato tubers

	Chromium	Copper	Zinc	Cadmium	Lead	Iron
WW	0.093	3.673	16.947	5.103	0.386	32.160
FW	0.052	3.084	8.691	0.104	0.065	29.138
BC	0.050	0.633	8.067	1.794	0.198	22.372
SAP	0.317	3.974	11.082	4.072	2.246	46.547
BC-SAP	0.047	2.076	10.031	2.884	0.239	27.921

Notes: Values are in mg/kg DM.

Table 3.2 Antioxidant Capacity values for potatoes before digestion.

	ABTS	DPPH	FC
WW	958.48 ± 32.42^{a}	793.86 ± 20.40^{a}	1458.33 ± 48.59^{a}
FW	594.85 ± 6.94^{b}	701.81 ± 20.83^{b}	558.50 ± 24.39^{c}
BC	358.79 ± 19.39^{c}	542.67 ± 8.52^{d}	498.74 ± 24.71^{c}
SAP	546.52 ± 32.10^{b}	667.97 ± 18.84^{bc}	705.05 ± 24.17^{b}
BC-SAP	600.15 ± 29.46^{b}	51.06 ± 20.85^{cd}	732.32 ± 45.11^{b}

Notes: Data is expressed as mean ± SE, $\alpha=0.05$. Means with same superscript in the same column are not significantly different. The values for ABTS and DPPH are expressed as mM Trolox/100 g DM and for FC as mg chlorogenic acid/100 g DM.

Table 3.3 *Lactobacillus* plate count expressed as Log10 number at baseline (0 h) and 24 h. Within same time point, values bearing different letter are significantly different. Within same treatment, values bearing different number are significantly different.

	WW	**FW**	**BC**	**SAP**	**BC-SAP**	**Blank**
0 h	4.86 ± 0.25^{a1}	4.86 ± 0.25^{a1}	4.86 ± 0.25^{a1}	4.86 ± 0.25^{a1}	4.86 ± 0.25^{a1}	4.86 ± 0.25^{a1}
24h	6.09 ± 0.54^{a2}	7.86 ± 0.98^{a2}	7.46 ± 0.19^{a2}	7.50 ± 1.30^{a2}	7.95 ± 0.93^{a2}	5.84 ± 0.09^{a2}

Notes: The values at baseline correspond to time zero values for the blank. The samples at time 0 h were taken after the addition of potato meals. This was done to exclude any effect of treatments on samples taken at time 0 h. Hence, the change was measured from a common baseline $\alpha=0.05$.

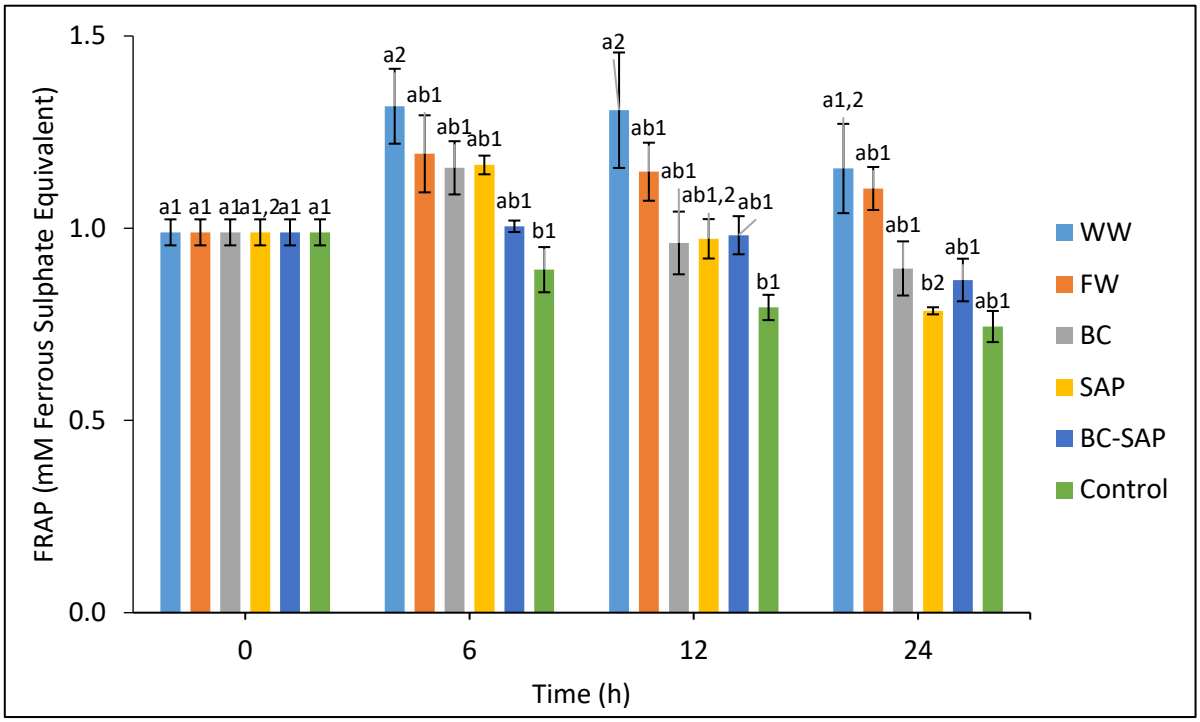

Figure 3.1 FRAP Antioxidant capacity values over a period of 24h in Batch reactors.

Notes: Data is expressed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different. Antioxidant capacity at time 0h is from the fecal samples, as the volunteer was not under restricted diet.

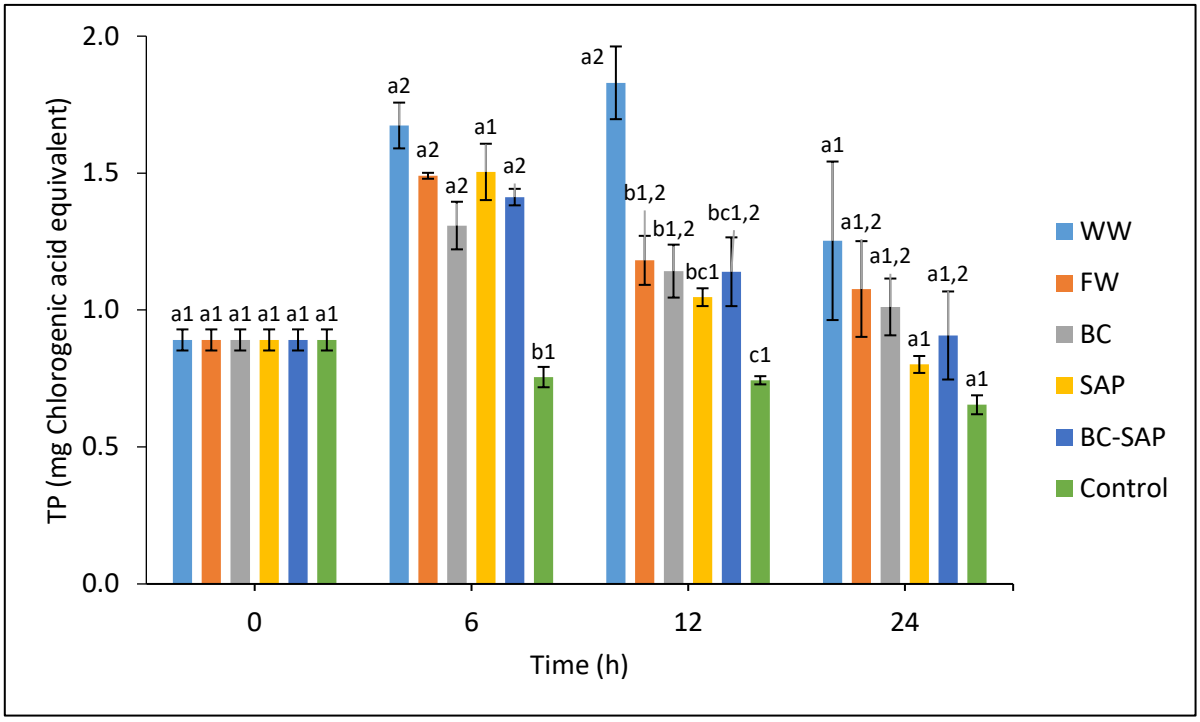

Figure 3.2 Total phenolics in the reactors over a span of 24 h.

Notes: Data is exprssed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different. TP at time 0 h are from the fecal samples, as the volunteer was not under restricted diet.

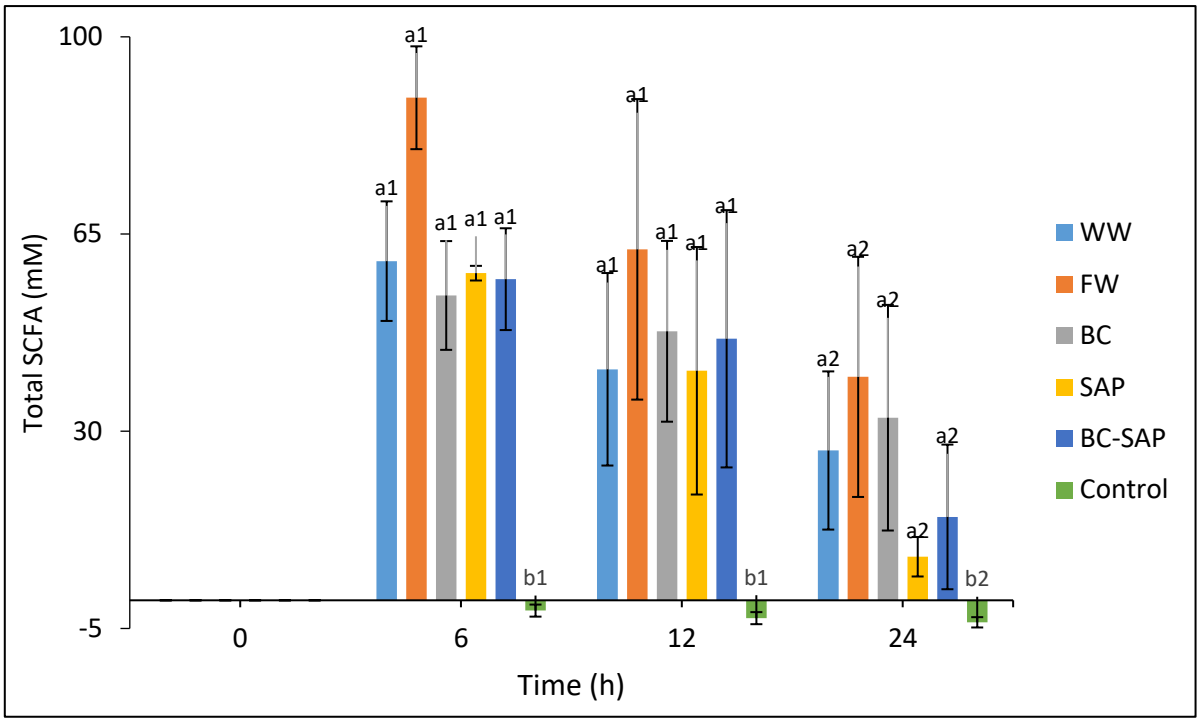

Figure 3.3 TSCFA changes in the batch reactors over 24 h time.

Notes: Data is expressed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different.

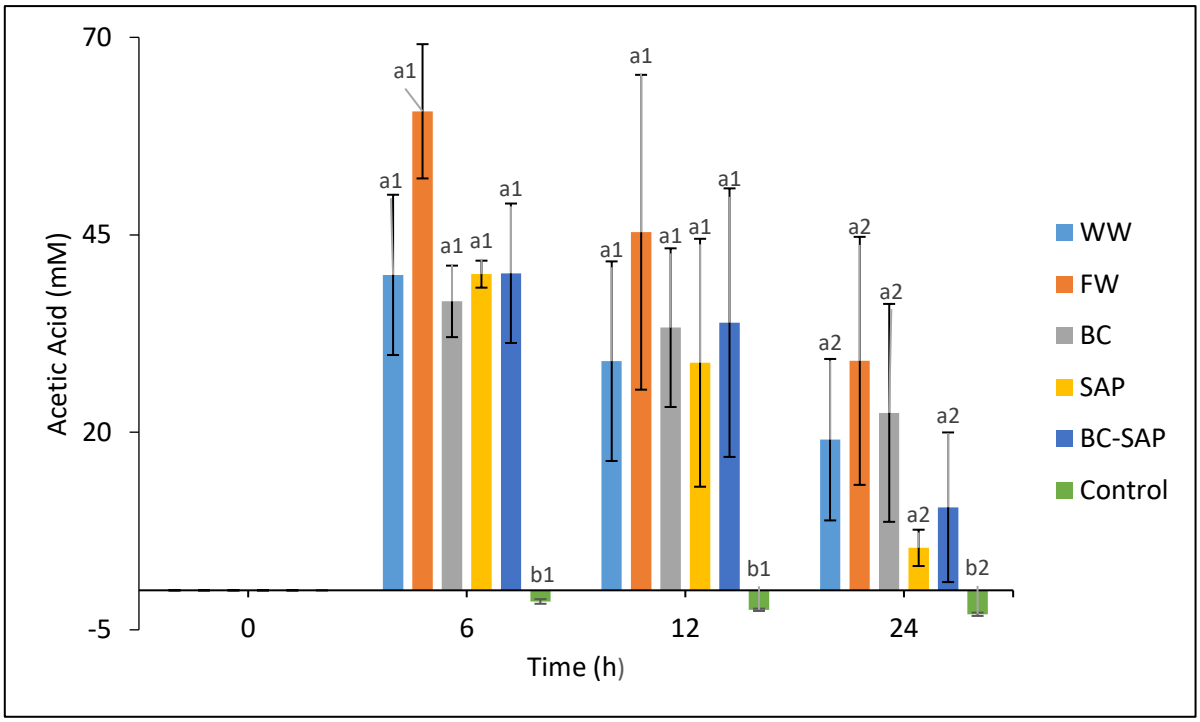

Figure 3.4 Acetic acid concentration in batch reactors during 24 h fermentation.

Notes: Data expressed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different.

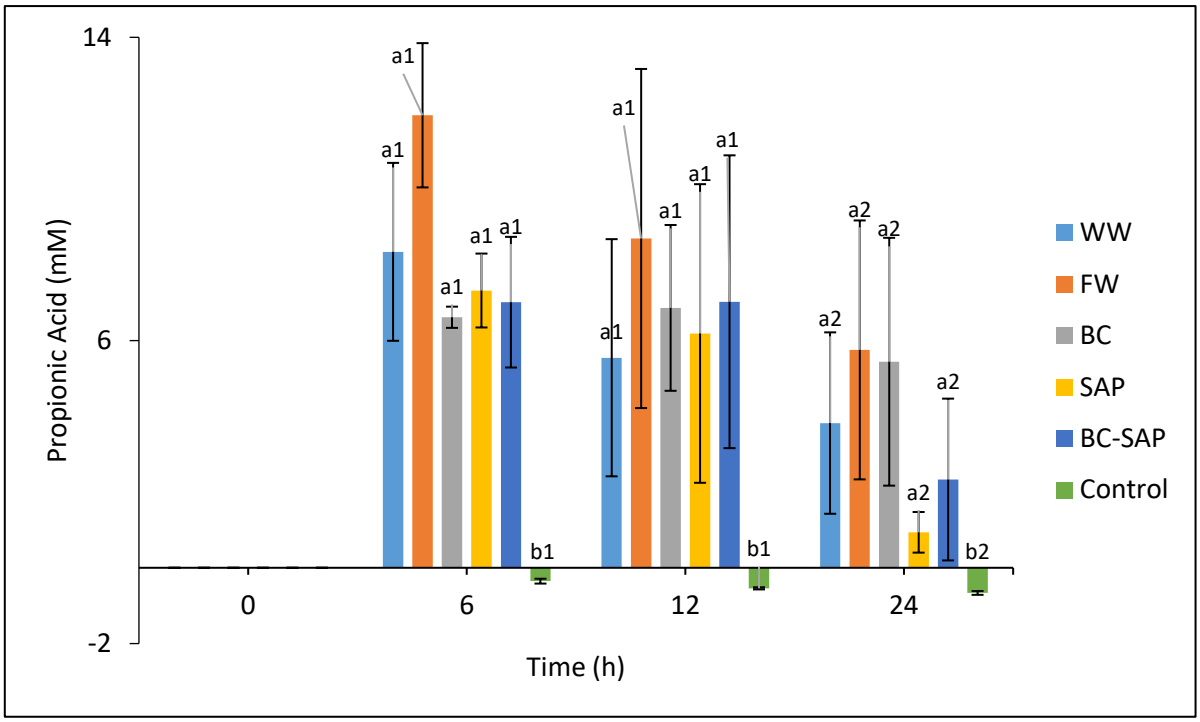

Figure 3.5 Propionic Acid concentration in batch reactors during 24 h fermentation.

Notes: Data is expressed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different.

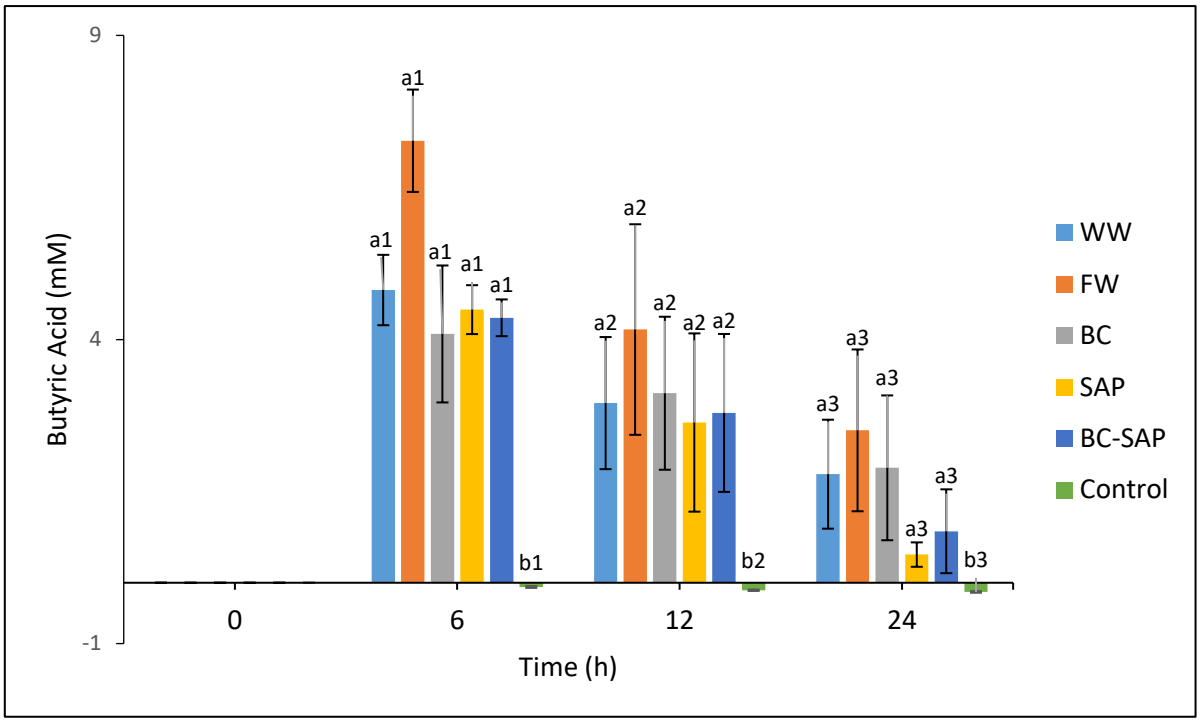

Figure 3.6 Butyric Acid concentration in batch reactors during 24 h fermentation

Notes: Data is expressed as mean ± SE, α=0.05. Values within same time point and bearing different letters are significantly different. Likewise, values within same treatment and bearing different number are significantly different.

CHAPTER 4
SUMMARY AND CONCLUSIONS

Heavy metals are the trace elements occurring naturally in earth's crust. Biological organisms are being exposed to them through various pathways, resulting from natural or anthropogenic activities. While some of the metals are essential for biological organisms in adequate amount, some heavy metals such as Pb, Cd, Ar, Hg, etc. are considered harmful and lethal even in trace amounts (Tchounwou et al., 2012). The heavy metals ions have the tendency to alter the composition gut microbiota and metabolism in the gastrointestinal tract (Jin et al., 2017). Gut microbiota has a symbiotic relationship with the host health. The role of microbiota in normal growth, development and maintenance of a healthy life has been proven decades ago through experimental studies on germ-free animals (Robles Alonso and Guarner, 2013). Therefore, any gut dysbiosis occurring due to the pollutants inside the gut can have direct health effects.

Contrary to the toxicity of heavy metals, polyphenols tend to have beneficial effects on the gut microbes and also found to induce the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Dolara et al., 2005; Tzounis et al., 2011). The various health promoting and therapeutic effects of potato-derived phenolics (Ezekiel et al., 2013) has been further suggested to be involved in decreased development of mammary cancer in a rat model by 23% (Thompson et al., 2009). Polyphenols have been found to induce the SCFA production (Sadeghi Ekbatan et al., 2016) that, in turn, are the primary fuel for the epithelial cells and enhance the gut morphology and functionality (Hijova and Chmelarova, 2007).

The present study was conducted to assess the potential impact of heavy metals in potatoes on gut metabolism and the beneficial bacteria, *Lactobacillus spp.* Four out of five treatments had heavy metals in different concentrations owing to the soil treatments and wastewater irrigation while one treatment was freshwater produced. The present thesis work involved a short-term study using batch fermentation reactors that was performed in triplicate

using a single daily serving of potatoes. The *Lactobacilli* numeration, antioxidant capacity and SCFA concentration was estimated over the 24 h batch fermentation of the potato meals. The wastewater and freshwater produced potatoes had similar fermentation trends in the batch reactors as indicated by a similar increase in SCFA concentrations. Also, the increase in antioxidant capacities in the batch fermenters was consistent with the pre-fermentation antioxidant capacities and total phenolics present in the different potato treatments. The *Lactobacilli* population also increased similarly in all the fermenters as compared to baseline. Potato-based resistant starch and chlorogenic acid have also been reported to induce SCFA production (Kleessen et al., 1997; Parkar et al., 2013). The SCFA production and the increased antioxidant capacity in the reactors can be linked with reduced oxidative damage, improved microbial composition and overall gut health (Dolara et al., 2005; Robert et al., 2013). Polyphenols from tea and red wine have also been reported to lower the tumor incidence in rats exposed to carcinogens and to decrease the DNA damage of the colonic mucosa (Dolara et al., 2005). Polyphenols modulate the intestinal ecology by promoting the growth of *Lactobacillus* and *Bifidobacterium spp.,* alter the balance of *Bacteroides/Firmicutes* and inhibit pathogenic bacteria such as *Clostridium histolyticum*, *Clostridium perfringens,* and *Clostridium difficile* reflecting a prebiotic effect (Cardona et al., 2013).

Overall, in this milieu of "polyphenol - microbiota" and "microbiota - SCFA" two way relationships associated with the potato feeding, the toxicity of heavy metals seems to be neutralized. Hence, wastewater irrigated potatoes despite being contaminated does not show significant difference in fermentation compared to freshwater irrigated ones. Also, the most contaminated treatment without any soil amendment had higher TP and AOC owing to heavy metal stress, which may have led to higher neutralising effect against the toxicity of metals. A study limitation is the wide range of variation in the three replicates, which could be due to underlying limitation of *in-vitro* gastrointestinal models. Therefore, the studies involving

chronic exposure of mice to the intake of potatoes contaminated with pollutants from natural uptake is needed to further validate the present thesis findings.

4.2 Future Recommendations

The present study presents results for the acute exposure of contaminated potatoes on the microbial metabolism in terms of SCFA, AOC and Lactobacilli plate count. While the phenolic metabolites formed after the digestion can also be examined to validate the change in AOC. Further studies are suggested to investigate a more detailed effect on the colonic microflora using microbiome analysis. The changes in the microbiome profiles can be assessed in this manner such as *Bacteroidetes* and *Firmicutes* ratio which has been associated with obesity and other diseases. The chronic exposure studies on the more complex models such as SHIME can be conducted to validate the present findings. It can also help observe the effects in the different colonic sections (ascending, transverse and descending).

The exposure effects of heavy metals in other food materials where antioxidants cannot help neutralise the toxicity can also be conducted. It will be interesting to look upon individual metal effects on the microbial profiles conducting studies with feeding the metals in food at different concentrations in the simulated human gastrointestinal models.

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